Maximising yeast health during the industrial brewing process

by

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Abbreviations

2-DE	Two-dimensional gel electrophoresis
Acetyl-CoA	Acetyl–coenzyme A
ACS	Acetyl-CoA synthetase
AO	Acridine orange
ADH	Alcohol dehydrogenase
ADY	Active dry yeasts
ALD	Acetyl aldehyde dehydrogenase
Ath1p	Acid trehalase 1
ATP	Adenosine triphosphate
AUD	Australian dollars
cFDA	Carboxyfluorescein diacetate
CO_2	Carbon dioxide
CFU	Colony forming unit
Crm1p	Exportin 1
C-terminal	Carboxy-terminal
CTT1	Cytosolic catalase T
DDA	Data dependent acquisition
DDR2	DNA damage-responsive gene
DHAP	Dihydroxyacetone phosphate
DIA	Data independent acquisition
DO	Dissolved oxygen
ESR	Environmental stress response
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
FAN	Free amino nitrogen
FASP	Filter-aided sample preparation
FDA	Fluorescein diacetate
FRET	Fluorescence energy resonance transfer
GAAC	General amino acid control
Gdb1p	Glycogen debranching enzyme
Glc3p	Glycogen branching enzyme
Glg1p/ Glg2p	Glycogenin
GLR1	Glutathione reductase
Glucose-6P	Glucose-6-phosphate

GPD1/ GPD2	Glycerol-3-phosphate dehydrogenase
Gph1p	Glycogen phosphorylase
GPX1/ GPX2	Glutathione peroxidase
Gpx3p/ Orp1p	Glutathione peroxidase (GPx)-like enzyme
GSH1	Glutamate-cysteine ligase
Gsy1p/ Gsy2p	Glycogen synthase
H_2O_2	Hydrogen peroxide
HOG	High osmolarity glycerol
HSPs	Heat shock proteins
Hxk2p	Hexokinase 2
HXT	Hexose transporters
ISD	In-solution digestion
iST	in-StageTip
iTRAQ	Isobaric tags for relative and absolute quantitation
LC	Liquid chromatography
MALx1	Maltose transporters
MALx2	Maltose permease
m/z	Mass-to-charge
MAPK	Mitogen-activated protein kinase
MB	Methylene blue
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS^1	Mass spectrum of precursor ions
MS^2	Mass spectrum of fragment ions
Msn2p and Msn4p	Multicopy suppressor of SNF1 mutation proteins 2 and 4
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NCR	Nitrogen catabolite repression
Nth1p	Neutral trehalase 1
$O_2^{\cdot -}$	Superoxide anions
OH^-	Hydroxy anions
PDC	Pyruvate decarboxylase
PDH	Pyruvate dehydrogenase

Pgm1p	Phosphoglucomutase 1
PKA	cAMP-dependent protein kinase A
°P	Plato
PI	Propidium iodide
PRM	Parallel reaction monitoring
Pse1p	Importin subunit beta-3
PTM	Post-translational modification
QqQ	Triple quadrupole
RT	Retention time
ROS	Reactive oxygen species
Sho1p	High osmolarity signaling protein SHO1
Sln1p	Osmosensing histidine protein kinase SLN1
SP3	Single-Pot Solid-Phase-enhanced Sample Preparation
SP4	Solvent precipitation SP3
SPEED	Sample Preparation by Easy Extraction and Digestion
SPS	Ssy1p/ Ptr3p/ Ssy5p
SRM	Selective reaction monitoring
STrap	Suspension trapping
STRE	Stress response element
SWATH-MS	Sequential window acquisition of all theoretical ions mass spectra
TCA	Tricarboxylic acid
TCEP	Tris(2-carboxyethyl)phosphine
TMT	Tandem mass tags
TOR	Target of rapamycin
TPP	Trehalose-6-phoshate phosphatase
TPS	Trehalose-6-phosphate synthase
Trehalose-6P	Trehalose-6-phosphate
TRR1	Thioreodoxin reductase
TRX2	Thioreodoxin 2
UDP-glucose	Uridine diphosphate-glucose
UFAs	Unsaturated fatty acids
Ugp1p	UDP-glucose pyrophosphorylase
Yap1p	bZip transcription factor

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Abstract

Brewing is an ancient practice and beer is still one of the most popular beverages in modern society. Bottle conditioning is a process where yeast is seeded into fresh beer that has been primed with a fermentable extract (sugar). The yeast performs a secondary fermentation for 2 – 4 weeks, resulting in carbonation of the beer and a product that is appreciated for its organoleptic and artisanal properties. This process relies on the yeast maintaining good physiological condition as a decline in yeast viability can lead to the production of off-flavours and a decrease in foam stability. Ultimately, this can impact the perceived quality of the product and potentially damage brand reputation.

The assessment of yeast quality at different brewing stages is important to ensure consistent batch-to-batch production of beer. Despite the availability of assays for assessing yeast quality, their implementation into a brewery setting can be difficult due to cost and time factors. Most breweries utilise methylene blue staining for the assessment of yeast quality. However, this method does not characterise the physiological state of the cell. A combination of viability and vitality measurements are necessary to gain an overall perspective of yeast quality. The beginning of this thesis focusses on the application of resazurin, a redox-sensitive dye, for the assessment of changes in yeast physiology during brewing propagation. Resazurin was able to show a decline in yeast metabolism at different stages of growth during propagation. Furthermore, we showed that a decline in metabolism occurred prior to the degradation of storage carbohydrates. This is important during propagation as selection of an optimal cropping time is necessary to promote consistent bottle conditioning. The use of resazurin can be applied within other brewery processes and industries to complement existing quality control measures.

The yeast utilised for bottle conditioning can be harvested from primary fermentation, freshly propagated, or an active dry yeast. However, preference is given to freshly propagated yeast as it can be pre-adapted to improve the longevity of the yeast during bottle conditioning and lower the risk of microbial contamination. Preliminary results in the brewery showed that the use of freshly propagated yeast did not improve their longevity during bottle conditioning compared to harvested yeast. However, it has been shown that the propagation time of yeast prior to transfer for primary fermentation can impact their fermentation ability. Therefore, we investigated the impact of propagating yeast to the logarithmic and post-diauxic growth phase prior to seeding for bottle

conditioning at an industrial scale (30 hL). We used a combination of markers for the assessment of yeast physiology and metrics for beer quality to investigate its influence on yeast survival and the shelf-life of the product. Additionally, we used sequential window acquisition of all theoretical ions mass spectrometry (SWATH-MS) to determine proteomic changes in the yeast that improved their longevity during bottle conditioning. It was observed that propagating to the post-diauxic phase led to an increase in the number of proteins associated with respiratory metabolism and general stress pathways. The upregulation of these pathways has been shown to improve cellular stress responses and yeast longevity. These results provide a more detailed understanding of proteomic changes in yeast during a brewery propagation and its downstream impacts on bottle conditioning.

It has also been shown that different yeast strains have different propensities for survival. Therefore, the choice of yeast strain that has better longevity will help improve the shelf-life of bottle conditioned beers. We investigated the performance of six different yeast strains from the brewing, distilling, and wine industries to survive during bottle conditioning and their influence on beer characteristics. Foam stability was only affected when the viability of the yeast strains declined below 50%. Interestingly, brewing strains produced a similar sensory aroma compared to the sparkling and distilling yeast strains. We observed that one brewing strain had improved longevity compared to the other yeast strains. To gain an understanding of the biological processes that were associated with improved longevity for specific yeast strains during bottle conditioning, we performed SWATH-MS. Proteins associated with oxidoreductase activity and mitochondrial ribosomes were increased in abundance in the yeast strain with superior survival and were able to respond to cellular stress more effectively, whereas proteins associated with cell wall modulation were increased in the strain with poor survival characteristics. Our results reveal the diversity between the proteome of yeast strains used in different beverage industries and how different biological processes can impact their longevity during bottle conditioning. Furthermore, we have shown that with careful selection of the yeast strain, it can have minimal influence on the aroma and foam stability of the product. This can allow brewers to select yeasts with improved longevity without effecting the final product and increase the shelf-life characteristics of the beer.

This thesis has utilised proteomics along with other yeast physiological markers and beer quality metrics to describe and improve our understanding of yeast metabolism during

brewing at an industrial scale. These insights into the yeast proteome will provide brewers with a better understanding of the impact of different brewery processes on yeast physiology. This can also be applied to other industries that utilise similar processes.

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Declaration

I declare that:

this thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.

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Thesis publications

Publications generated from this thesis are listed below.

Aim 1:

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Aim 2:

Dilmetz, B.A., Desire, C.T., Meneses, J., Klingler-Hoffmann, M., Young, C., & Hoffmann, P. (2024). Influence of propagation time on yeast physiology during bottle conditioning on an industrial scale. *Food Chemistry*, 435: 137655. https://doi.org/10.1016/j.foodchem.2023.137655.

Aim 3:

Dilmetz, B.A., Brar, G., Desire, C.T.; Meneses, J., Klingler-Hoffmann, M., Young, C., Hoffmann, P. (2024). Performance of Different *Saccharomyces* Strains on Secondary Fermentation during the Production of Beer. *Foods,* 13, 2593. https://doi.org/10.3390/foods13162593.

Other published work during candidature

Sebben, D.A., Semple, S.J, Condina, M.R., **Dilmetz, B.A.,** Hoffmann, P, Claudie, D.J., Krasowska, M., Beattie, D.A. (2024). Extraction and Surface Activity of Australian Native Plant Extracts: *Alphitonia excelsa*. *Colloids and Interfaces*. *8,* 46. https://doi.org/10.3390/colloids8040046

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1 Introduction

Yeasts are unicellular microorganisms of the fungi kingdom that undergo cell division by budding or fission[1]. Archaeologists have found evidence that their use in fermented products has been documented to occur as early as the Neolithic times (7000 BC – 1700 BC)[2]. However, the first time that yeast was visualised under the microscope wasn't until the 17th century by Antoni van Leeuwenhoek[3]. It wasn't until almost two centuries later that Louis Pasteur first demonstrated that yeast was the microorganism responsible for alcohol formation from sugars[4]. The importance of *Saccharomyces cerevisiae* as a model microorganism was first demonstrated in the 1996 landmark study, where it was the first microorganism to have its genome sequenced[5]. This made *S. cerevisiae* amenable to modifications (such as gene disruption and mutations) and led to its use for fundamental research, and in a range of industrial processes including, but not limited to, fermented food and beverages, fuels, chemicals, and for the synthesis of recombinant proteins[6,7]. The synthesis of recombinant proteins by *S. cerevisiae* are useful for medical purposes such as human insulin production and hepatitis vaccines[7].

There are thousands of *S. cerevisiae* strains, with just over 2,500 strains having been sequenced, revealing a large genetic diversity and complex population structure[8,9]. A phylogenomic study that investigated the genome evolution of 1,011 *S. cerevisiae* strains observed that most of the strains group into 26 well-defined clades[9]. A clade is a group of organisms that share a common ancestor with each other[10]. This study also supported that the origin of all domesticated *S. cerevisiae* strains was most likely from China[9]. Furthermore, they and others have observed that geographic location, industry application and the degree of human association correlate with the different lineages[9,11,12]. For example, ale brewing strains were observed to originate from two independent lineages, with one exclusive beer clade showing that the strains were distributed by geographical locations[11]. Additionally, brewing yeasts have high genetic diversity, suggesting that their primary mode of growth was through asexual reproduction, leading to beer brewing

strains thriving in man-made environmental niches[11]. In contrast, domesticated *S. cerevisiae* wine yeasts have been shown to originate from a single lineage and have less genetic diversity compared to brewing yeasts, allowing them to survive better in different environments[13].

General overview of yeast metabolism

Sugars, mainly glucose, are the major source of energy for cellular growth in *S. cerevisiae*. These sugars can be metabolised through fermentative and respiratory metabolic pathways depending on the external environment. Glucose is transported into the cell by hexose transporters (HXTs) through passive diffusion (Figure 1)[14]. Maltose is the main carbohydrate in brewing and is transported into the cell by maltose transporters (*MALx1*) using an active transport process[15]. Maltose is hydrolysed into two glucose molecules by maltose permease (*MALx2*)[15]. In the cell, glucose is converted into pyruvate resulting in the production of two molecules of adenosine triphosphate (ATP) and two molecules of reduced nicotinamide adenine dinucleotide (NADH). This process is called glycolysis, and is also known as the Emben-Meyerhof-Parnas pathway[16]. These reactions are catalysed by multiple enzymes that are regulated at different levels, such as transcription and post-translational modifications (PTM)[17]. The final product of this pathway, pyruvate, is a key metabolite in carbon metabolism and is the branch point for fermentative and respiratory metabolism.

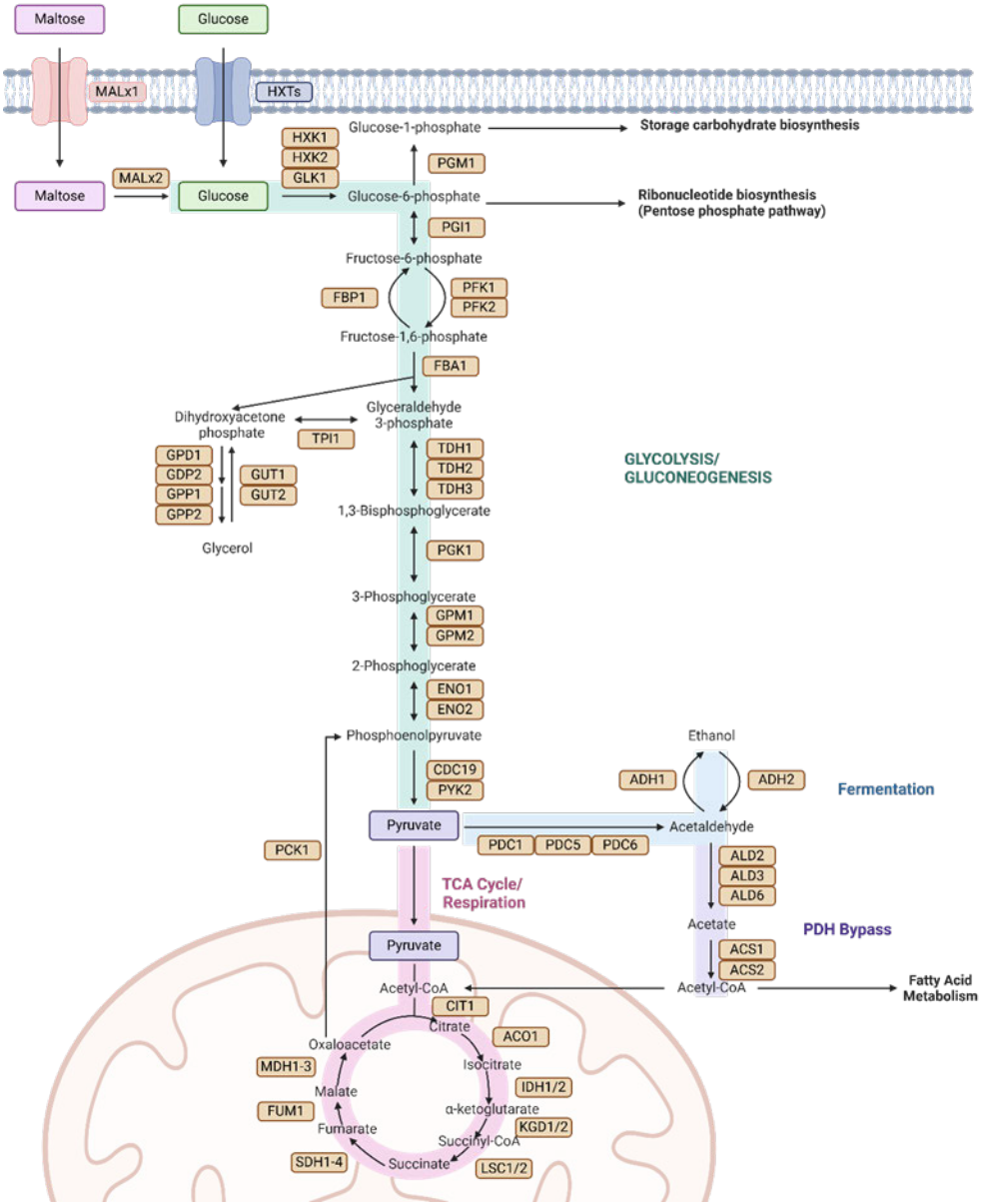


Figure 1 Schematic overview of glycolysis, fermentation, and tricarboxylic acid cycle pathways in *Saccharomyces cerevisiae*. Glucose is transported into the cell by passive diffusion whereas maltose is transported intact by an active process. Maltose is converted to glucose before it is used for energy production. S. cerevisiae have two pathways for energy production: fermentation and respiration. The start of both pathways begins with glycolysis where glucose is converted to pyruvate. Pyruvate is used to produce ethanol during fermentation (anaerobic conditions and aerobic conditions if the glucose concentration is high). Pyruvate can also be utilised in the TCA cycle during aerobic conditions when glucose levels are low and when using ethanol as a carbon source. Figure adapted from[18,19]. Created in Biorender®.

Glucose rich conditions (>0.1 g/L) in *S. cerevisiae* results in the carbon flux being directed towards fermentative metabolism irrespective of oxygen availability[20]. This causes the repression of genes involved in respiratory metabolism[19,20]. This phenomenon is known as the Crabtree effect and results in respiro-fermentative metabolism[21]. The advantage of preferential dissimilation of pyruvate to ethanol has also been suggested to provide *S. cerevisiae* with a selective advantage to outcompete other microorganisms[22]. During fermentation, pyruvate synthesised during glycolysis is decarboxylated to acetaldehyde by pyruvate decarboxylase (PDC). Acetaldehyde is then reduced to ethanol by alcohol dehydrogenase (ADH), which regenerates the nicotinamide adenine dinucleotide (NAD^+) that was previously reduced during glycolysis[23].

S. cerevisiae can also metabolise pyruvate through respiratory pathways, which is energetically more favourable, producing between 16 and 36 ATP molecules per glucose molecule[23,24]. Pyruvate is imported into the mitochondrial matrix by protein transporters and subsequently oxidised to acetyl–coenzyme A (acetyl-CoA) by the mitochondrial pyruvate dehydrogenase (PDH) complex[25]. Acetyl-CoA can also be produced through the PDH bypass in the cytosol of *S. cerevisiae*. The PDH bypass is a series of reactions where acetaldehyde is converted to acetate by acetaldehyde dehydrogenase (ALD) and then into acetyl-CoA by acetyl-CoA synthetase (ACS)[26]. Acetyl-CoA is utilised in the tricarboxylic acid (TCA) cycle for the synthesis of precursors that are utilised in alternative metabolic pathways such as amino acid biosynthesis. The co-factors, flavin adenine dinucleotide ($FADH_2$) and NADH, that are produced during the TCA cycle provide electrons to the multi-subunit protein complexes of the electron transport chain (ETC), leading to their replenishment[27].

The ETC in *S. cerevisiae* consists of a peripheral membrane NADH dehydrogenase and three additional protein complexes located in the inner mitochondrial membrane[27]. This leads to the generation of a proton gradient across the inner mitochondrial membrane which drives ATP-synthase and ATP generation[28]. The depletion of glucose from the environment results in *S. cerevisiae* utilising ethanol as a substrate for energy production. This only occurs in aerobic conditions and is known as the diauxic shift. *S. cerevisiae* switches to gluconeogenic pathways (redirection of carbon flux through glycolysis) and utilises ethanol as the carbon source[29]. This pathway involves the oxidation of ethanol to

acetaldehyde, acetaldehyde oxidation to acetate, and then the conversion of acetate to acetyl-CoA that can be utilised in different metabolic pathways.

Yeast growth during aerobic and anaerobic conditions

During aerobic conditions, and with a high amount of sugar, *S. cerevisiae* has six distinct growth phases (Figure 2). After inoculation or seeding into the medium, a lag phase occurs where the yeast has a period of adjustment to adapt to the growth medium[30]. The cells do not undergo cell division during this stage, but are metabolically active[30,31]. This is followed by a period of accelerating growth until the cells enter logarithmic growth phase. Logarithmic growth is where the yeast population increases at a constant rate, sugars are consumed, and ethanol is produced[32]. As nutrients in the medium become limiting, the cells enter the diauxic shift. The diauxic shift is characterised by cells momentarily arresting cell growth and a switch from fermentative to respiratory metabolism, consuming ethanol as the primary carbon source (post-diauxic shift)[32]. Cellular growth during the post-diauxic shift occurs at a much slower rate[33]. The yeast cells then enter stationary phase, where they cease growth. After extended starvation, the cells have a decrease in viability and enter death phase, resulting in a decrease in the population[34]. Typically, the growth of yeast in aerobic conditions only occurs during yeast propagation within a brewing setting. On the other hand, most yeast utilises anaerobic metabolism during brewery handling. Anaerobic growth by *S. cerevisiae* has a lag phase, logarithmic phase, stationary phase, and death phase. After a lag phase, the yeast population increases at a constant rate, depletes any remaining oxygen, and creates an anaerobic environment. As the sugars are consumed and the ethanol concentration increases in the environment, the yeast enters stationary phase. The absence of oxygen results in the yeast being unable to metabolise ethanol as a carbon source. After extended starvation, the cells have a decrease in viability and enter death phase, resulting in a decrease in the population. Anaerobic growth occurs during primary fermentation and bottle conditioning during brewing.

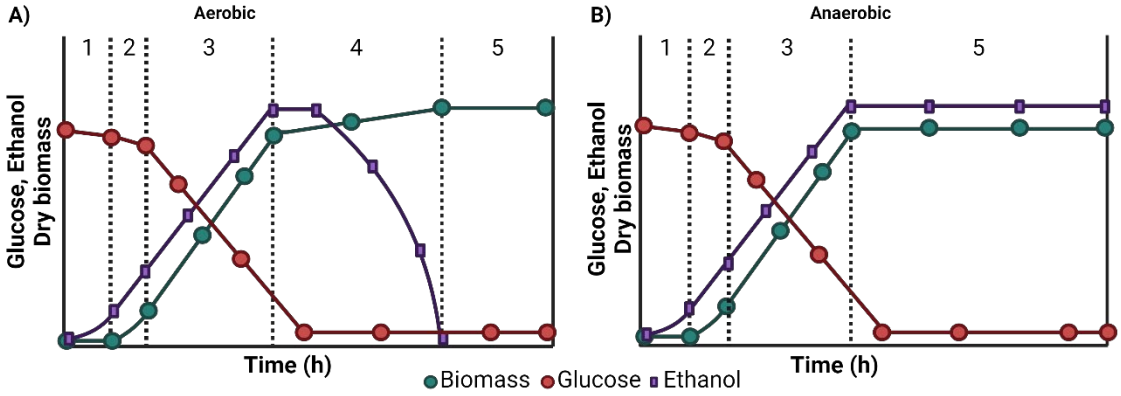


Figure 2. Growth phases in aerobic and anaerobic batch cultures. Biomass (green circle), glucose (red circle), and ethanol (purple square) concentration during different stages of growth in A) aerobic and B) anaerobic conditions of batch cultures. Death phase not shown in figure. 1) Lag phase, 2) accelerating growth phase, 3) logarithmic phase, 4) post-diauxic shift, and 5) stationary phase. Created in Biorender®.

Beer brewing in Australia

The production of beer is one of the oldest biotechnologies, with evidence of brewing dating to the Predynastic era (5500 BC – 3100 BC)[35]. Today, beer continues to be one of the most popular beverages worldwide and holds economical and sociocultural importance in modern society[36]. In Australia, the brewing industry generated $5.1 billion (AUD) in revenue, with craft breweries contributing $1 billion (AUD)[37,38]. Moreover, the industry supports 7,581 jobs within Australia[37]. The Australian market share is dominated by the large brewing companies, Asahi Holdings (Australia) Pty Ltd. and Lion Pty Ltd. This is followed by several other breweries, including Coopers Brewery Ltd. which has a market share of 4%, making it the largest wholly South Australian owned brewery[37]. The global prominence of beer and the increase in the number of craft breweries has resulted in the need to produce consistent and high-quality beers to maintain customer satisfaction and competition with other breweries.

Overview of brewing process

Historically, the fermentation of food and beverages was a spontaneous process that relied on yeasts and bacteria that were naturally present in the raw materials such as grape musts and cereals[39]. Despite spontaneous fermentations being used for thousands of years, there are numerous challenges associated with it, such as variable and unpredictable fermentation that can lead to inconsistent product quality[39]. This led to most industrial

fermentations utilising pure starter cultures, mainly *Saccharomyces* yeast, for the fermentation of beer, bread, and wine.

The primary raw ingredients used to brew beer are cereal grains, predominately malted barley (*Hordeum vulgare)*, hops, water, and yeast. The first stage of the brewing process is malting. Malting is comprised of three stages, 1) steeping, 2) germination, and 3) kilning. The primary aim of malting is to synthesise and activate hydrolytic enzymes within the grain in a controlled environment[40]. The steeping process involves a series of repeating steps; the grain is immersed in water (12 °C), followed by a period of air-rest where the water is drained, and then removal of carbon dioxide (CO_2) from the chamber for 2 – 4 days[40]. The removal of water and CO_2 is required to minimise fermentative metabolism and maximise aerobic metabolism in the grain and therefore germination[40]. Hydrolytic enzymes such as amylases and proteases are synthesised and activated during germination, which are required for mashing. This process aims to increase the moisture content from 12% – 14% to 42% – 48%[41]. To prevent degradation and loss of starch and proteins from the grain, the germination process is stopped by heating the grain gradually from 50 °C to 85 °C for around 21 h[42]. This process is known as kilning and typically the moisture content of the grain is reduced to below 5%. The malt is then milled to form the grist prior to mashing.

The production of beer is comprised of several key steps (Figure 3). Mashing is the first step in the production of beer. During mashing, the grist is added to water which is heated at different temperatures to stimulate enzymatic activity, and to hydrolyse the starch and proteins into fermentable sugars and amino acids, respectively[41]. The resultant liquor is termed 'wort'. The wort is separated from the spent grain using a lauter tun or mash filter and transferred to the kettle[41]. In the kettle, the wort is boiled to remove unwanted volatiles, coagulate proteins and polyphenols (hot break), isomerise α-acids from hops (resulting in bitterness), and inactivate any enzymatic activity[41]. Additionally, it is at this stage where various types of hops can be added. Hops are the flowers produced from the *Humulus lupulus* vine that impart bitterness, specific flavours and aromas, and aid in sterilisation of the wort[43]. The wort is cooled through a heat exchanger to temperatures required for fermentation. Yeast is pitched into the aerated wort and as fermentation proceeds, oxygen is depleted and most of the available sugars are consumed and converted to ethanol. The beer can be left unfiltered or filtered, unpasteurised or

pasteurised prior to being aged, carbonated, and packaged. Some breweries also use bottle conditioning which is also known as bottle refermentation.

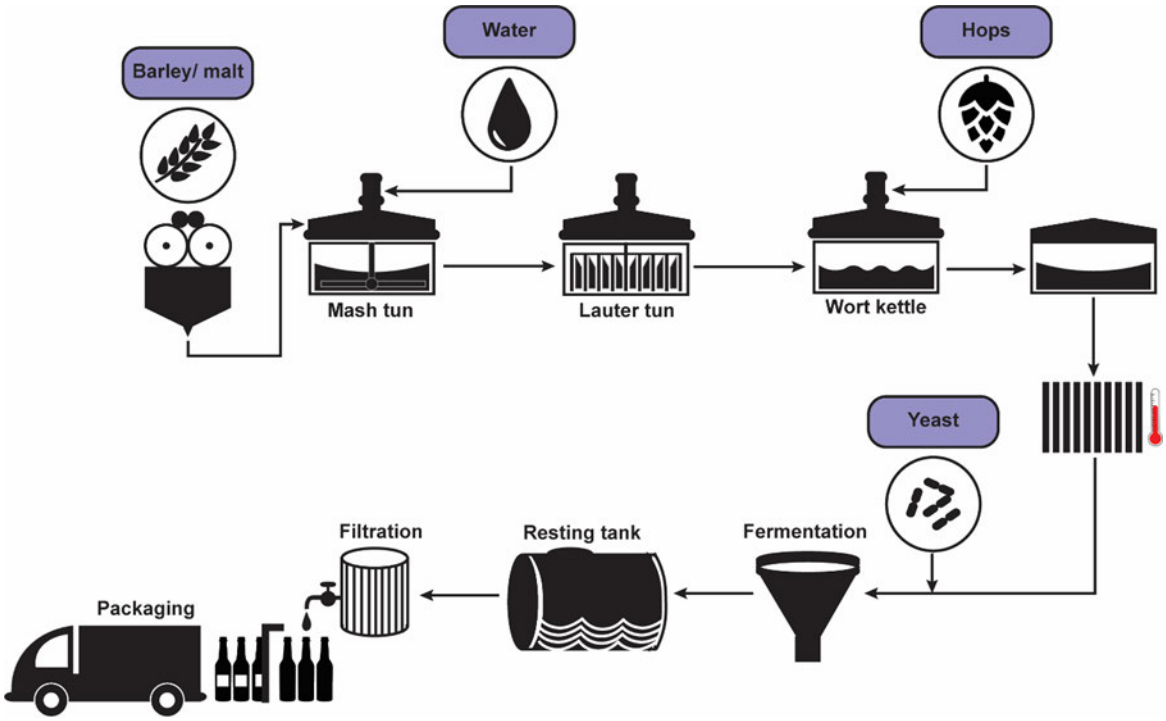


Figure 3. Overview of the brewing process. The grist (malted barley) is placed into the mash tun with water and heated at different temperatures to stimulate the activity of different enzymes and the solubilisation of nutrients to generate wort. The insoluble material is separated from the wort through a mash tun or lauter filter and placed into the wort kettle. The wort is boiled and hops (Humulus lupulus) are added to aid in sterilisation and flavour. The wort is cooled, transferred to the fermentation vessel, and then yeast is added. The yeast ferments the wort to produce ethanol and is then transferred for downstream brewing processes.

Wort composition

The composition of wort is a complex mixture of different components such as sugars, dextrins, amino acids, peptides, proteins, vitamins, ions, and nucleic acids[41]. Typically the carbohydrate portion is comprised of sucrose (1% – 2%), fructose (1% – 2%), glucose (10% – 15%), maltose (50% – 60%), maltotriose (15% – 20%) and dextrins (20% – 30%)[44].

Nitrogen compounds have been estimated to constitute 4% – 5% in wort[45]. Assimilable nitrogen utilised by yeast is called free amino nitrogen (FAN) and is typically composed of amino acids, ammonium ions and small peptides[46]. The concentration of FAN in standard brewery wort can vary greatly depending on the raw materials used and malting

and mashing parameters[45]. It has been estimated that a minimum concentration of 100 ppm FAN is required for successful fermentation[47].

Vitamins and ions are also an important component of wort. Vitamins such as thiamine and metal ions such as zinc are essential co-factors in various biological processes within yeast. For example, thiamine is a co-factor of PDC in the decarboxylation of pyruvate to acetaldehyde[48] and zinc ions are required for ADH activity during fermentation[49]. Insufficient levels of vitamins and metal ions can lead to sluggish or stuck fermentations[50].

Brewing yeasts

There are two main types of yeasts utilised in beer production. The production of an ale beer uses *S. cerevisiae* that rises to the top of the fermentation vessel (top fermenting) during fermentation, whereas lager beer uses *S. pastorianus* that sediments to the bottom of the fermentation vessel (bottom fermenting)[51]. Historically, lager yeast strains were known as *S. carlsbergensis*, however, it is now established that *S. pastorianus* is a hybrid of *S. cerevisiae* and *S. eubayanus*[52]. There are some differences between ale and lager yeasts (Table 1); the main difference being that fermentation with ale yeast is usually performed at a temperature of 18 °C – 25 °C whereas with lager yeast it is performed at 8 °C – 15 °C[53]. This results in shorter fermentation times for ale beers, usually 4 – 5 days, compared to lager beers that can take up to 7 days[51,53]. Despite the predominant use of *Saccharomyces* strains in brewing, there are a range of non-conventional yeasts such as *Dekkera/ Brettanomyces*, *Wickerhamomyces anomalus* and *Torulaspora delbrueckii* that are also used in brewing to provide distinct aroma profiles and therefore a specialised beer[54].

Table 1. Characteristics of ale and lager yeast strains. Adapted from[53]

Characteristic	Ale	Lager
Strain	*Saccharomyces cerevisiae*	*Saccharomyces pastorianus*
Fermentation temperature	18 °C – 25 °C	8 °C – 15 °C
Maximum growth temperature	37 °C or higher	Cells cannot grow at 37 °C or higher
Flocculation	Top	Bottom
Sugar utilisation	Cannot ferment the disaccharide melibiose	Ferments the disaccharide melibiose

Propagation and fermentation in beer production

Propagation

The quantity of yeast required for primary fermentation and downstream processing, such as bottle conditioning, in an industry setting requires the use of large vessels to obtain yeast of sufficient concentration ($200x10^6$ cells/mL)[55]. Propagation is comprised of a series of volumetric scale-up steps that occur first in the laboratory and then in brewery propagation plant (Figure 4)[56]. For example, a propagation process can be initiated by the inoculation of a stored culture into 10 mL of wort. The culture is serially sub-cultured to generate a 20 L culture in a stainless-steel vessel. After sufficient cell growth has occurred, the culture is transferred into the brewery, where it is placed into a 10 hL prior to transfer into a 60 hL propagation tank.

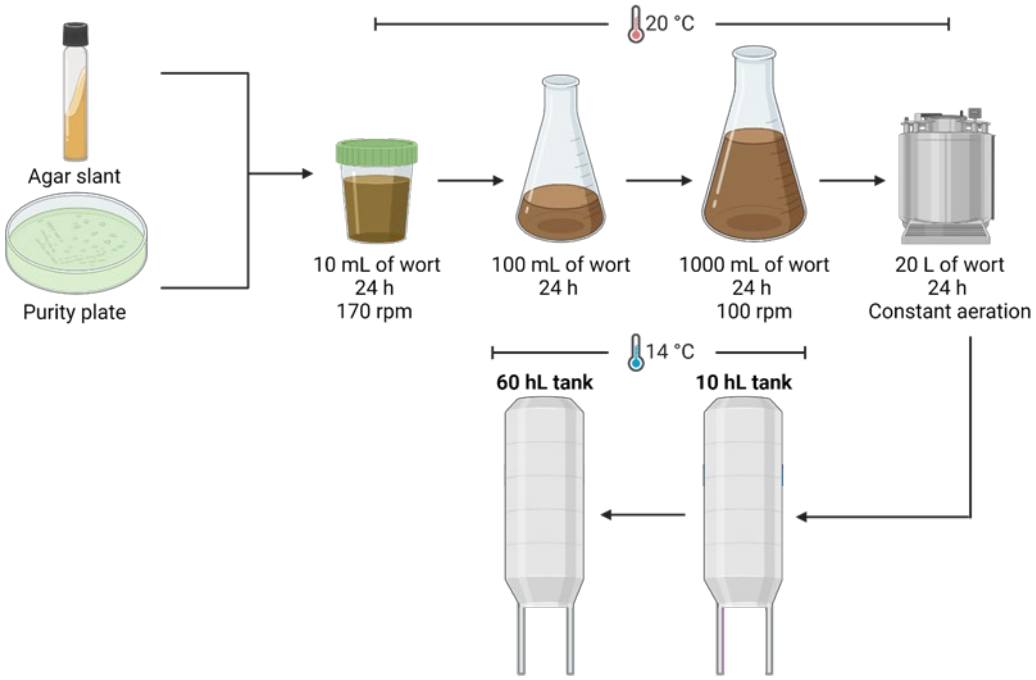


Figure 4. Typical industrial propagation of yeast for beer brewing. Pure yeast stocks are obtained, and colonies inoculated into wort. The volume of yeast is sequentially upscaled into larger volumes, reaching 20 L, and grown in aerobic conditions in the laboratory. The yeast culture is transferred into a 10 hL tank and then into a 60 hL tank and incubated using pre-determined settings. Created in Biorender®.

Propagation is performed in aerobic conditions to obtain optimal biomass generation. The amount of dissolved oxygen (DO) necessary during propagation varies between 4 ppm – 40 ppm and is highly strain dependent[57,58]. As *S. cerevisiae* preferentially utilises fermentative metabolism when glucose is in excess despite the presence of oxygen (Crabtree effect)[21,59,60], other industries usually employ highly aerated fed-batch (consistent low dose of sugar) systems that maximise yeast growth through aerobic respiration and minimise ethanol production[61]. Using a fed-batch approach results in a yield factor of 54 g of dry yeast produced for every 100 g of sugar consumed (54%)[62]. However, unlike other industries, brewery propagations occur in standard brewery wort and contain high sugar levels as this is easy to access and cost effective. This leads to a lower biomass yield compared to fed-batch, with a study showing that yeast propagated in wort containing a sugar concentration of 12°Plato (°P) results in a yield factor of 10 g of dry yeast produced for every 100 g of sugar consumed (5% – 10%), regardless of the amount of oxygen supplied[62]. This is due to the high sugar content in wort driving metabolic flux towards fermentative processes and the production of alcohol[63]. After the glucose levels reach sufficiently low levels, *S. cerevisiae* will enter the diauxic shift and switch from fermentative to respiratory metabolism in the presence of oxygen, consuming

ethanol (post-diauxic shift) (Figure 2)[32]. Cellular growth during the post-diauxic shift occurs at a much slower rate[33]. The yeast cells then enter stationary phase, where they cease growth.

Fermentation

Fermentation in beer production by *S. cerevisiae* requires wort aeration to enable the yeast to synthesise sterols and unsaturated fatty acids (UFAs) that are necessary for anaerobic growth and cell division[64]. Yeast is transferred into the wort and rapidly consumes the oxygen available before entering logarithmic growth, a process described as pitching. This occurs in an anaerobic environment, resulting in fermentative metabolism and the production of ethanol and CO_2[23]. As the nutrients in wort become limiting, the yeast enters stationary phase and flocculate. The yeast is separated from the beer, for example using a centrifuge, and stored at low temperatures of 3 °C – 4 °C prior to use in subsequent fermentations[56]. The re-use of yeast in multiple fermentation cycles is commonly known as serial repitching. The viability and vitality of the yeast can deteriorate during serial repitching and the number of times a yeast is reused in fermentations is dependent on brewery processes[65].

Bottle conditioning in beer

Bottle conditioning is a widely used process to naturally carbonate the beer. This process involves the addition of yeast and fermentable extract, also known as priming sugar, to the so called 'green' beer prior to filling[66]. The yeast performs a secondary fermentation for a period of 2 – 4 weeks at ambient temperature prior to distribution. The fermentation process results in the consumption of excess oxygen by the yeast and the production of ethanol and CO_2. Fermentation in bottle conditioning can be challenging for yeast as beer is a poor environment for cellular growth due to its low pH and oxygen, high ethanol, and poor nutrients[66]. Despite this, bottle conditioning has been shown to provide flavour stability by decelerating the formation of ageing compounds and thereby increasing the shelf-life of the beer[67]. During bottle conditioning the yeast acts as an oxygen scavenger and minimises the production of oxygen-dependent off-flavours[68,69]. Aldehydes in beer are associated with off-flavours and during bottle conditioning are reduced by yeast into less flavour-active alcohols[66,70]. The positive effect yeast exerts on beer during bottle conditioning is reliant on the yeast maintaining good physiological condition for an extended period of time. The autolysis of yeast results in the secretion of compounds such

as fatty acids[71] and proteolytic enzymes[72] into the beer, ultimately leading to changes in its sensory profile and characteristics[66].

Factors affecting bottle conditioning in beer

The quality and consistency of the bottle conditioning process is primarily dependent on the 1) fermentable extract, 2) yeast strain, and 3) production process utilised.

The addition of a fermentable extract to the beer is required during bottle conditioning to increase the alcohol and CO_2 content. Commercially available extracts for bottle conditioning exists as syrups and granulated sugars[73]. However, granulated sugars are not typically used as they are more prone to microbial contamination. The type of fermentable extract used can have an influence on the final mouthfeel of the product, with extracts containing simple sugars (glucose, fructose, or sucrose) having a higher fermentability, producing a beer with a low residual extract content and a 'drier' beer[74]. It has been shown that the use of maltose and maltotriose, as the fermentable extract, results in negligible fermentation, and was strongly dependent on the yeast strain and beer type[74,75].

The ability of yeasts to withstand the harsh conditions of bottle conditioning is expected to be strain dependent. However, studies that assess the viability of different yeast strains during bottle conditioning in beer are limited[74-76]. It was observed that the viability of the yeast strains varied, with some showing a decrease after 1 month, whilst others maintained their viability for 3-4 months[76]. Ideally, a brewer would select the yeast strain that exhibits superior longevity. However, other characteristics such as their aggregation and sedimentation (flocculation) ability and aroma formation are also strain dependent[74,77]. This makes brewers hesitant to choose a different yeast for bottle conditioning, as a change in aroma profile will alter the characteristics of the beer and potentially damage brand reputation with the consumer.

Yeast for bottle conditioning can be produced in different ways. Yeast from primary fermentation can be harvested and used for bottle conditioning[76]. However, as this is usually cropped and re-used in multiple fermentations as described above, it can cause deterioration of the yeast[78]. It was observed that this can lead to the secretion of intracellular compounds into the surrounding medium and impact the final characteristics of the beer[79]. Moreover, there is a higher risk of microbial contamination when using

yeast from primary fermentation compared to freshly propagated yeast due to the increased number of handling steps and serial repitching.

Active dry yeasts (ADY) are an attractive alternative to yeast used in main fermentation or freshly propagated yeast as they can be stored conveniently, be prepared rapidly, and have a long shelf-life[80]. It has been shown that rehydration of ADY has a direct impact on their viability and therefore the final product[80]. Recent studies have shown that optimal rehydration conditions and adaption to beer types can improve yeast viability[81,82].

In breweries, the preferred production method of yeast for biomass is the use of a propagation plant[56]. The advantage of using a propagation process is that yeast cells can be pre-adapted to the conditions experienced in the beer, with a recent study showing that yeast adapted to high ethanol and acidic conditions enabled the yeast to more efficiently perform bottle conditioning[83]. Additionally, as the yeast is propagated regularly, it ensures yeast of the same quality is used in each bottle, improving batch-batch consistency.

Sources of stress in the brewery

Yeast cells need to maintain an optimal balance of internal conditions for cellular growth and function. The ability to respond to environmental perturbations and maintain cellular homeostasis is critical to cell survival[84]. Industrial brewing practices expose yeast to a range of sub-optimal conditions that includes high ethanol, cold shock, nutrient deprivation, and oxidative and osmotic stress[65]. Exposure of yeast cells to these conditions can lead to many physical and metabolic perturbations and consequently lead to a decrease in yeast performance, impacting the characteristics of the final product.

The environmental stress response

Yeast cells respond to stress by the upregulation of different response mechanisms that allow a rapid adjustment of cellular resources[65]. The cumulative response to adverse environmental conditions that involves many common genes in *S. cerevisiae* is known as the environmental stress response (ESR). Genomic studies of the ESR in *S. cerevisiae* estimated that it results in a common response in approximately 10% – 14% of the yeast genome[85,86]. It was found that the expression of ~300 and ~600 genes were induced and repressed, respectively, in response to varying stress, corresponding to ~14% of the yeast genome[86]. Upregulated genes in the ESR were involved in carbohydrate metabolism,

energy generation, and cellular stress, whereas genes involved in cellular growth, ribosome and nucleotide biosynthesis were repressed[86].

Many of the genes implicated in the ESR contain a common stress response element (STRE, pentameric core of CCCCT) in their upstream-regulatory DNA region. The STRE was first identified in the promoter of cytosolic catalase T (*CTT1*) and a DNA damage-responsive gene (*DDR2*)[87,88]. Subsequently, the expression of genes in the ESR were found to be coordinated through the expression of two partially redundant zinc finger transcription factors, multicopy suppressor of SNF1 mutation proteins 2 and 4 (Msn2p and Msn4p)[89]. These transcription factors are regulated by multiple nutrient signalling pathways including cAMP-dependent protein kinase A (PKA)[90] and target of rapamycin (TOR)[91]. Msn2p and Msn4p are maintained in the cytosol under non-stressed conditions and are rapidly shuttled into the nucleus in response to stress[90]. The activation of the ESR enables yeast to rapidly respond to external perturbations and maintain cellular homeostasis[92].

Nutritional stress

Carbohydrate metabolism

Typical wort sugars are sucrose (1% – 2%), fructose (1% – 2%), glucose (10% – 15%), maltose (50% – 60%), maltotriose (15% – 20%) and dextrins (20% – 30%)[44]. There is a preference for uptake of sugars from the wort in *S. cerevisiae* and this depends on diffusion rates, intracellular localisation of relevant enzymes, and gene regulation (Figure 5)[44].

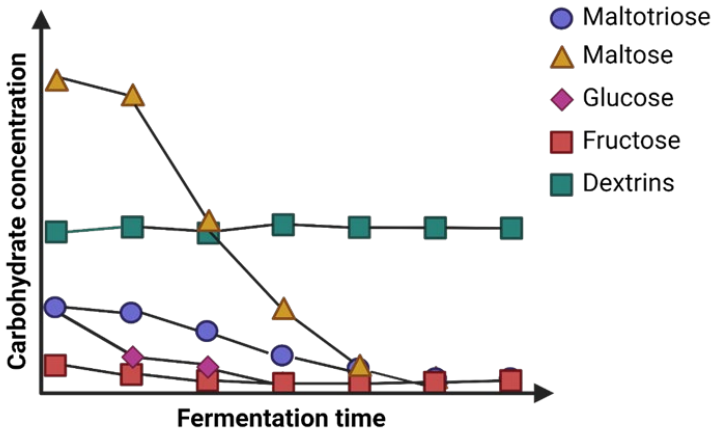


Figure 5. Carbohydrate utilisation during fermentation of standard brewery wort. Glucose is typically consumed first, followed by fructose, and maltose and maltotriose. Figure adapted from[44]. Created in Biorender®.

For example, sucrose is hydrolysed first into the monosaccharides, glucose and fructose, by the enzyme invertase situated in the periplasmic space of the cell wall[93]. Glucose and fructose are subsequently transported into the cell by facilitated diffusion through multiple hexose transporters[94]. In contrast, maltose and maltotriose require energy expenditure for uptake by the cell, which is mediated by the expression of the maltose transport system, which is encoded by five unlinked *MAL* loci[95]. Interestingly, the *MAL* genes are subject to repression by glucose such that metabolism of maltose and maltotriose only occurs after glucose levels decline[96]. The depletion of fermentable carbohydrates in the wort results in the synthesis of the carbohydrates, glycogen and trehalose, which are used in the cell as a source of carbohydrate during nutritional stress and to protect the cell from environmental perturbations, respectively[97].

Nitrogen metabolism

Nitrogen is an important nutrient for yeast growth, with inadequate nitrogen supply leading to poor yeast growth and stalling fermentations[98]. It has been well established that the assimilation of FAN from wort in brewing occurs in a specific order (Table 2)[99]. The mechanism by which yeast regulate nitrogen metabolism is complex and a result of various integrated mechanisms such as the Ssy1p/ Ptr3p/ Ssy5p (SPS) sensor[100], TOR[101], nitrogen catabolite repression (NCR)[102], and the general amino acid control (GAAC)[103] pathways.

Table 2. Classification and speed of assimilation of nitrogenous compounds from wort during fermentation. (Reproduced from[104]).

Group A (Immediately assimilated)	**Group B (Gradually assimilated)**	**Group C (Assimilated after Group A)**	**Group D (Little or no assimilation)***
Glutamic acid	Valine	Alanine	Proline
Aspartic acid	Histidine	Glycine	
Asparagine	Tryptophan	Ammonia	
Glutamine	Tyrosine		
Serine	Phenylalanine		
Threonine			
Lysine			
Arginine			
Methionine			
Isoleucine			
Leucine			

*Cysteine or cysteic acid has not been detected in wort

Although *S. cerevisiae* can grow on various nitrogen sources, they do not equally provide the nutrients necessary to promote high growth rates[105]. For example, in the presence of preferred nitrogen sources such as glutamic acid and glutamine, yeasts activate the NCR pathway to repress the expression of genes for less preferred nitrogen sources[105,106]. It has been shown that nitrogen limitation during propagation leads to defects in wort attenuation during subsequent fermentations[63]. Attenuation in brewing is the amount of sugar the yeast consumes during fermentation. Additionally, the consumption of FAN has a direct impact on the final sensory profile of the beer[107].

Ethanol stress

The primary purpose of fermentation in brewing is to produce ethanol from sugars. During brewery fermentations, the concentration of ethanol increases and results in the yeast cells being exposed to increasing concentrations of ethanol. Bottle conditioning results in the immediate exposure of yeast cells to elevated concentrations of ethanol that could affect their viability. During standard brewing, ethanol concentrations can range between 3% – 6% (v/v) but during high- and very high gravity brewing, ethanol concentrations can exceed 10% (v/v)[108]. Ethanol has been shown to inhibit cell growth and viability[109], perturb protein function[110], and reduce fermentation rates[111].

Ethanol primarily impacts yeast physiology at the plasma membrane[112,113]. Ethanol can permeabilise the plasma membrane by altering the interaction and position of lipids and proteins, leading to a passive influx of protons and cytosolic acidification[114]. *S. cerevisiae* has been shown to respond to increased ethanol concentrations by altering the UFAs and ergosterol content in the plasma membrane[115-117]. Oleic acid (Δ^9Z-$C_{18:1}$) and palmitoleic acid (Δ^9Z-$C_{16:1}$) are the primary mono-UFAs found in the plasma membrane and it was observed that an increase in oleic acid was important for overcoming the toxic effects of ethanol[115]. Additionally, it has been shown that yeast increases the activity of plasma membrane H^+-ATPase activity, to pump protons from the cell and restore intracellular pH levels[118].

Ethanol can also perturb protein folding and lead to the aggregation of proteins within the cell[113]. It has been found that heat shock proteins (HSPs) are upregulated in response to ethanol exposure to overcome this[119]. HSPs mainly act as chaperones to ensure the proper folding or refolding of nascent or denatured proteins to maintain their function[120]. For example, Hsp104p, Hsp70p, and Hsp26p were upregulated above 4% ethanol[121].

Additionally, upregulation of trehalose synthesis has been shown to assist in coping with ethanol stress[122].

To overcome the toxic effect of ethanol, it has been shown that pre-adaptation of yeast to mild ethanol stress increases their ability to adapt to subsequent ethanol stress[123]. Therefore, exposure of yeast cells during propagation to ethanol may reduce ethanol shock during bottle conditioning and improve their longevity during long-term storage.

Osmotic stress

During brewing, yeast cells are exposed to high concentrations of sugar at the beginning of propagation and fermentation[65,124]. Hyperosmotic stress can lead to water efflux and cell shrinkage[125] and as a consequence a decrease in cellular growth and viability[126,127].

How *S. cerevisiae* responds and adapts to osmotic changes by increasing the glycerol concentration of the cells and decreasing glycerol permeability via the High Osmolarity Glycerol (HOG) [mitogen-activated protein kinase (MAPK)] pathway (for comprehensive reviews see[128,129]) is now understood in greater detail. The HOG pathway is mediated through two branches that contain the transmembrane osmosensors, Sln1p (Osmosensing histidine protein kinase SLN1) and Sho1p (High osmolarity signaling protein SHO1) (Figure 6)[130]. Activation of these sensors leads to activation of the downstream MAPK signalling pathway and phosphorylation of the transcription factor, Hog1p (MAPK)[128].

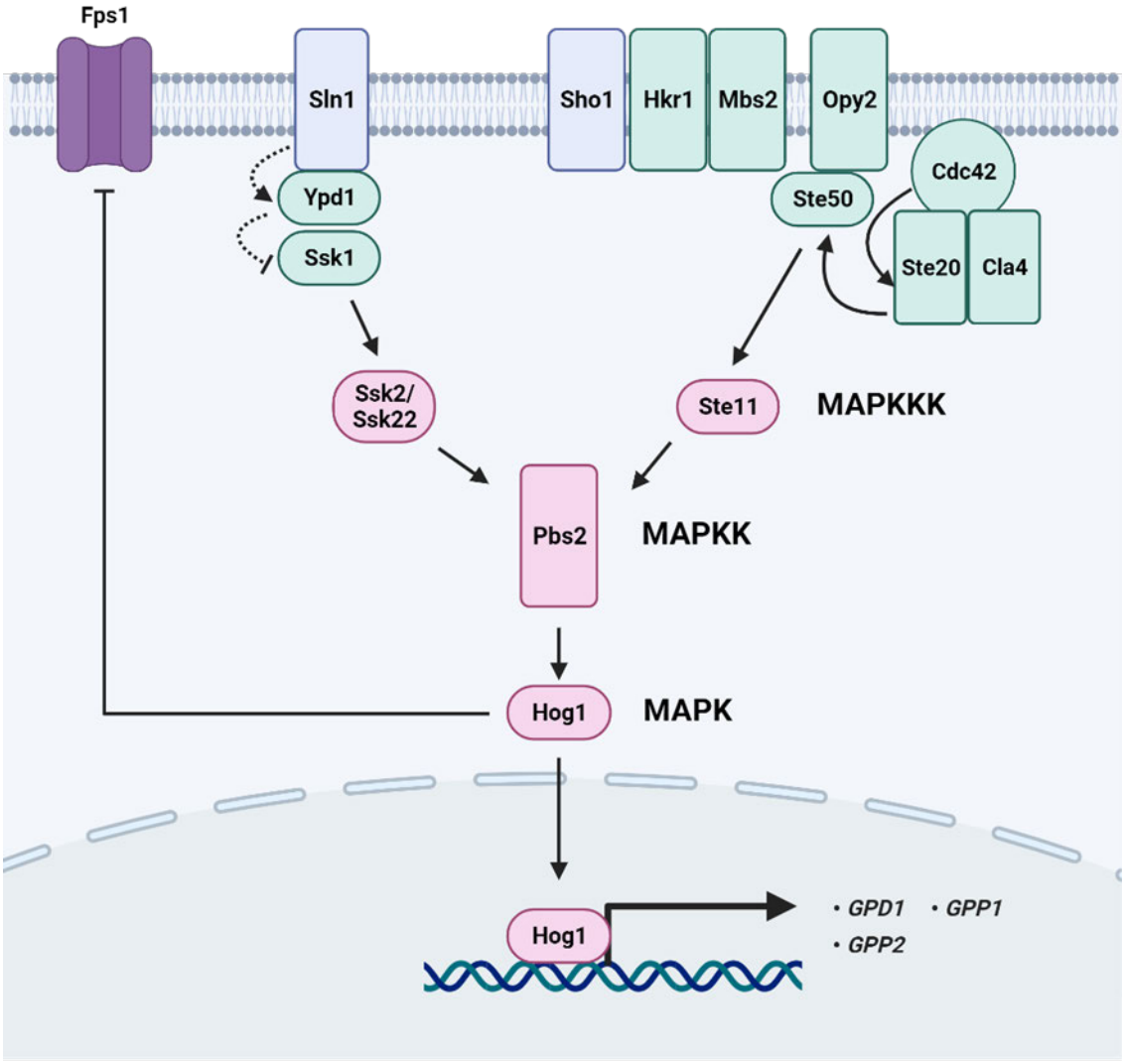


Figure 6. Response of *Saccharomyces cerevisiae* after changes in external osmolarity through the high osmolarity glycerol (HOG) pathway. The HOG pathway has two independent upstream osmosensors, Sln1p and Sho1p. This results in activation of the mitogen-activated protein kinase (MAPK) cascade consisting of Ssk2p/Ssk22p or Ste11p (MAPKKK), Pbs2p (MAPKK), and Hog1p (MAPK). Figure was adapted from[131]. Created in Biorender®.

Activated Hog1p is partially translocated into the nucleus and acts as a transcription factor for target genes[132]. For example, Hog1p results in the upregulation of NAD-dependent glycerol-3-phosphate dehydrogenase (*GPD1* and *GPD2*). Gpd1p and Gpd2p are enzymes in *S. cerevisiae* involved in the synthesis of glycerol from dihydroxyacetone phosphate (DHAP), an intermediate of glycolysis[132]. Glycerol is a small molecule that *S. cerevisiae* uses to overcome changes in osmolarity[133]. Hog1p also prevents glycerol export by phosphorylating Rgc2p glycerol channel regulator which in turn phosphorylates the

aquaglycoporin, Fsp1p which inhibits secretion of glycerol into the external environment[134].

Sugar in wort and increased ethanol concentration can both cause osmotic stress[124]. It was shown that high osmolarity primarily impacted cell membrane fluidity and resulted in a decrease in viability and vitality, with stationary phase cells more resistant to changes in osmolarity[124].

Oxidative stress

S. cerevisiae is a facultative anaerobe and can therefore utilise anaerobic and aerobic metabolisms. A supply of oxygen during brewery propagations and at the start of fermentation is required to enable the biosynthesis of UFAs and sterols, which are required for yeast growth during anaerobic conditions[135]. These UFAs and sterols that are formed during aerobic growth are diluted amongst daughter cells during anaerobic fermentation, with growth ceasing if the sterol content drops below 0.1%[49,135]. The aeration regime selected by brewers is critical to produce a consistent product as under-aeration can lead to reduced cell growth, viability, and beer flavour changes, whilst over-aeration can lead to toxic effects from reactive oxygen species (ROS)[136].

Oxidative metabolism results in the generation of ROS such as superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxy anions (OH^-). ROS can lead to the oxidation of lipids, proteins, and DNA[137]. The main pathway for eliciting an oxidative stress response in yeast is well understood and involves Yap1p (bZip transcription factor) (Figure 7)[138]. For example, in non-stressed conditions Yap1p is located within the cytoplasm of the cells by exportin 1-mediated (Crm1p) nuclear export. However, elevated levels of H_2O_2 lead to accumulation of Yap1p in the nucleus[138]. This is due to its nuclear export signal being masked through the action of glutathione peroxidase (GPx)-like enzyme (Gpx3p or Orp1p), allowing its translocation via importin subunit beta-3 (Pse1p)[138]. In the nucleus, Yap1p acts as a transcription factor for multiple genes associated with oxidative stress, such as glutamate-cysteine ligase (*GSH1*), glutathione reductase (*GLR1*), and glutathione peroxidase (*GPX1* and *GPX2*)[139]. A second transcription factor, Skn7p, is constitutively nuclear and acts synergistically with Yap1p to upregulate expression of oxidative stress genes such as thioredoxin 2 (*TRX2*) and thioredoxin reductase (*TRR1*)[140]. As ROS levels subside Yap1p is reduced by thioredoxin and Yap1p translocates into the cytoplasm in its inactive state[138].

Although ROS has been linked to cellular ageing[141], it has been well established that mitochondria and therefore oxidative respiration have an important role in the extension of yeast chronological lifespan[142]. More specifically, that activation of mitochondrial respiration above a certain threshold induces stress resistance of yeast cells[143]. Therefore, propagation of yeast in aerobic conditions (such that oxidative respiration occurs) may increase its ability to withstand stress and improve longevity in downstream brewery processes.

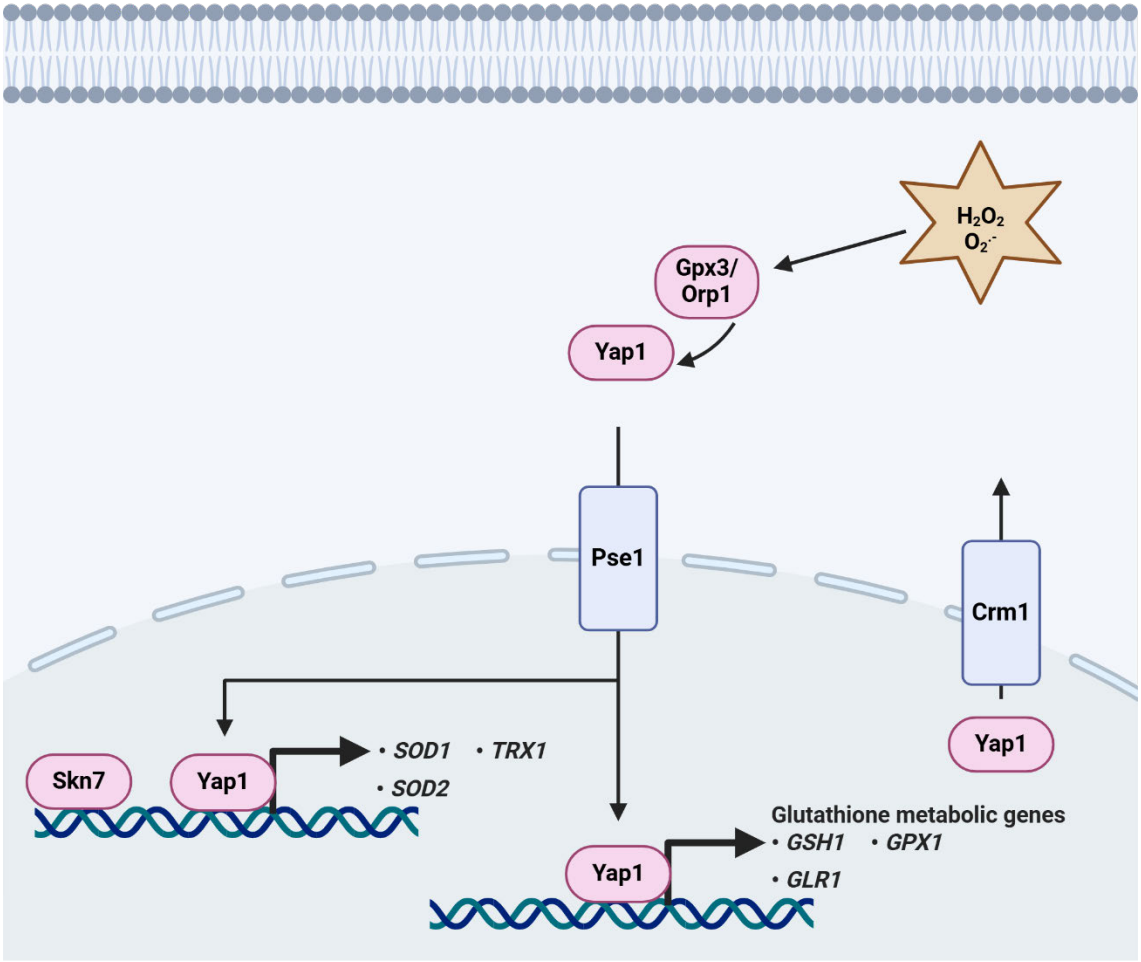


Figure 7. Oxidative stress response of *Saccharomyces cerevisiae*. Exposure to oxygen, ethanol, and high sugar can lead to the generation of reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). *S. cerevisiae* upregulates enzymatic and non-enzymatic detoxification through the Yap1p (bZip transcription factor) pathway. Yap1p is exported from the nucleus by exportin-1 (Crm1p). Increased oxidative stress leads to the nuclear export signal of Yap1p being masked by Gpx3p (glutathione peroxidase (GPx)-like enzyme or Orp1p). Yap1p is translocated into the nucleus of the cell by the importin subunit beta-3 (Pse1p), where it binds to its target genes and upregulates their expression. Figure adapted from[112]. Created in Biorender®.

Storage carbohydrates in yeast during the brewing process

Glycogen

Glycogen is a branched glucose polysaccharide containing α-(1,4) and α-(1,6) glycosidic linkages and is a major storage carbohydrate in yeast cells[97]. It can accumulate up to 20% – 30% of the dry weight of yeast at different stages of growth[144]. At the beginning of

fermentation, glycogen reserves are rapidly depleted to be used for sterol biosynthesis[145]. Glycogen then accumulates in yeast cells during the later stages of fermentation when glucose concentrations are reduced by 50% in wort[144,145]. Subsequent dissimilation of glycogen occurs during nutrient limitation and long-term storage of yeast[145]. The monitoring of glycogen in yeast during brewing is important as it has been linked to the yeast's ability to maintain high viability and vitality as well as their fermentation performance following storage[144,146,147].

The metabolism of glycogen in yeast is complex and mediated through multiple major nutrient sensing pathways such as PKA and TOR (for comprehensive reviews see[97,148,149]). The major enzymes involved in glycogen synthesis are Glg1p and Glg2p (glycogenin), Ugp1p (uridine diphosphate-glucose (UDP-glucose) pyrophosphorylase) and Gsy1p and Gsy2p (glycogen synthase) (Figure 8)[148]. Initially, glucose in the cell is converted to glucose-6-phosphate (glucose-6P) and then glucose-1P by Hxk2p (hexokinase) and Pgm1p (phosphoglucomutase 1), respectively. UDP-glucose is subsequently synthesised from glucose-1P by Ugp1p and is utilised by Glg1p/ Glg2p and Gsy1p/ Gsy2p[148]. As UDP-glucose is also required for cell wall β-glucan synthesis, Ugp1p localisation is controlled through phosphorylation[150]. Phosphorylation of Ugp1p leads to localisation of the enzyme to the plasma membrane and preferential use of UDP-glucose for β-glucan synthesis[150]. Initiation of glycogen synthesis occurs through Glg1p and Glg2p which are self-glucosylating initiator proteins[151]. These proteins provide an oligosaccharide primer that enable Gsy1p/ Gsy2p to synthesise glycogen[151]. The major isoform, Gsy2p, catalyses the synthesis of the α-(1,4) linkages from the oligosaccharide primer and Glc3p (glycogen branching enzyme) synthesises α-(1,6) glycosidic linkages[152]. Gsy2p is inhibited by phosphorylation; however, the effect of phosphorylation can be overcome by the binding of glucose-6P, an allosteric activator of the enzyme[153]. Glycogen is hydrolysed by Gph1p (glycogen phosphorylase) through the removal of glucose-1P from the nonreducing ends of the α-(1,4) chains[148]. To allow further break down of glycogen, Gph1p requires Gdb1p (glycogen debranching enzyme) to convert the α-(1,6) branch points to α-(1,4) linkages[149]. Gph1p activity is activated by phosphorylation[154].

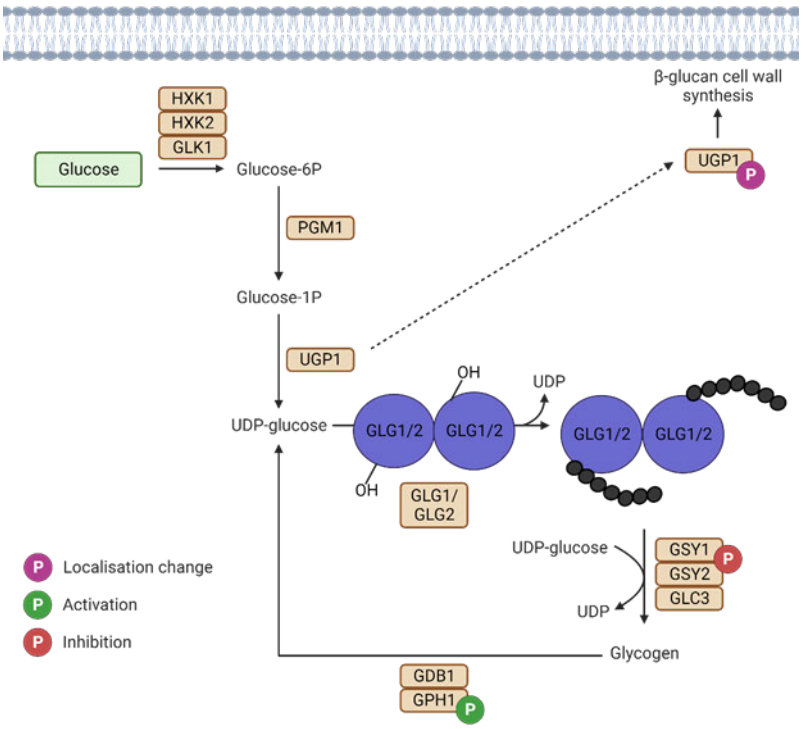


Figure 8. Pathway of glycogen metabolism in *Saccharomyces cerevisiae*. Glucose is phosphorylated to glucose-1P and subsequently into UDP-glucose by Upg1p. Glycogenin is an initiator protein that utilises UDP-glucose to synthesise an oligosaccharide primer. This allows Gsy1p and Gsy2p to extend α-(1,4) linkages and Glc3p to synthesise α-(1,6) branch points. Gph1p and Gdb1p degrade glycogen into UDP-glucose. Figure adapted from[148]. Created in Biorender®.

Trehalose

Trehalose is a non-reducing disaccharide comprised of two glucose moieties linked via an α-(1,1) linkage[97]. Trehalose content in yeast cells varies significantly according to growth and environmental conditions[149]. The accumulation of trehalose only occurs after glucose has been consumed and in response to different types of cellular stress including heat shock[155], high ethanol concentrations[156], oxidative stress[157], desiccation[158], and entry into stationary phase[144]. The differential accumulation pattern of trehalose compared to glycogen indicates that each carbohydrate has a specific function within the cell. Trehalose has been implicated in fermentation performance, with higher trehalose levels being shown to have higher cell viability upon pitching for primary fermentation[159]. This suggests that seeding yeast for bottle conditioning that contains a higher trehalose content may promote yeast longevity.

The synthesis of trehalose is catalysed by the TPS (trehalose-6-phosphate synthase) and TPP (trehalose-6-phoshate phosphatase) complex (Figure 9)[160]. TPS catalyses the

synthesis of trehalose-6 phosphate (trehalose-6P) from glucose-6P and UDP-glucose and TPP dephosphorylates trehalose-6P to trehalose. The complex is comprised of four subunits that are encoded by the *TPS1*, *TPS2*, *TSL1*, and *TPS3* genes[97]. *TPS1* and *TPS2* are the catalytic domains of TPS and TPP, respectively, and *TSL1* and *TPS3* are the regulatory domains[160,161]. During normal conditions, the TPS/ TPP complex does not form as Tsl1p is not synthesised[162]. Under stress conditions, there is an increase in Tsl1p abundance, leading to the formation of the TPS/TPP complex and synthesis of trehalose[162]. During stress recovery, Tps3p is phosphorylated and inhibits Tps2p activity[163]. This results in an accumulation of trehalose-6P that allosterically inhibits Tps1p[162,163]. Trehalose can be hydrolysed in the cell through the action of two enzymes, Nth1p (neutral trehalase 1) and Ath1p (acid trehalase 1)[163]. Nth1p is located in the cytoplasm and functions optimally at neutral pH, whereas Ath1p is suggested to be located in the vacuole and the cell surface and functions optimally in acidic conditions[164,165]. Nth1p activity is controlled through phosphorylation on multiple sites by multiple pathways and is most active during the early stages of cellular growth[165,166].

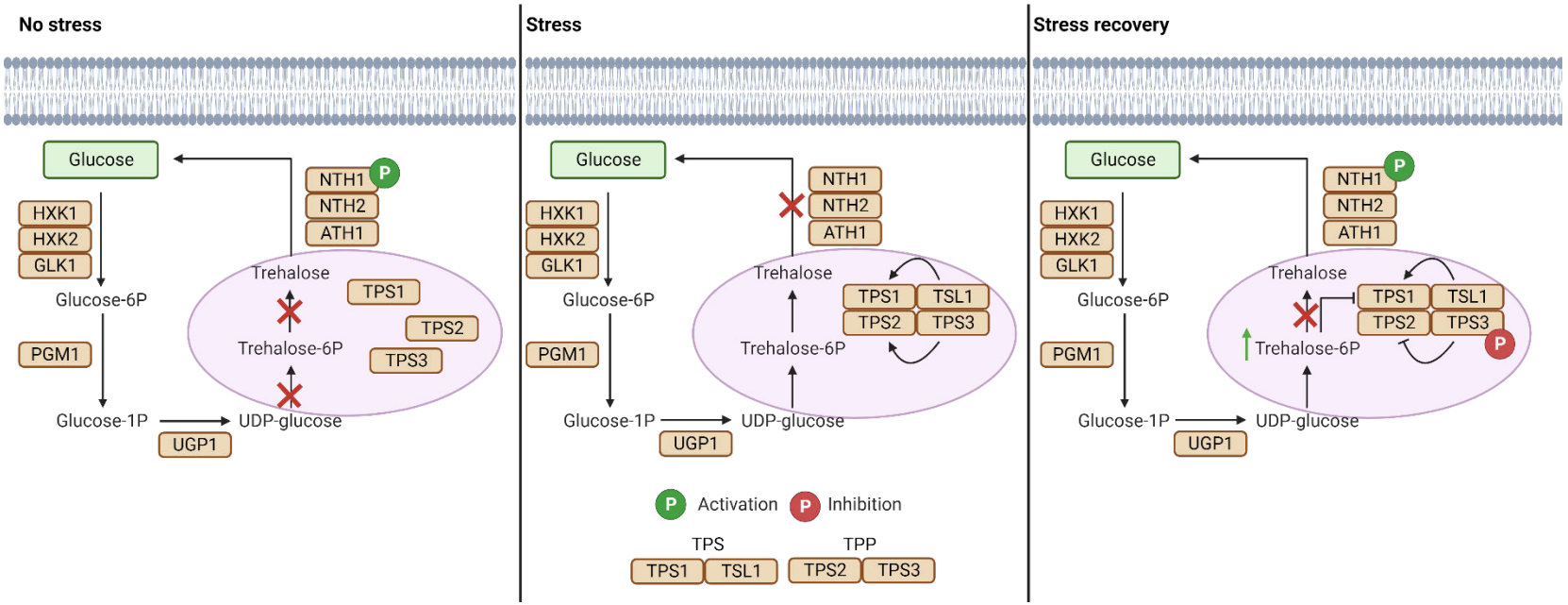


Figure 9. Pathway of trehalose metabolism in *Saccharomyces cerevisiae*. During normal cellular conditions, the trehalose phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) is not completely formed and trehalose is not synthesised. TPS catalyses the synthesis of trehalose-6P from glucose-6P and UDP-glucose and TPP dephosphorylates trehalose-6P to trehalose. *TPS1* and *TPS2* are the catalytic domains of TPS and TPP, respectively, and *TSL1* and *TPS3* are the regulatory subunits of TPS and TPP, respectively. Stress conditions lead to an increase in abundance of Tsl1p, association of the complex, and trehalose synthesis. During stress recovery, the Tps3p subunit is phosphorylated and leads to inhibition of Tps2p. There is an accumulation of trehalose-6P that allosterically inhibits Tps1p. Trehalose is then degraded through the action of Nth1p. Figure adapted from[162]. Created with BioRender.com.

Methods for the assessment of yeast quality in brewing

The ability to routinely monitor and further characterise physiological changes of yeast during the brewing process is highly valuable. Monitoring yeast quality during brewing can enable process improvements, ensuring a high-quality product and reducing costs. The overall quality of yeast during brewing processes should be determined using a combination of viability and vitality techniques[167].

Cell Viability

Cell viability refers to the percentage of live cells in a whole population that are able to grow and reproduce[78]. There are several methods available that allow viability determination. Growth in liquid (or on solid media), capacitance, zone of growth inhibition and spot tests have the advantage of being low cost and easy to use, however, they do require extensive time to culture the yeast[168]. This led to the development of alternative methods to monitor yeast viability, which involve colorimetric and fluorescent stains.

Agar plating

Agar plating is an easy and cost-effective method to determine yeast health. Traditionally, the spread-plate technique involved diluting the yeast to various unknown concentrations and then spreading a known amount of diluted yeast onto the agar plate[167]. The number of colony forming units (CFU) are counted after incubation and then the concentration adjusted by multiplying by the dilution factor. Furthermore, the optimal dilution is usually plated in triplicate to ensure robust counting. A modern version of agar plating is called 'spot plating' which enables six 10-fold dilutions to be applied to a single agar plate, increasing throughput[169]. Another adaptation of this method involves spiral plating where accurate volumes of yeast are added in decreasing volumes in an Archimedes spiral onto the surface of a rotating agar plate. The plate is then incubated and the CFU determined[167]. This plating method is easy to implement in breweries and cost-effective. The main drawback of this method is the time required to enumerate colonies (> 24 h).

Capacitance

A capacitance probe can be used as an offline or online method for determining yeast viability in a brewery[170]. Yeast cells when they have an intact membrane are non-conducting and can build charge in the presence of an electric field. In contrast, cells that

have a compromised membrane are not able to build charge and are unable to contribute to the capacitance signal. The advantage of this method is that it is rapid (<1s) and other components within the media such as trubs and gas molecules do not interfere with the reading. The main disadvantage of this method is that it must be performed with other methods that allow total cell concentration determination as the capacitance only detects viable cells. Additionally, this method may underestimate cell viability as damaged membranes can be repaired by the cell.

Colorimetric and fluorescent dyes

There are two categories of dyes commercially available that are differentiated based on their mechanism of action: cell membrane dependent and independent dyes.

Cell membrane dependent dyes

At physiological pH, these dyes are negatively charged and unable to penetrate the intact cell membrane. As the cellular membrane deteriorates it becomes compromised and the dye can readily diffuse into the cell, leading to staining of the cytoplasm or nucleus. Commonly used dyes include trypan blue[171], ethidium bromide[172], and propidium iodide (PI)[173].

PI is a nucleic acid membrane integrity stain that can only enter cells with a compromised cell membrane to detect non-viable cells[173]. Acridine (AO) is a cationic nucleic acid stain that can enter viable and non-viable cells. The dual staining of non-viable yeast cells with AO and PI results in the fluorescence emission from AO being transferred to PI due to fluorescence energy resonance transfer (FRET)[173]. This results in viable and non-viable cells staining green and red, respectively. The dual use of these stains is advantageous in the brewing industry as it overcomes the interference of debris and non-specific particles[174]. Furthermore, this method can be performed on commercially available and easy-to-use automated cell counters such as the Cellometer X2™. This is advantageous as it eliminates the user-to-user variability, and it more accurately measures the viability of yeast cells with lower viability compared to other stains such as methylene blue (MB). The main disadvantage of this methods is that initial cost investment required for a flow cytometer or automated cell counter, which can be cost inhibitory for smaller breweries.

Cell membrane independent dyes

Cell membrane independent dyes do not readily enter live and dead cells. MB is an industry standard dye that involves the exclusion or reduction of the dye into a colourless

product, whilst dead cells are stained blue[175]. Therefore, despite MB being considered a viability measurement, it also measures vitality[176]. This method can result in an overestimation of cell viability when the proportion of live cells decreases (<90%) and potentially have poor correlation with fermentation performance[176].

Cell vitality

Methods that measure yeast vitality are based on monitoring different physiological and metabolic aspects of the cell[78]. These tests include the acidification power test[177], intracellular ATP content[178], staining with rhodamine 123[179], and enzymatic activity assays such as esterases staining with fluorescein diacetate (FDA)[180].

Acidification power test

The acidification power test is a simple method that has been used by brewers to predict a successful fermentation[177]. It measures the decrease in extracellular pH of a yeast suspension after the addition of glucose. The decrease in pH after the addition of glucose occurs as it is metabolised by the yeast and leads to the excretion of protons into the beer and a concomitant decrease in pH[181]. This method is well suited to detecting large differences in yeast vitality, however, it requires multiple time points to be analysed[182]. Additionally, it is time intensive as it requires extensive washing of the cells, and the presence of intracellular carbohydrates can make the output ambiguous.

Intracellular ATP content

The measurement of intracellular ATP content has recently been demonstrated to be a sensitive indicator of yeast cell physiology[178]. This assay requires ATP extraction from cells and its reaction with luciferin to produce a bioluminescent signal[183]. It was found that the ATP content of yeast decreased as the length of yeast storage increased, despite no changes in cellular viability, indicating that ATP content reflected declining cell vitality. However, this method can be cost inhibitory for many breweries.

Enzymatic stains

Fluorescein diacetate

FDA is a non-fluorescent product, is taken into the cell and is cleaved in the cell by intracellular enzymes to fluorescein, a fluorescent product[184]. To improve the sensitivity of the stain, a more stable derivative of fluorescein, carboxyfluorescein diacetate (cFDA), was developed and utilised in assays. It has been shown that cFDA could be used to

monitor changes in vitality of yeast during brewery fermentations[173]. The main disadvantage of this method is the initial investment cost required for a flow cytometer or automated cell counter, which can be cost inhibitory for smaller breweries.

Despite the various methods for determining cell vitality being available, their implementation in a brewery setting is limited, and no recommended vitality method exists.

Resazurin

Tetrazolium salts such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide) are a popular method for assessing cell physiology in a wide range of applications[185]. MTT is reduced to a violet-blue and water-insoluble product, formazan, by mitochondrial enzymes[185]. The formation of formazan crystals results in cell death and the requirement of a cell lysis and a formazan-dissolving step[186]. These additional steps reduce the speed of the assay. Furthermore, as cell death occurs, this assay cannot be used as a real-time assay.

Resazurin or Alamar Blue is a blue dye that is non-toxic and routinely employed as a biochemical assay for measuring cellular metabolism[187]. Resazurin has been used in a wide range of applications such as food contamination[188], cellular cytotoxicity and activity[187], and drug discovery[189]. Additionally, it has been used across various species including bacteria[188], fungi[190], plants[191], and mammalian cells[192].

The measurement of cell vitality involves the addition of resazurin to cells, its uptake into the cell and subsequent reduction to resorufin, a highly fluorescent pink product (Figure 10)[187]. Resorufin can be further metabolised to dihydroresorufin, a non-fluorescent product. The reduction of resazurin is mediated by multiple intracellular enzymes in the cytosol and mitochondria[193].

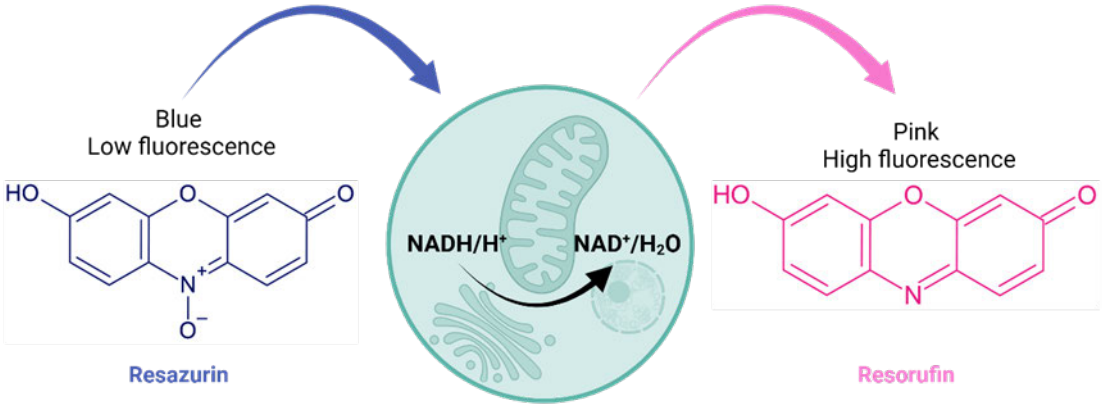


Figure 10. Resazurin reduction to resorufin in cells. Resazurin (weakly fluorescent, blue) is converted to resorufin (highly fluorescent, pink) by reducing enzymes within the cell. Created with BioRender.com.

The main advantages of resazurin as a cell vitality marker is that it is inexpensive, rapid, non-toxic, and can be measured using both colorimetry and fluorimetry. Furthermore, it could be used for a range of brewery applications such as cellular proliferation and toxicity.

"Omics" technologies for evaluating yeast metabolism

Overview of technologies

The term "omics" can be used to describe the quantification of entire sets of biological molecules[194]. For example, genes (genomics), mRNA (transcriptomics), proteins (proteomics), and metabolites (metabolomics) and their context within a biological system fall under the term "omics". The central dogma of molecular biology was that information in the cell flows unidirectional from genes to transcript to proteins[195]. Therefore, each of these tools provides a snapshot of the cell at a given time.

Genomics involves the sequencing of an organism's genome and is an important tool for understanding their evolutionary history and similarities within different environments[196]. Furthermore, genomics has been used to determine the evolutionary adaptation of yeast to cellular stress within different contexts such as brewing[197]. However, not all genes are transcriptionally active in a cell or tissue at one time such that genomics is not sufficient to elucidate the functional phenotype.

Transcriptomics is the measure of mRNA within the cell and numerous studies have elucidated the regulation of transcription within the yeast cell[86]. Specifically, transcriptomics of yeasts has been a powerful tool for understanding the genetic

regulation of yeast metabolism during wine[198] and brewing processes[199]. Although transcriptomics is a powerful tool for understanding functional mechanisms, its use alone is insufficient for understanding biological systems[200]. This is due to a variety of post-transcriptional processes such as alternative splicing and PTM and subcellular environments (for example, subcellular domains have different chemical environments that affect proteins)[201]. Proteomics was used in this study to elucidate yeast responses to industrial brewing processes and is discussed in the following section.

Metabolomics is a measure of all metabolites within a biological system[202]. The metabolome complements other 'omics' technologies as it reflects gene expression within a specific environment[203]. Metabolomics has been useful within the beverage industry to inform brewers on quality aspects such as taste, colour and aroma[204]. For example, the formation of different volatile compounds at different stages of beer production and their impact on the final product was evaluated[205]. Metabolomics has also been utilised in conjunction with proteomics within the brewing context to show that enzymes for starch degradation in malting with sorghum and barley resulted in a different sugar profile[206]. The analysis of metabolites within yeast has been challenging as the metabolome is sensitive to the sampling regime and extraction method[207]. Nevertheless, metabolomics can provide complementary information to other 'omics' techniques.

Proteomics

The term proteome was proposed by Marc Wilkins at the symposium "2D Electrophoresis: from protein maps to genomes" in Siena, Italy in 1994 and was later published in 1995 to describe the total complement of the genome[208,209]. Proteomics is defined as the analysis of protein and peptide expression and their localisation, interactions, modifications, and functions within the cell[210]. Protein levels within cells are highly dynamic, as genes and the environment influence their abundance and activity[210]. The feasibility of analysing hundreds of proteins was discovered in the 1970s using two-dimensional gel electrophoresis (2-DE)[211]. Modern proteomics now utilises liquid chromatography (LC) prior to mass spectrometry (MS) to enable the identification of thousands of proteins[212]. The study of the yeast proteome during industrial brewery propagation and bottle conditioning will provide a more holistic understanding of the effect of these processes on yeast metabolism. Additionally, these findings could lead to process improvements and an improved final product.

Proteomic analysis can be applied using a bottom-up (also known as shotgun), middle-down, or top-down approache[213]. These three methodologies refer to the way proteins are analysed. A bottom-up approach means that after protein solubilisation, the proteins are unfolded and digested into peptides by an enzyme. The peptide mixture is then analysed and can be related back to the protein of origin. This method is favoured over other approaches as peptides can be more easily solubilised and separated prior to MS and the fragmentation of peptides for identification is more feasible[214]. The bottom-up approach is most commonly applied in proteomics for the identification of proteins and associated PTM from complex mixtures. In bottom-up proteomics, the main enzyme used for proteolytic digestion is trypsin which cleaves at the carboxy-terminal (C-terminal) of arginine and lysine residues. This makes these peptides amenable to standard chromatographic separation, peptide fragmentation, and search algorithms. However, the majority of tryptic peptides have a short length (56% of all generated peptides are ≤ 6 residues), and are usually not identified in MS[215]. This results in only a small part of the proteome being analysed, and the protein identification being inferred from a limited number of tryptic peptides in bottom-up proteomics[215]. This leads to difficulty in obtaining proteoform and PTM characterisation across all proteins.

Diverse forms of proteins are termed proteoforms and are derived from single protein-encoding genes due to mutations, RNA splicing, and PTM[216]. Top-down proteomics is a powerful tool used for the analysis of proteoforms and it involves the analysis of intact proteins without digestion. The accurate measurement of the intact molecular mass and subsequent controlled fragmentation in the gas phase is required[217]. The advantage of a top-down approach is that accurate protein identification and PTM localisation can be achieved as peptides are not used to infer the protein identification (bottom-up). Although increasing in popularity, top-down proteomics has many challenges such as protein solubility, dynamic range, and data analysis that have limited its use for large-scale proteomic analysis[218].

The limitation of analysing proteoforms in bottom-up proteomics and difficulties in analysing proteoforms larger than 50 kDa in top-down proteomics has led to the emergence of middle-down proteomics[219]. Middle-down proteomics combines the strengths of bottom-up and top-down proteomic workflows by analysing longer peptides (>3 kDa)[215]. This can be achieved through specific proteases, limited digestion strategies, or chemical cleavage[220]. This method increases the likelihood of obtaining proteoform

information that would be lost in bottom-up proteomics. Middle-down proteomic analysis has been limited to specific applications such as the characterisation of recombinant monoclonal antibodies[221] and histone modifications[222]. The application of middle-down proteomics in other research applications has not been fully explored and is currently limited in high-throughput applications[223]. The follow sections focus on bottom-up proteomic approaches as this workflow was utilised in this thesis.

Sample preparation

Sample preparation is a critical step to achieving reproducible results in bottom-up proteomic analysis as it can greatly affect the downstream processes. There are numerous bottom-up sample preparation strategies available and it generally consists of several steps: 1) cell lysis, 2) extraction and solubilisation of proteins, 3) denaturation of proteins, 4) enzymatic digestion, and 5) peptide clean-up (removal of salts)[224]. Traditional sample preparation strategies include in-gel and in-solution digestion (ISD)[225]. An ISD begins with sample lysis using detergents such as sodium dodecyl sulphate or sodium deoxycholate. Chaotropic agents, such as urea or guanidine hydrochloride, are also utilised to extract and solubilise the proteins[226]. Disulfide bonds on cysteine residues in the proteins are reduced with chemicals (e.g. dithiothreitol) and then alkylated (e.g. iodoacetamide) to disrupt disulfide bonds and protect free thiols, respectively. This also prevents the regeneration of peptides with disulfide bonds, which are more difficult to fragment and identify[225]. Detergents and salts commonly used during sample preparation are generally not compatible with downstream MS analysis[227]. The most common methods for their removal and/ or concentration of the proteins in proteomics is the use of acetone[228], trichloroacetic acid[229] or chloroform/ methanol precipitation[230]. Re-solubilisation of the protein is performed in an appropriate buffer such as ammonium bicarbonate or urea, with the latter requiring dilution prior to the addition of proteolytic enzyme(s). The incubation of proteins at high temperatures with urea can lead to enzyme denaturation and carbamylation of proteins and peptides through their reaction with isocyanic acid, the breakdown product of urea[231]. Trypsin is the most common enzyme utilised as it cleaves at the C-terminal of lysine and arginine residues, and leads to more efficient ionisation in the MS and promotes *y*-ion production during fragmentation[232]. The resulting peptide mixture is then desalted prior to analysis using LC-MS.

There have been numerous improvements to sample preparation methods for bottom-up proteomics to increase robustness and throughput. This includes a novel ISD strategy that

exclusively uses trifluoroacetic acid for protein extraction and solubilisation without the requirement of detergents called Sample Preparation by Easy Extraction and Digestion (SPEED)[233] as well as in-stageTip (iST)[234], filter-aided sample preparation (FASP)[235], Single-Pot Solid-Phase-enhanced Sample Preparation (SP3)[236], solvent precipitation SP3 (SP4)[237], and suspension trapping (STrap)[238]. These different strategies have been compared and it appears that there is no universal method that can be applied for all sample types[225,227,239].

Data acquisition

The abundance of peptides and their corresponding proteins can be determined using many different methods in a bottom-up approach and is dependent on the sample preparation and MS strategy utilised. This includes isobaric labelling of peptides[240,241], data dependent acquisition (DDA)[242], data independent acquisition (DIA)[243], and targeted methods (Figure 11)[244].

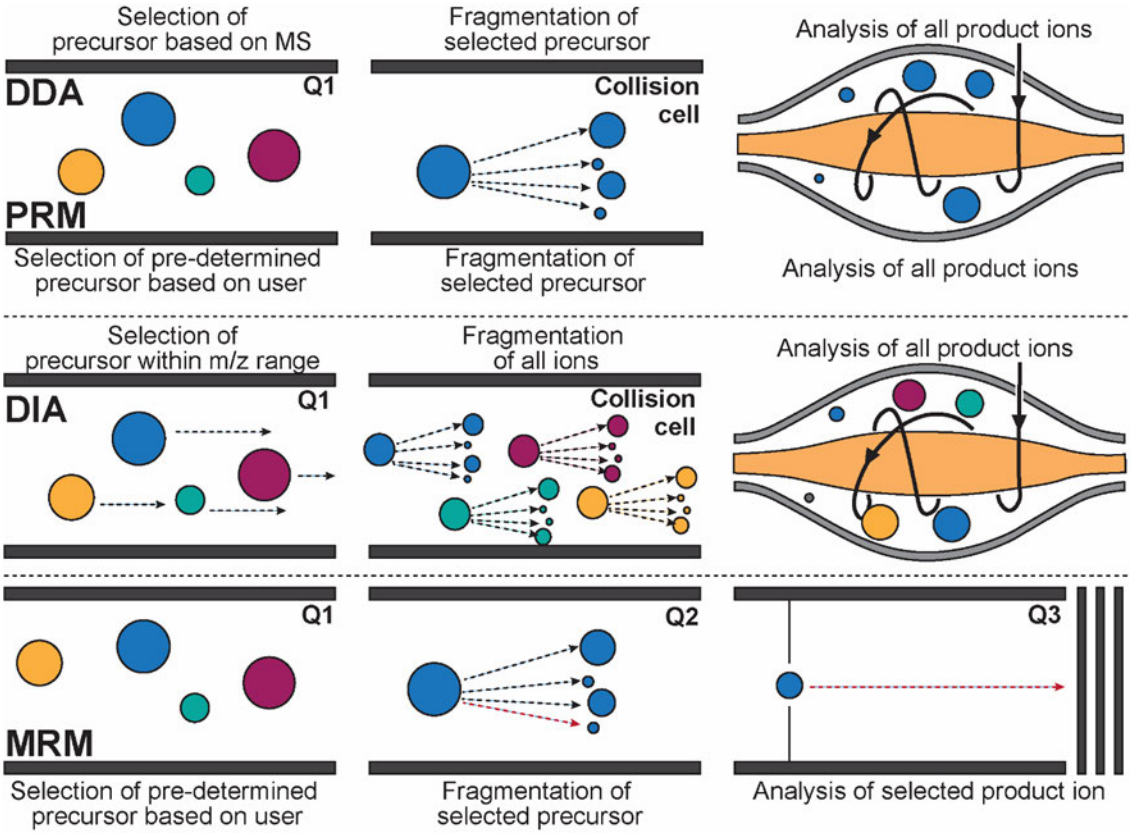


Figure 11. Schematic representation of different acquisition types by mass spectrometry. Data dependent acquisition (DDA): a mass spectrum (full scan or MS1) of all co-eluting ions (precursor ions) at a specific point in the LC-gradient is acquired. The MS selects a single precursor for fragmentation in the collision cell and the product ions are analysed in the analyser. Data independent acquisition (DIA): all precursor ions that are within a pre-defined mass-to-charge (*m/z*) and retention time (RT) window are filtered in quadrupole 1 (Q1), fragmented in the collision cell and the product ions analysed in the analyser at the same time (independent of the precursor). Parallel reaction monitoring (PRM): A specific precursor ion is filtered in Q1, fragmented in the collision cell and all the product ions are analysed in the analyser. MRM: the first and third Q act as mass filters for the predefined precursor ion and its product ions within a defined m/z and RT window, respectively. The precursor ions are fragmented in the second quadrupole (Q2). Figure adapted from[245].

Isobaric peptide labelling methods such as tandem mass tags (TMT)[240] or isobaric tags for relative and absolute quantitation (iTRAQ)[241] allows for accurate quantitation of peptides at the MS^2 (spectrum of ions produced from the precursor ion) level. The main advantage of isotopic labelling is that different samples can be combined and analysed within a single LC-MS measurement, reducing the experimental variability that is introduced during separate measurements and improves relative quantitation[246]. Despite their success these workflows require extended sample preparation and are limited in the number of samples that can be analysed due to the number of isotopic tags available. For

example, TMT can currently only accommodate 18 different samples[246]. However, the main disadvantage is the high cost associated with the use of these labelling methods.

In DDA, a mass spectrum of all co-eluting precursor ions (MS^1) at a specific time in the LC-gradient is acquired[247]. The instrument then selects a predefined number of precursor ions, usually the most abundant ions, for fragmentation and acquires the MS^2 [247]. Dynamic exclusion is usually applied to prevent the successive re-selection of the same precursor ions for fragmentation, minimising redundancy and increasing proteome coverage[248]. A major disadvantage of DDA is the poor run-to-run reproducibility between replicate analyses and leads to the issue of "missing values"[249]. A variety of imputation methods have been developed to overcome the issues associated with missing values and improve downstream statistical analyses[250].

DIA-based methods such as sequential window acquisition of all theoretical mass spectra (SWATH-MS) overcome the limitations associated with DDA[243]. The use of DIA has gained significant momentum in recent years due to advances in instrumentation speed and sensitivity and data analysis pipelines[251,252]. In DIA, all precursor ions that are within a pre-defined mass-to-charge (*m/z*) ratio and retention time (RT) window are fragmented at the same time, independently of the intensity[253]. This minimises the "missing value" problem that occurs with DDA by selecting specific precursors. A major challenge that was associated with DIA methods was the generation of multiplexed MS^2 spectra, as the direct correlation between precursor and fragment ions is lost[253]. Data processing approaches for the analysis of DIA spectra include spectrum-centric and peptide-centric. Spectrum-centric approaches involve the creation of "pseudo-MS/MS spectra" where fragment ions are associated with the precursor ion that they are most likely to be derived from and are subsequently matched against a database of *in silico* digested protein sequences similar to DDA[254,255]. In contrast, peptide-centric approaches search for evidence in the data of a peptide that is based on the precursor ions and its different characteristics such as fragment ions and retention time. This information is provided as a spectral library (fractionated DDA data) or generated *in silico* from a protein sequence database[256-259]. A FASTA file contains a list of protein sequences that are known for the organism of interest. The proteins contained within the FASTA file are *in silico* digested to peptides based on the predicted cleavage sites of the enzyme that is specified (e.g. trypsin). The RT and MS^2 fragment intensities of different peptides are predicted using deep learning approaches[260,261]. The deep learning models are trained using MS^2 data

from specific instruments[260] or MS^2 data of synthetic peptides[261]. It has been demonstrated that SWATH DIA-based MS can detect a higher number of peptides and has improved measurement reproducibility compared to DDA-based MS[262]. This highlights that DIA workflows are a good compromise between DDA and targeted MS acquisition methods.

Targeted MS workflows are typically used to validate and quantify specific candidates identified from discovery approaches in a sensitive, specific, and reproducible manner[244,263]. The selection of the precursor ion of interest and its product ions are usually identified from previous DDA/ DIA experiments. Multiple reaction monitoring (MRM) (also known as selected reaction monitoring (SRM))[244] and parallel reaction monitoring (PRM)[264] are targeted MS approaches that provide high precision and reproducibility compared to DDA and DIA methods. Briefly, MRM approaches are performed using a triple quadrupole (QqQ) MS, where the first and third quadrupoles act as mass filters for the predefined precursor ion and its product ion within a defined *m/z* and RT window (scheduled MRM), respectively[244]. The second quadrupole acts as a collision cell. PRM is usually performed on high resolution instruments (such as quadrupole-orbitrap and quadrupole-time-of-flight) and simultaneously monitors all fragment ions from a specific precursor[264]. A predefined precursor is selected and filtered in the quadrupole at a specific *m/z* and RT, fragmented in the collision cell and all product ions are monitored in the Orbitrap mass analyser.

Proteomic analysis of yeast during brewery fermentation and propagation

The proteome of *S. cerevisiae* is one of the most well studied, with approximately 90% of the proteome estimated to be characterised[265]. Proteomics can therefore be leveraged to improve our understanding of yeast metabolism during brewery production processes and improve production parameters.

Proteomics within the context of brewery fermentation and propagation remain scarce. Initial studies demonstrated the applicability of 2-DE in combination with MS to generate a proteome map of different industrial lager strains[266,267]. This highlighted the potential of using MS to evaluate physiological changes of yeast during brewery fermentation. Two subsequent studies evaluated changes in ale and lager yeast proteomes during an industrial-scale fermentation using 2-DE[268,269]. The study on ale yeast was used to evaluate proteome changes after the transfer of yeast from the propagation vessel to the

fermentation vessel[268]. The yeast was then repitched for three generations to evaluate the impact of serial fermentation. They found that the transfer of yeast from propagation to fermentation led to large proteome changes associated with adaptation to anaerobic conditions. They also observed that as the yeast was serially repitched, the proteome changes were minimal as the number of fermentations proceeded, suggesting adaptation to those environmental conditions. Since then, the evaluation of metabolic changes in *S. cerevisiae* at the proteome level during the transition from logarithmic to stationary phase in anaerobic conditions have mostly been limited to 2-DE coupled MS within a wine context[270-273]. These studies showed that entry into stationary phase is accompanied by an increase in stress response proteins. Proteomics has been used to evaluate protein changes in yeast during bottle conditioning of sparkling wines[274-279]. It was observed that proteins related to aerobic respiration were upregulated during secondary fermentation, despite minimal oxygen being present[276]. Additionally, there was an increase in oxidative stress response proteins that was hypothesised to be a result of increased ethanol concentrations.

In contrast, there have been many studies that have utilised proteomics to obtain a better understanding of dynamic and temporal protein changes and therefore yeast metabolism in response to oxygen availability within a laboratory setting[24,29,280-283]. For example, the metabolism of yeast in aerobic and anaerobic conditions under steady-state conditions showed that proteins involved in the TCA were upregulated in aerobic conditions whilst proteins in glycolysis were upregulated in anaerobic conditions[281]. This was expected as anaerobic cultures utilise fermentative pathways for energy production, whereas aerobic cultures utilise respiratory pathways. Another study that performed temporal proteomics on yeast during aerobic conditions also showed that there was an increase in proteins associated with the glyoxylate cycle during the diauxic shift[29,282]. The upregulation of the glyoxylate cycle is required to fuel gluconeogenesis and enable yeast growth on ethanol when glucose is exhausted. These results were supported by a recent study that investigated proteome dynamics throughout different yeast growth stages in aerobic and anaerobic conditions[284]. They showed that during anaerobic adaptation to low glucose levels, smaller proteome changes occurred compared to aerobic adaptation to low glucose levels. Moreover, the anaerobically grown yeast lacked the ability to sufficiently respond to starvation conditions. This was also illustrated in a transcriptomics study that found that anaerobic yeast cultures do not have time to remodel their metabolism to robustly face starvation and therefore have a more rapid decline in viability[285].

Despite there being a vast number of proteomic studies that have investigated yeast metabolism, it has not been fully explored within a brewery context. More specifically, an understanding of the impact of brewery propagation on yeast metabolism in downstream production processes is limited. To gain a more holistic understanding of these processes, further study and experimentation with the latest technology is required.

Aims

The overall aim for this study was to evaluate the influence of industrial brewing processes on yeast physiology and the quality of the product produced. The following aims were developed to achieve this:

Aim 1: To evaluate the feasibility of using resazurin for the assessment of yeast vitality in a brewery setting.

Aim 2: To determine the impact of propagation time on yeast longevity during bottle conditioning.

Aim 3: To determine the propensity of different yeast strains to survive during bottle conditioning.

Aim 1: Overview and key objectives

There is an unmet need for a rapid and cost-effective marker of yeast vitality for different brewing processes at Coopers Brewery Ltd. Typically, a high viability (> 99%) of yeast is usually observed during propagation using MB. Therefore, a method that determines a decline in metabolic activity is required. Current methods for the assessment of yeast vitality (such as the ATP assay or acidification power test) are either cost inhibitory or time consuming. Therefore, the first aim was to evaluate and optimise the feasibility of using the redox-sensitive dye, resazurin, for the assessment of yeast vitality and its application in a brewery propagation.

Key objectives

1. Determine the number of yeast cells, concentration of resazurin, incubation time required for reduction of resazurin within a short timeframe.
2. Determine whether resazurin can successfully differentiate between different growth stages within a laboratory setting.
3. Assess the feasibility of using the resazurin assay for monitoring yeast vitality during an industrial scale propagation.

Aim 2: Overview and key objectives

The yeast used during bottle conditioning will have a direct effect on the sensory profile of beer. It has been suggested that the optimal time for transfer of yeast between brewing propagation and fermentation tanks is in the logarithmic growth phase prior to the onset of stationary phase. To gain a better understanding of the importance of propagation time for yeast prior to seeding for bottle conditioning, the second aim was to investigate the influence of propagation time on secondary fermentation characteristics and yeast longevity.

Key objectives

1. Determine the influence of propagation time on yeast physiology during secondary fermentation during bottle conditioning.
2. Determine the influence of propagation time on yeast longevity in bottle conditioning.
3. Use proteomics to evaluate the underlying physiological changes in yeast during propagation that influence their ability to survive longer during bottle conditioning.

Aim 3: Overview and key objectives

It has been shown that yeast longevity is dependent on the yeast strain. Here, we aimed to gain an overall perspective of the behaviour and longevity of brewing yeasts compared to other industrial yeast strains during bottle conditioning. This experiment will provide additional insight into how the type of yeast affects bottle conditioning and may highlight potential biological processes that contribute to longevity in the bottle.

Key objectives

1. Evaluation of yeast strain fermentation performance during bottle conditioning.
2. Determine the impact of different yeast strains on beer quality (e.g., foam stability, sensory evaluation).
3. Use proteomics to determine key biological processes that allow certain yeast strains to survive longer during bottle conditioning.

2 Assessment of yeast physiology during industrial-scale brewing practices using the redox-sensitive dye resazurin

The information presented in this Chapter is reproduced from an article published in Yeast:

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All **Tables** and **Figures** that correspond to the supplementary documentation within this chapter can be accessed via the appropriate Digital Object Identifier (DOI) listed above.

Summary

The ability to monitor yeast quality during brewery processes is critical for product quality. The overall physiology of yeast should be determined using a combination of viability and vitality techniques. Currently there is no standard method available in breweries to determine yeast vitality. The following study aims to show the applicability of using resazurin, a cost-effective and rapid dye, to detect changes in yeast physiology before any discernible changes in viability. We initially compared resazurin to the commercially available BacTiter-Glo assay within a laboratory setting and then applied resazurin for the measurement of yeast metabolism during a brewery propagation.

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RESEARCH ARTICLE

Yeast WILEY

Assessment of yeast physiology during industrial-scale brewing practices using the redox-sensitive dye resazurin

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Abstract

Beer refermentation in bottles is an industrial process utilized by breweries where yeast and fermentable extract are added to green beer. The beer is refermented for a minimum of 2 weeks before distribution, with the physiological state of the yeast a critical factor for successful refermentation. Ideally, fresh yeast that is propagated from a dedicated propagation plant should be used for refermentation in bottles. Here, we explored the applicability of the fluorescent and redox-sensitive dye, resazurin, to assess cellular metabolism in yeast and its ability to differentiate between growth stages. We applied this assay, with other markers of yeast physiology, to evaluate yeast quality during a full-scale industrial propagation. Resazurin allowed the discrimination between the different growth phases in yeast and afforded a more in-depth understanding of yeast metabolism during propagation. This assay can be used to optimize the yeast propagation process and cropping time to improve beer quality.

KEYWORDS

bottle conditioning, brewing, metabolic activity, Resazurin, *Saccharomyces cerevisiae*, yeast

1 | INTRODUCTION

The production of beer is an ancient practice and is still one of the most popular beverages worldwide (Statista, 2021). In 2020, Australia generated $4.3 billion (AUD) in revenue and despite beer consumption in Australia decreasing, the craft brewing industry is projected to grow 5.9% over the next 5 years (Cloutman, 2021; Oo, 2021). The increase in craft breweries has been driven by consumer demand for beers with various flavor profiles and production techniques (Aquilani et al., 2015; Donadini et al., 2016). This has resulted in the requirement of consistent and high-quality products to attract and retain customers. The quality and flavor of beer is linked to the raw materials utilized such as malt, water, hops, yeast, and adjuncts (J. Wang, Ding, et al., 2019). Specifically, the physiological state of the yeast has been associated with the consistency and efficiency of fermentation and can directly impact the productivity of the brewery (Michel et al., 2020). A decline in yeast health can lead to a decrease in the attenuation of the wort, the secretion of intracellular components (enzymes, amino acids, and fatty acids) into the medium, as well as alterations to the sensory profile of the beer (Cooper et al., 2000; J. Wang, , Ding, et al., 2019).

Achieving flavor stability in beer is arguably one of the most challenging factors that breweries encounter (Lehnhardt et al., 2019).

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The development of staling flavors in beer are influenced by the production, packaging, and storage parameters (Vanderhaegen et al., 2006). Beer staling can be minimized using cooled transport and storage and reducing the presence of oxygen during brewing and bottling (Vanderhaegen et al., 2006; Wauters et al., 2021). Alternative measures to reduce beer ageing is bottle refermentation (or bottle conditioning). Bottle refermentation is a process that involves secondary fermentation through the addition of fermentable extract (priming sugar) and yeast to the bottle (Wauters et al., 2021). The practice of bottle refermentation is a traditional process used to naturally carbonate the beer, provide flavor stability, and potentially decelerate the aging process (Saison et al., 2010, 2011). Yeast influences the flavor stability by acting as an oxygen scavenger to decrease oxidative reactions and reducing aldehydes and ketones to their less flavor-active alcohol counterparts during refermentation (Saison et al., 2010; Vanderhaegen et al., 2003). It is imperative that the yeast maintains a healthy state for an extended period after refermentation is complete, as yeast autolysis leads to the formation of negative beer attributes such as poor foam stability and decreased shelf-life (Marconi et al., 2016). It has been shown that the longevity of yeast during bottle refermentation depends on the yeast strain (Qin & Lu, 2006), the priming sugar (Vanbeneden et al., 2006), and the strategy to produce the yeast (Van Landschoot et al., 2004).

The preferred method of yeast production for bottle refermentation is the use of a propagation plant, as it allows the preadaptation of yeast to conditions they will encounter in bottle refermentation, such as low pH, high ethanol, and nutrient availability (Rogers et al., 2016) and the lower risk of microbial contamination (Boulton & Quain, 2006). Typically, brewery propagations are a batch process that involve the volumetric scale-up of yeast from laboratory agar slants (Lodolo et al., 2008). The medium used for brewery propagations is usually wort as it is cost-effective and provides the necessary nutrients for yeast to grow. Despite the tanks being aerated during propagation, the metabolic flux in yeast is driven towards fermentation pathways due to the high sugar content in wort, while the oxygen only supports lipid synthesis (Moutsoglou & Dearden, 2020). After reaching the desired yeast count, the yeast is pitched into a vessel for primary beer fermentation or bottles for refermentation. It has been suggested that the growth phase of yeast for seeding should result in a minimal lag phase during refermentation to prevent prolonged exposure of the beer to oxygen and oxidative reactions (Derdelinckx et al., 1992). Despite these findings, the characterization of yeast physiology during a brewery propagation before bottle refermentation at an industrial scale remains scarce.

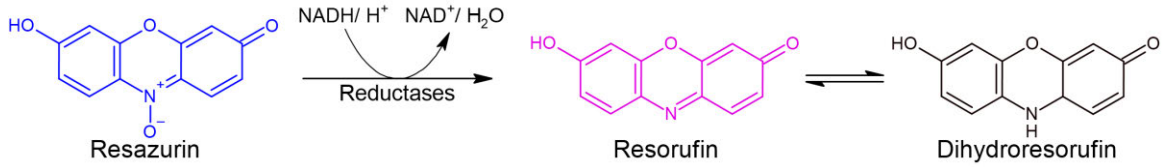
Many yeast biomarkers have been investigated to determine the propensity of yeast to perform optimally (Kwolek-Mirek & Zadrag-Tecza, 2014). Current approaches employed by breweries for the evaluation of yeast physiology involve staining techniques in combination with direct microscopy. Methylene blue is classified as the "industry standard" for the determination of live and dead cells (Painting & Kirsop, 1990). It is a cell membrane permeable dye that penetrates both cell types, where live cells reduce the dye into a colorless product, but dead cells remain blue. Alternative dyes, such as trypan blue, are dependent on the integrity of the cell membrane and only enter dead cells with a compromised membrane (McGahon et al., 1995). The sole use of methylene blue or trypan blue as an indicator of yeast physiology is insufficient (Layfield & Sheppard, 2015), as the brewing process exposes yeast cells to environmental changes such as varying oxygen levels or pH, which can impact different physiological parameters and the quality of the assay (Gibson et al., 2007). Evaluating the quality of the yeast population and their performance capabilities in different brewing processes requires the use of multiple complementary assays (cell number, pH, and the intracellular components trehalose and glycogen) (Layfield & Sheppard, 2015; Tapia et al., 2015). Specifically, it has been shown that the accumulation or utilization of intracellular glycogen can provide an overview of the nutritional status of the cell, with glycogen accumulation occurring in yeast upon the limitation of carbon, nitrogen, sulfur, or phosphorus (Lillie & Pringle, 1980). Likewise, trehalose accumulation occurs in response to multiple environmental stresses including nutrient deprivation (Lillie & Pringle, 1980), desiccation (Tapia & Koshland, 2014), heat shock (Pan et al., 2019), oxidative stress (Herdeiro et al., 2006), and ethanol toxicity (Mansure et al., 1994). There are numerous assays commercially available that exploit different cellular processes to measure cellular viability and vitality (Crouch et al., 1993; Kuhn et al., 2003; Levitz et al., 1985). The BacTiter-Glo Microbial Cell Viability Assay has been used to estimate cell viability in bacteria and yeast (Doll et al., 2016; Z. Wang, Wang, et al., 2019). This assay relies on the extraction of adenosine triphosphate (ATP) and its subsequent reaction with luciferin in the presence of Mg^{2+} cations to produce a bioluminescent signal (Crouch et al., 1993). Despite the sensitivity of monitoring ATP content in cells, it might be cost-inhibitory.

Fluorescence can also be used to determine the metabolic activity of yeast cells that do not have compromised cellular membranes. For example, fluorescence in conjunction with resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) has been used as a rapid, cost-efficient, and nontoxic alternative for monitoring cell metabolism for mammalian cells (O'Brien et al., 2000). Resazurin is a blue dye, that when added to live cells, is converted to the reduced pink form (Scheme 1) (Uzarski et al., 2017). The reduction of resazurin to resorufin is mediated by multiple intracellular enzymes (diaphorases) and can be monitored using colorimetry or fluorimetry (O'Brien et al., 2000). Resorufin can be reduced further to the nonfluorescent product, dihydroresorufin (Chen et al., 2018). The reduction of

Take-away

- Resazurin can discriminate between different growth phases in yeast.
- Resazurin permits deeper understanding of yeast physiology during a brewery propagation.
- Yeast metabolic activity was decreased at the end of an industrial-scale propagation.

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SCHEME 1 The conversion of resazurin to resorufin and dihydroresorufin. Resazurin (weakly fluorescent, blue) is converted to resorufin (highly fluorescent, pink) by reducing enzymes within the cell. Resorufin can be further reduced to dihydroresorufin (nonfluorescent, colorless).

resazurin can be used to indicate impairment or changes to cellular metabolism (Czekanska, 2011).

In this study, a cost-effective technique to assess the reductive capacity of yeast within a laboratory setting and compare its reliability to the BacTiter-Glo assay is described. The resazurin assay was used to assess the reductive capacity of yeast during a full-scale industrial propagation. Together with measurements of yeast quality (methylene blue and storage carbohydrates), it was possible to successfully differentiate between lag, logarithmic, and stationary growth phases using resazurin that preceded any detectable changes in viability. This method has the potential to be applied in other food and beverage contexts, where a high-quality product is required.

2 | MATERIALS AND METHODS

2.1 | Chemicals and yeasts

Acetic acid (for molecular biology), amyloglucosidase from *Aspergillus niger* (lyophilized powder, ~70 U/mg), glucose (GO) assay kit, phosphate-buffered saline (PBS), sodium acetate (anhydrous, reagent plus), sodium carbonate (BioXtra), and trehalase from porcine kidney (≥1.0 units/mg protein) were purchased from Sigma-Aldrich. BacTiter-Glo Microbial Cell Viability Assay was purchased from Promega. Oxoid agar (Agar No. 1, bacteriological), Oxoid dextrose (bacteriological), Oxoid peptone (bacteriological), Oxoid yeast extract powder, resazurin (sodium salt), and Wallerstein Laboratory Nutrient (WLN) agar were purchased from Thermo Fisher Scientific. Sulfuric acid (analytical grade) was obtained from Chem-Supply (Adelaide) while trypan blue solution was purchased from Merck. Methylene blue and trisodium citrate were purchased from Aim Scientific (Adelaide) and Rowe Scientific (Adelaide), respectively.

The ale yeast strain (*Saccharomyces cerevisiae*) was supplied by Coopers Brewery Ltd. (Adelaide) on an agar slant. A single colony was grown in yeast extract peptone dextrose (YPD) media (yeast extract [1%], peptone [2%], and dextrose [2%]) overnight at room temperature. The sample was stored in glycerol at −80°C until required.

2.2 | Yeast growth

A single colony of yeast was inoculated into YPD medium and incubated overnight at 25°C. The culture was diluted to ~A_{600} of 0.02 in YPD medium and incubated for 24 h with samples obtained at 1, 3, 5.5, 8, and 24 h.

An ale yeast growth curve was determined by diluting an overnight culture to ~A_{600} of 0.02 using YPD medium. Samples were aliquoted in quadruplicate into a 96-well clear flat bottom plate from Corning. Samples were analyzed using a *VICTOR*^{3} 1420 Multilabel Counter (PerkinElmer) and measured every 30 min for 24 h. The absorbance of wells containing YPD without yeast cells was subtracted from sample absorbances. Specific gravity was measured using an EasyDens device (Anton Paar).

2.3 | Industrial propagation

Yeast from agar slants were streaked onto WLN agar and incubated at 27°C for 3 days. The plate was stored at 4°C until required. A single colony was inoculated into 1 mL of sterilized wort and incubated at room temperature for 24 h with shaking. The yeast culture was serially subcultured to obtain a 1 L culture. The culture was placed into a Carlsberg flask containing 9 L of sterilized wort and incubated at room temperature for 24 h with continuous aeration. The yeast was transferred to a 10 hL CONTI PROP tank (ESAU & HUEBER) containing sterilized wort at 14°P and incubated at 14°C for 24 h. Yeast was subsequently transferred to a 60 hL CONTI PROP tank containing 40 hL of sterilized wort with an initial gravity of 14°P. Propagation was performed at 14°C with 13 min homogenization, 7 min aeration, and 20 min rest for a period of 47.5 h.

2.4 | Cell counts

Yeast cell counts were performed in duplicate using a hemocytometer. Samples were diluted in an appropriate amount of PBS buffer and mixed 1:1 with trypan blue (0.2% final concentration). For brewery counts, methylene blue was dissolved in 2% (*w*/*v*) trisodium citrate and filtered to obtain a 0.01% (*w*/*v*) solution. Sample duplicates were diluted 1:10 with methylene blue solution and counted with a hemocytometer.

2.5 | Glycogen and trehalose analysis

Glycogen and trehalose analysis was performed as previously described with minor modifications (Shi et al., 2010). Yeast cells (1×10^7 cells) were harvested from the propagator by centrifugation

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at 4000*g* for 5 min. Cells were washed with PBS and immediately stored at −80°C until required. Cell pellets were slowly defrosted on ice before the addition of 0.25 or 0.125 mL of 0.25 M sodium carbonate for glycogen and trehalose analysis, respectively. The samples were incubated at 95°C for 2 h. Samples required for glycogen analysis were acidified by the addition of 0.15 mL of 1 M acetic acid and 0.6 mL of 0.2 M sodium acetate. Samples required for trehalose analysis were acidified by the addition of 0.075 mL of 1 M acetic acid and 0.3 mL of 0.2 M sodium acetate. Half of the sample was transferred to a fresh collection tube as a control and the remaining sample was incubated with 1 U/mL of amyloglucosidase at 57°C or 0.025 U/mL of trehalase at 37°C for 18.5 h with shaking. Samples were centrifuged at 20,000*g* for 10 min and the supernatant analyzed using the glucose (GO) assay kit in a 96-well plate format. The absorbance was measured at 540 nm using a Multiskan FC (Thermo Fisher Scientific) and SkanIt Software (ver 4.1).

2.6 | Alcohol content, apparent extract, and pH

Propagation samples were filtered through a 0.4 µm filter and sonicated for 10 min before the analysis of alcohol content and apparent extract (AE). Alcohol content (%) and AE (%) were determined using an Alcolyzer: Beer Analyzing System (Anton Paar). The pH was determined using a pH meter from Mettler Toledo. These measurements were performed as a single replicate.

2.7 | ATP determination

ATP content of cells was determined using the BacTiter-Glo Microbial Cell Viability Assay according to manufacturer instruction. Briefly, the BacTiter-Glo buffer and substrate were equilibrated to room temperature. The buffer was added to the substrate and equilibrated at room temperature for 15 min before use. Yeast cells (0.5×10^6 cells) were harvested by centrifugation at 4000*g* for 5 min in triplicate, washed with 0.1 mL of PBS, and resuspended in 0.1 mL of PBS. The samples were added to a 96-well opaque plate (Corning). An equal volume of the substrate was added, and the samples incubated for 5 min at room temperature. Luminescence was measured on a GloMax® 96 Microplate Luminometer (Promega) with an integration time of 0.3 s. A blank sample (PBS) was used as a control and subtracted from sample readings in each experiment.

2.8 | Resazurin assay

2.8.1 | Effect of media on resazurin fluorescence

Resazurin was dissolved in PBS and the resulting 4 mM stock solution was sterile filtered using a syringe filter with 0.45 µm pores. Pale ale beer was filtered and centrifuged at 20,000*g* for 5 min to remove any particulates. Pale ale beer, YPD, and PBS were mixed with resazurin to obtain a final concentration of 40 µM resazurin in triplicate. Plates were incubated at room temperature for 1 h before measurement on a BMG LABTECH FLUOstar Omega plate reader (Ortenberg). Excitation and emission were performed at 570 nm (±10 nm) and 590 nm (±10 nm), respectively, with automatic gain set at 1000.

2.9 | Evaluation of resazurin concentration

The stock solution was diluted in PBS to give 10, 20, and 40 µM resazurin before addition of 1×10^6 or 2×10^6 yeast cells in triplicate. The plates were incubated with the various resazurin concentrations with a final volume of 0.2 mL from 1 to 4 h in the dark.

2.10 | Evaluation of cell number and incubation time with resazurin

Approximately 0.125×10^6 to 3×10^6 cells were resuspended in PBS containing 40 µM resazurin in triplicate. The samples were incubated at room temperature in the dark and the fluorescence monitored between 0.5 and 4 h.

2.11 | Final assay conditions

Yeast cells (1×10^6 cells) were harvested from the propagator by centrifugation at 4000*g* for 5 min in triplicate (Figure 1). Cells were washed with PBS and resuspended in 0.2 mL of PBS containing 40 µM resazurin. Samples were placed into a 96-well flat-bottom plate with 0.2 mL of PBS between samples to prevent cross-fluorescence in each experiment. A blank sample (PBS containing resazurin) was used as a control and subtracted from sample readings in each experiment. A more detailed description of the usage of resazurin is provided as a lab protocol in the supplementary materials.

3 | RESULTS AND DISCUSSION

3.1 | Evaluation of resazurin assay conditions for the assessment of yeast growth

As incubation time, cell density, and resazurin concentration can influence the formation of resorufin, we evaluated the impact of these parameters on brewing yeast cells grown in the laboratory before their use on cells grown at an industrial scale.

3.2 | Interaction of brewing media with the resazurin assay

To elucidate the potential impact of culture medium differences (pH and ethanol content), we compared the response of resazurin

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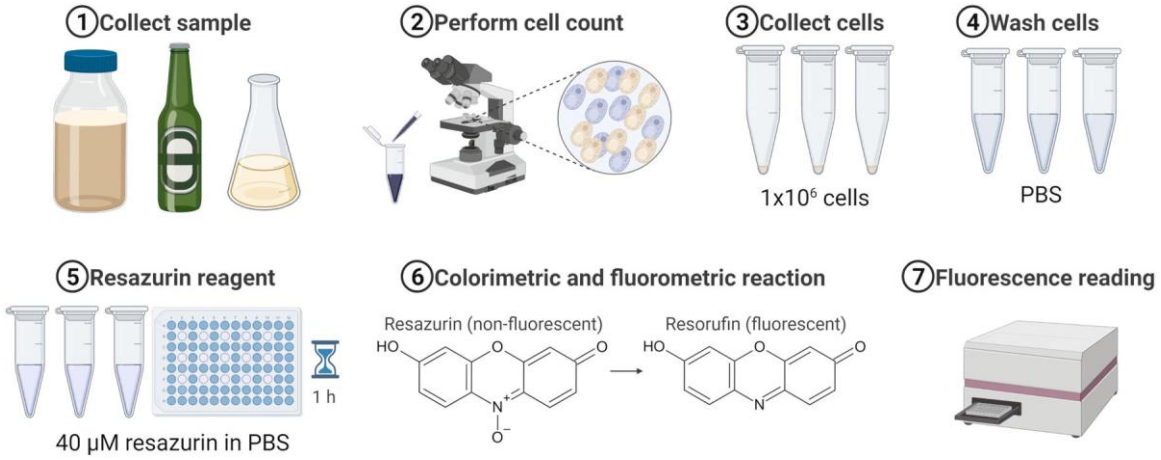


FIGURE 1 Experimental workflow for the evaluation of yeast metabolism during brewery processes. Yeast cells (1×10^6) were collected, counted, washed with PBS, and seeded into a 96-well flat-bottom plate in PBS containing 40 µM resazurin. Samples were incubated at room temperature for 1 h and fluorescence measured using a multimode microplate reader. Created with BioRender.com. PBS, phosphate-buffered saline.

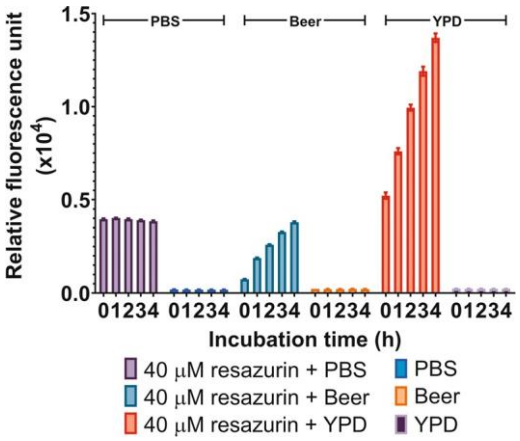


FIGURE 2 Effect of medium on resorufin production over incubation time. PBS, Beer, and YPD medium in the presence of 40 µM resazurin was incubated at room temperature for up to 4 h. Data shown as mean fluorescence of triplicate wells and error bars represent standard deviation. PBS, phosphate-buffered saline; YPD, yeast extract peptone dextrose.

with PBS, beer, and YPD culture medium (Figure 2). While PBS in the presence of resazurin produced a consistent background fluorescence, YPD showed a higher background fluorescence that increased with longer incubation times. Compared to resazurin in PBS, the quenching of fluorescence in beer containing resazurin was most likely due to the low pH (Bueno et al., 2002). The fluorescence also increased with longer incubation times but remained below that of resazurin in PBS until 4 h. As there were two factors influencing the background readings of resazurin with beer, subsequent brewery samples were washed in PBS and resuspended in the desired resazurin concentration to ensure consistent readings.

3.3 | Evaluation of cell density and incubation time for quicker resorufin production

Resazurin is considered nontoxic to mammalian cells (Ansar Ahmed et al., 1994). To ensure resazurin was nontoxic to yeast cells, ale yeast was grown in YPD medium, and 1×10^6 cells incubated with or without 100 µM resazurin. The cell viability was evaluated using the trypan blue exclusion method at 1 and 4 h postresazurin addition. As cell viability remained the same irrespective of the addition of resazurin, it demonstrated 100 µM resazurin was nontoxic to yeast cells up to 4 h (Supporting Information: Figure S1).

A rapid and inexpensive assay that determines yeast physiology is essential in a brewery setting, as corrective action can be performed before the pitching of yeast. For example, culture-based methods require incubation periods of 24-48 h. Therefore, sufficient resorufin production within a short incubation time is required for the assay. To ensure that resazurin was not a limiting factor, the dye was added at 10, 20, or 40 µM to yeast cells and resorufin production measured from 1 to 4 h (Supporting Information: Figure S2). At 10 and 20 µM resazurin, the rate of resorufin production began to slow within 3 h. A consistent reduction was observed for 4 h incubation with 40 µM resazurin, indicating that this concentration of resazurin was not a limiting factor and suitable for subsequent experiments.

As the fluorescence signal of resazurin with beer was most likely affected by pH (pH ~4) and compounds in beer that causes the formation of resorufin, it was not suitable for the assessment of yeast metabolism during brewing processes. Further, as YPD may potentially alter the metabolism of the yeast obtained from brewery processes, we assessed the suitability of incubating cells obtained from laboratory cultures in PBS containing resazurin. Yeast cells were isolated at the logarithmic phase of growth and incubated with 40 µM resazurin containing PBS (Figure 3). The fluorescence was higher as the number of cells increased, irrespective of the incubation time. This was expected as a higher number of cells

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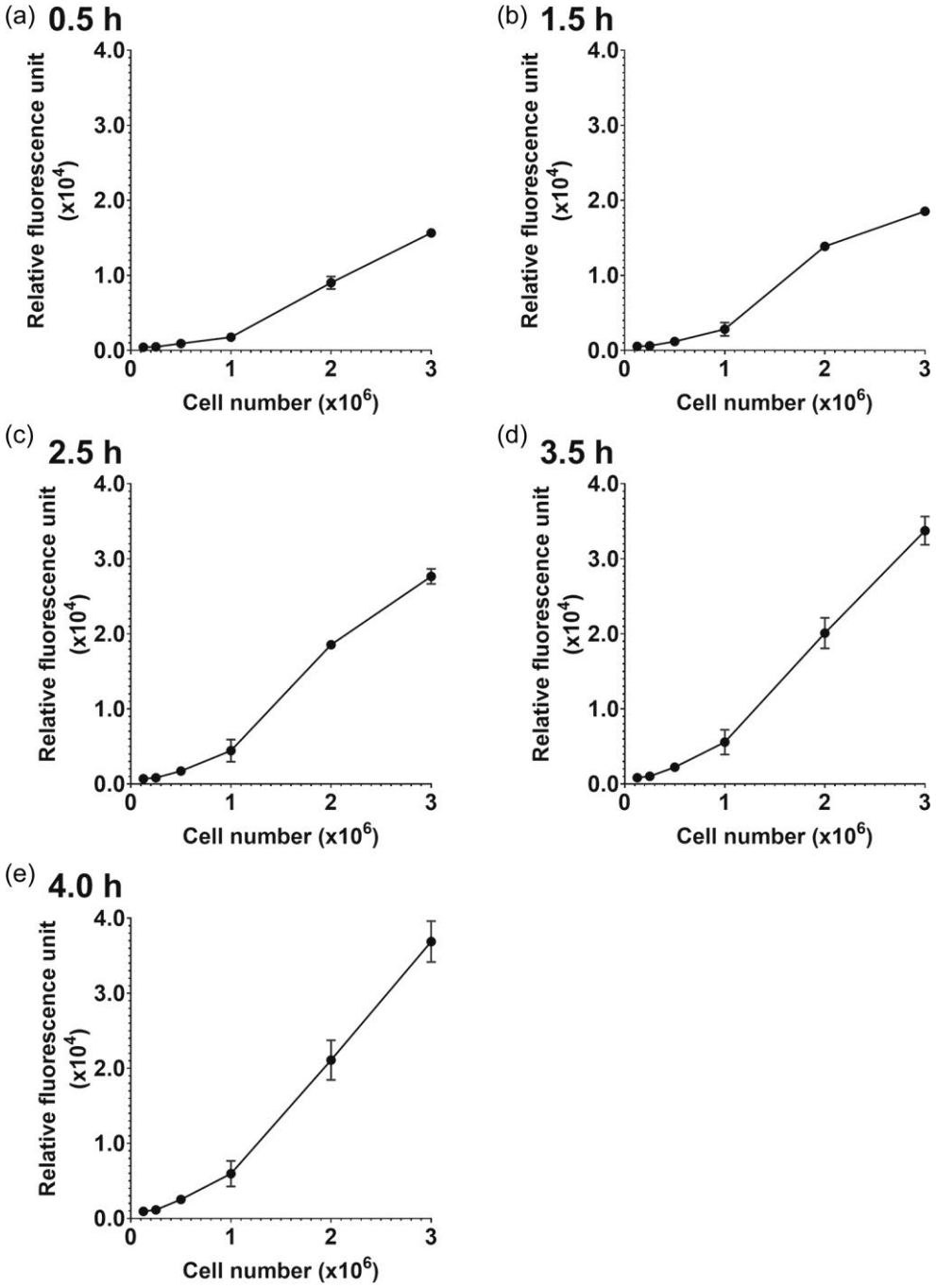


FIGURE 3 Evaluation of cell number on resazurin reduction in PBS. Ale yeast was grown in YPD medium at room temperature. 0.125×10^6 to 3×10^6 yeast cells were obtained by centrifugation, washed with PBS, and resuspended with PBS containing 40 μM resazurin. Samples were incubated in the dark for (a) 0.5 h, (b) 1.5 h, (c) 2.5 h, (d) 3.5 h, and (e) 4 h prior to measurements. Data shown as the mean fluorescence of triplicate wells and error bars represent standard deviation. PBS, phosphate-buffered saline; YPD, yeast extract peptone dextrose.

should produce an increased amount of resorufin. This has been demonstrated with previous studies that have assessed the formation of resorufin from resazurin in mammalian cells (Gong et al., 2020). A linear increase of fluorescence was observed at cell densities of 1×10^6 to 3×10^6 from 0.5 to 4 h, indicating fluorescence formation can directly reflect cell vitality. Additionally, the variability of the fluorescence measurements was generally small (within a coefficient of variation of 20%) for all cell numbers and incubation times (Supporting Information: Figure S3). This showed that the measurements were consistent across the observed range of fluorescence values and cell numbers.

To demonstrate the reliability of the resazurin assay, we performed the BacTiter-Glo Microbial Cell Viability Assay in parallel. Yeast cells were isolated at the logarithmic phase of growth and resuspended in PBS before the addition of BacTiter-Glo reagent. A linear relationship between

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luminescence and cell density from 0.0625×10^6 to 1×10^6 was observed (Supporting Information: Figure S4). This was expected as it has been demonstrated that ATP content relates directly to the number of cells present in culture (Crouch et al., 1993).

To ensure consistency in subsequent experiments, we utilized a cell density of 1×10^6, a resazurin concentration of 40 μM, and a 1 h incubation time as this enabled a rapid determination of cell vitality within the linear range. It is possible that the optimal resazurin concentration could be above 40 μM, but a rapid increase in fluorescence within a short incubation time was preferred to enable implementation in a brewery setting.

3.4 | Resazurin can indicate differences in metabolism of yeast at different stages of growth

Since the resazurin assay provides an indication of the reductive capacity of yeast, it may be possible to differentiate cellular growth phases during the brewery process. Typically, yeast cells grown in glucose-rich media in aerobic conditions have distinct growth phases including the lag and logarithmic phases, diauxic shift, and stationary phase (Werner-Washburne et al., 1993).

Yeast grown in YPD showed a lag phase from 0 to 3 h, followed by a logarithmic phase from 3 to 12 h, and then entered stationary phase (Figure 4a). The specific gravity (SG) corresponds to the density of dissolved sugars in the media. The sugars were consumed by the yeast during logarithmic phase, which correlated to a decrease in SG. The SG did not change during stationary phase as the yeast was not in active growth.

Yeast cells (1×10^6 cells) were obtained at the lag (1 and 3 h), logarithmic (5.5 and 8 h), and stationary phases (24 h). Interestingly, the fluorescence of yeast cells in the early lag phase (1 h) was higher than all other time points (Figure 4b). However, the fluorescence observed during the late lag (3 h) and early logarithmic (5.5 h) phases could not be easily differentiated from each other, but both were significantly higher than late logarithmic phase (8 h), while the fluorescence of stationary phase (24 h) cells was lower than lag or logarithmic phase yeast cells. The decline in fluorescence over time was most likely due to a decrease in the redox potential of the yeast cells. It has been reported that the rapid decline of redox potential correlated to the faster yeast growth and reached a minimum at stationary phase (Lin et al., 2010).

The ATP content of the yeast cells was compared to resorufin production. A lower ATP content during early lag phase (1 h) was observed (Figure 4c). The ATP content increased at the late lag phase (3 h) and showed similar levels throughout the logarithmic growth phases (5.5 and 8 h) before decreasing at stationary phase (24 h). It has been shown that ATP content increases during cellular growth and rapidly decreases after glucose exhaustion (Sato et al., 2000). The different trends observed between resorufin fluorescence and ATP luminescence

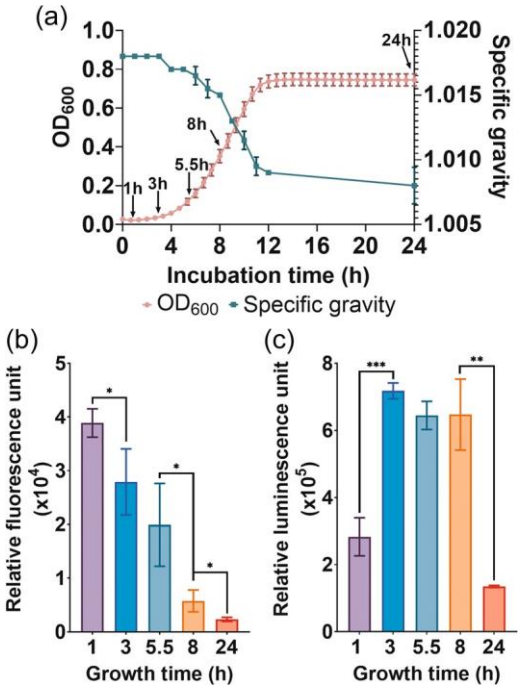


FIGURE 4 Evaluation of the resazurin assay to differentiate between yeast cell growth phases. (a) Yeast growth was measured by monitoring the optical density at 600 nm ($n = 4$) and the specific gravity of YPD was measured ($n = 2$) up to 24 h. Data shown as mean value with error bars representing standard deviation. Yeast (1×10^6 cells) obtained from a laboratory culture at the lag, logarithmic, and stationary phases were resuspended in PBS containing either (b) 40 μM resazurin and incubated for 1 h or (c) ATP bioluminescence reagent and incubated for 5 min. Data shown as the mean fluorescence of at least $n = 3$ replicates with error bars representing standard deviation. Statistical analyses were performed using *t*-tests: $^*p \le 0.05$, $^{**}p \le 0.005$, $^{***}p \le 0.0005$. ATP, adenosine triphosphate; PBS, phosphate-buffered saline; YPD, yeast extract peptone dextrose.

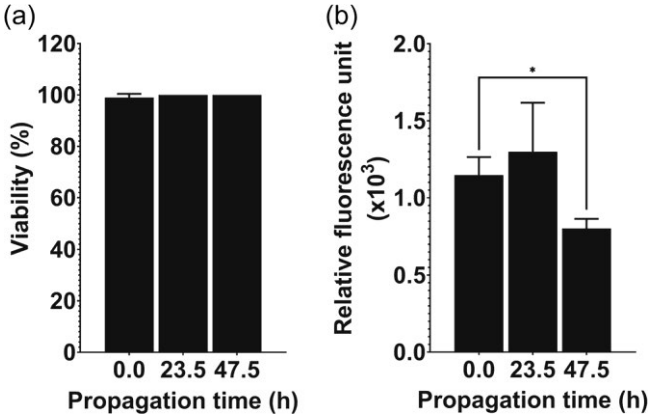


FIGURE 5 Yeast showed a decrease in metabolism during propagation. Yeast was obtained from a propagation plant at different time points before their use for bottle refermentation. (a) Viability of yeast cells using methylene blue ($n = 2$). (b) Cells (1×10^6 cells) were washed with PBS, resuspended in PBS containing 40 μM resazurin and were incubated at room temperature for 1 h ($n = 3$). Data shown as the mean value and error bars represent standard deviation. Statistical analyses were performed using *t*-tests: $^*p \le 0.05$. PBS, phosphate-buffered saline.

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most likely occurred due to resorufin fluorescence reflecting cellular metabolism whilst ATP concentration measures energy production. However, we have shown that resazurin was able to better differentiate between different stages of yeast growth.

3.5 | Application of the resazurin assay to evaluate an industrial-scale propagation process

Next, we evaluated the applicability of the resazurin assay to monitor changes in yeast metabolism during a brewery propagation. Yeast samples were obtained from an industrial-scale propagator at regular intervals and changes to yeast metabolism were monitored using resazurin, storage carbohydrates, yeast cell counts, and methylene blue assays.

The viability of the yeast cells remained above 95% for the duration of propagation (Figure 5a). The formation of resorufin was consistent between the samples collected at 0 and 23.5 h (Figure 5b), but a decrease in fluorescence was observed in the 47.5 h samples collected at the end of propagation. These results indicate that metabolism decreased towards the end of propagation and suggests the yeast may have entered stationary phase. This was also observed for laboratory-grown yeast cells in stationary phase (Figure 4b).

It was expected that the yeast would be driven towards fermentative metabolism during propagation, despite being supplied with oxygen at regular intervals as the concentration of sugars in the wort were high (Moutsoglou & Dearden, 2020). Limiting factors in the wort such as nutrient deprivation (assimilable nitrogen or zinc ions), the production of ethanol and carbon dioxide, and a high cell density results in slower yeast growth and entry into the stationary phase (Hulse, 2003).

To gain a more detailed insight of yeast metabolism during propagation, we evaluated the cellular levels of the storage carbohydrates glycogen and trehalose. The monitoring of storage

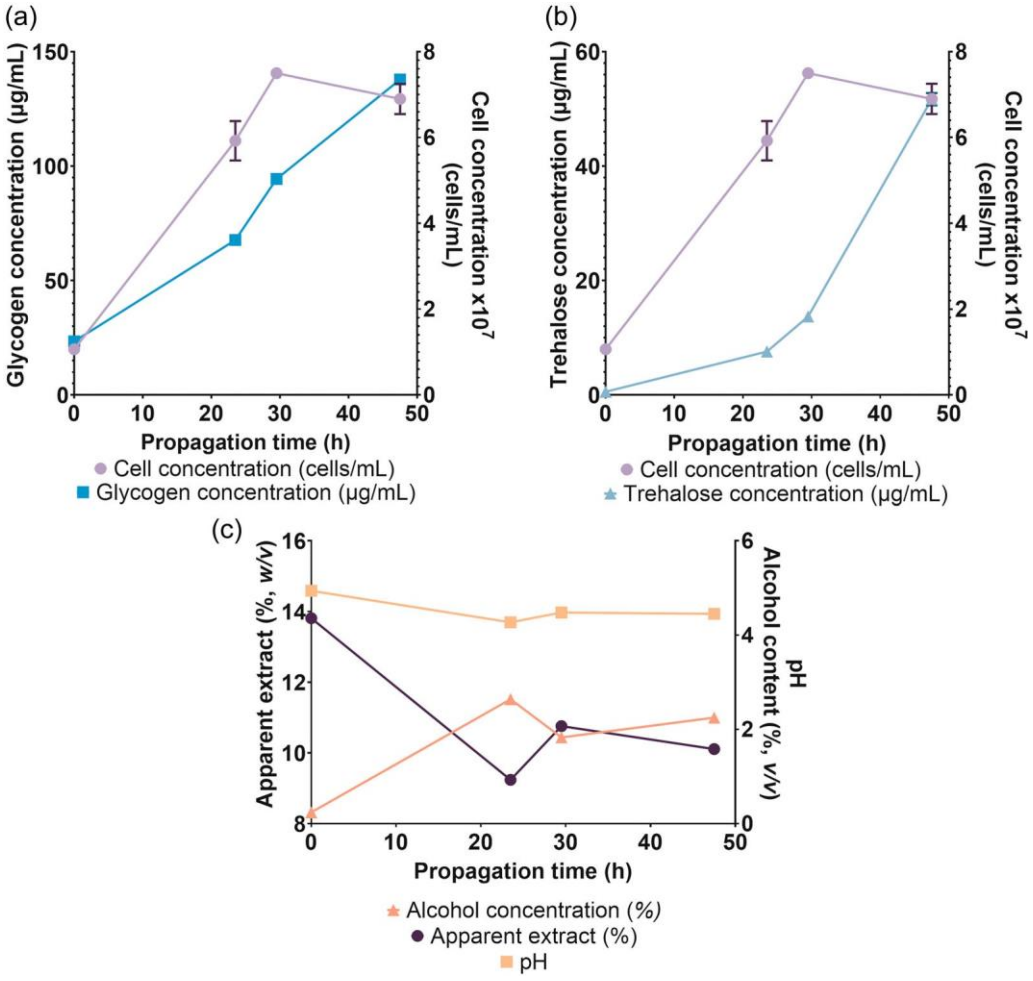


FIGURE 6 Monitoring of different parameters during industrial-scale propagation. Ale brewing yeast cells (1×10^7 cells) were obtained from a propagator at different times before seeding for bottle refermentation. Extracted carbohydrates were digested with amyloglucosidase or trehalase to determine (a) glycogen or (b) trehalose concentrations, respectively. Data shown as the mean absorbance of triplicate wells and error bars represent standard deviation. Yeast obtained from the propagator were mixed with methylene blue and counted using a hemocytometer. Data shown as the mean count of replicate samples with error bars representing standard deviation. (c) Propagation samples were filtered through a 0.4 µm filter and degassed before the measurement of alcohol concentration (*v*/*v*,%), apparent extract (*w*/*v*,%), and pH.

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carbohydrates provides complementary insights to the resazurin assay regarding cellular growth and metabolism.

The concentration of glycogen was 23.4 ± 0.4 μg/mL at the start of propagation (Figure 6a). Glycogen began to accumulate immediately after it was transferred into the propagator and reached 67.7 ± 1.2 μg/mL after 23.5 h and continued to increase to 137.8 ± 1.8 μg/mL at the end of propagation (47.5 h). The immediate accumulation of glycogen during propagation was expected as the yeast was transferred into the propagation tank during logarithmic growth such that no lag phase was observed. The accumulation of glycogen during logarithmic growth has been shown to occur during fermentation on wort and is in response to the depletion of essential nutrients, such as glucose (Ouain et al., 1981). The amount of glycogen present in the yeast before seeding for bottle conditioning is important as it has been reported that yeast with lower glycogen content will perform less well (Quain, 1988). Therefore, poor glycogen content could result in a slower refermentation rate and a longer lag phase during bottle conditioning. This is undesirable as it can lead to the presence of unwanted flavors (Derdelinckx et al., 1992; Hulse, 2003).

Trehalose levels were low at the start of propagation (0.54 ± 0.1 μg/mL) and remained relatively low at 7.6 ± 0.04 μg/mL until 23.5 h (Figure 6b). Nonetheless, the amount of trehalose rapidly increased from 13.7 ± 0.2 μg/mL at 29.5 h to 51.7 ± 1.1 μg/mL at 47.5 h. This accumulation of trehalose towards the end of propagation was expected as trehalose synthesis does not usually occur in logarithmic phase unless the yeast cells are exposed to stress (Eleutherio et al., 2015). As the amount of yeast cells in the propagator began to plateau between 29.5 and 47.5 h, along with the AE and alcohol content (Figure 6c), it suggested that trehalose synthesis was initiated by energy limitations and in preparation towards a different growth stage. This has been shown to occur in yeasts during the shift from logarithmic to stationary phase under wine-making conditions (Novo et al., 2005).

In summary, the lower metabolic activity observed with resazurin and the concomitant increases in glycogen and trehalose indicate that the yeast reached stationary phase after 47.5 h. As resazurin can detect changes in metabolic activity, it can provide breweries with a useful method to rapidly detect the onset of stationary phase before the degradation of glycogen and trehalose. This is important during propagation as selecting an optimal cropping time before storage carbohydrate degradation occurred would promote consistent bottle refermentation.

In this study, we demonstrated the application of resazurin to detect changes in the metabolic activity of yeast under laboratory and industrial settings. Although cell number, incubation time, resazurin concentration, and media type influence assay measurements, they can be successfully used for the assessment of cell vitality, provided appropriate control experiments are performed to determine their influence on fluorescence. After evaluation of these parameters, a cell density, incubation time, and resazurin concentration showed a linear relationship with fluorescence production that allowed an evaluation of changes in yeast vitality within a rapid timeframe.

We successfully showed that a decrease in metabolic activity using resazurin preceded any discernable decrease in storage carbohydrates and viability during propagation. This is important as propagation time and the cropping of yeast for use in downstream production processes can directly impact product quality. This rapid method can be implemented by breweries to monitor changes in yeast metabolic activity in combination with other techniques to assess yeast quality.

AUTHOR CONTRIBUTIONS

Brooke A. Dilmetz, Clifford Young, Jon Meneses, Manuela Klingler-Hoffmann, and Peter Hoffmann conceived the experimental design of this study. Brooke A. Dilmetz conducted all experiments and performed the data analysis. Brooke A. Dilmetz interpreted the observed data and Christopher T. Desire, Leigh Donnellan, Clifford Young, Jon Meneses, Manuela Klingler-Hoffmann, and Peter Hoffmann provided advice regarding data interpretation and manuscript preparation. Brooke A. Dilmetz wrote the manuscript with input from all authors. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Ansar Ahmed, S., Gogal, Jr., R. M., & Walsh, J. E. (1994). A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: An alternative to [3H]thymidine incorporation assay. *Journal of Immunological Methods*, *170*(2), 211-224. https://doi.org/10.1016/0022-1759(94)90396-4

Aquilani, B., Laureti, T., Poponi, S., & Secondi, L. (2015). Beer choice and consumption determinants when craft beers are tasted: An exploratory study of consumer preferences. *Food Quality and Preference*, *41*, 214–224. https://doi.org/10.1016/j.foodqual.2014.12.005

Boulton, C., & Quain, D. (2006). Yeast management. In C. Boulton, & D. Quain (Eds.), *Brewing yeast and fermentation* (pp. 468-509). Blackwell Science.

10970061, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/yea.3851 by University Of South Australia, Wiley Online Library on [15/05/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Bueno, C., Villegas, M. L., Bertolotti, S. G., Previtali, C. M., Neumann, M. G., & Encinas, M. V. (2002). The excited-state interaction of resazurin and resorufin with aminesin aqueous solutions. photophysics and photochemical reaction. *Photochemistry and Photobiology*, *76*(4), 385-390. https://doi.org/10.1562/0031-8655(2002)0760385TESIOR2.0.CO2

Chen, J. L., Steele, T. W. J., & Stuckey, D. C. (2018). Metabolic reduction of resazurin; location within the cell for cytotoxicity assays. *Biotechnology and Bioengineering*, *115*(2), 351-358. https://doi.org/10.1002/bit.26475

Cloutman, N. (2021). *Craft beer production in Australia (O5071)*. https://my.ibisworld.com/au/en/industry-specialized/od5071/about

Conway, J. Statista. (2021). *Global beer market*. https://www.statista.com/statistics/270275/worldwide-beer-production/

Cooper, D. J., Stewart, G. G., & Bryce, J. H. (2000). Yeast proteolytic activity during high and low gravity wort fermentations and its effect on head retention. *Journal of the Institute of Brewing*, *106*(4), 197-202. https://doi.org/10.1002/j.2050-0416.2000.tb00057.x

Crouch, S. P., Kozlowski, R., Slater, K. J., & Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *Journal of Immunological Methods*, *160*(1), 81-88. https://doi.org/10.1016/0022-1759(93)90011-u

Czekanska, E. M. (2011). Assessment of cell proliferation with resazurin-based fluorescent dye. *Methods in Molecular Biology*, *740*, 27-32. https://doi.org/10.1007/978-1-61779-108-6_5

Derdelinckx, G., Vanderhasselt, B., Maudoux, M., & Dufour, J. (1992). Refermentation in bottles and kegs. A rigorous approach. *Brauwelt International*, *2*, 156-164.

Doll, K., Jongsthaphongpun, K. L., Stumpp, N. S., Winkel, A., & Stiesch, M. (2016). Quantifying implant-associated biofilms: Comparison of microscopic, microbiologic and biochemical methods. *Journal of Microbiological Methods*, *130*, 61-68. https://doi.org/10.1016/j.mimet.2016.07.016

Donadini, G., Fumi, M. D., Kordialik-Bogacka, E., Maggi, L., Lambri, M., & Sckokai, P. (2016). Consumer interest in specialty beers in three European markets. *Food Research International*, *85*, 301-314. https://doi.org/10.1016/j.foodres.2016.04.029

Eleutherio, E., Panek, A., De Mesquita, J. F., Trevisol, E., & Magalhães, R. (2015). Revisiting yeast trehalose metabolism. *Current Genetics*, *61*(3), 263-274. https://doi.org/10.1007/s00294-014-0450-1

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. *FEMS Microbiology Reviews*, *31*(5), 535-569. https://doi.org/10.1111/j.1574-6976.2007.00076.x

Gong, X., Liang, Z., Yang, Y., Liu, H., Ji, J., & Fan, Y. (2020). A resazurin-based, nondestructive assay for monitoring cell proliferation during a scaffold-based 3D culture process. *Regenerative Biomaterials*, *7*(3), 271-281. https://doi.org/10.1093/rb/rbaa002

Herdeiro, R. S., Pereira, M. D., Panek, A. D., & Eleutherio, E. C. A. (2006). Trehalose protects *Saccharomyces cerevisiae* from lipid peroxidation during oxidative stress. *Biochimica et Biophysica Acta (BBA)*, *1760*(3), 340-346. https://doi.org/10.1016/j.bbagen.2006.01.010

Hulse, G. A. (2003). Yeast propagation. In K. Smart (Ed.), *Brewing yeast fermentation performance*. https://doi.org/10.1002/9780470696040.ch23

Kuhn, D.M., Balkis, M., Chandra, J., Mukherjee, P. K., & Ghannoum, M. A. (2003). Uses and limitations of the XTT assay in studies of Candida growth and metabolism. *Journal of Clinical Microbiology*, *41*(1),506-508. https://doi.org/10.1128/JCM.41.1.506-508.2003

Kwolek-Mirek, M., & Zadrag-Tecza, R. (2014). Comparison of methods used for assessing the viability and vitality of yeast cells. *FEMS Yeast Research*, *14*(7), 1068-1079. https://doi.org/10.1111/1567-1364.12202

Van Landschoot, A., Vanbeneden, N., Vanderputten, D., & Derdelinckx, G. (2004). Effect of pitching yeast preparation on the refermentation of beer in bottles. *Cerevisia*, *29*(3), 140-145.

Layfield, J. B., & Sheppard, J. D. (2015). What brewers should know about viability, vitality, and overall brewing fitness: A mini-review. *MBAA TQ*, *52*(3), 132-140.

Lehnhardt, F., Gastl, M., & Becker, T. (2019). Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review. *Critical Reviews in Food Science and Nutrition*, *59*(16), 2642-2653. https://doi.org/10.1080/10408398.2018.1462761

Levitz, S. M., & Diamond, R. D. (1985). A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. *The Journal of Infectious Diseases*, *152*(5), 938-945. https://doi.org/10.1093/infdis/152.5.938

Lillie, S. H., & Pringle, J. R. (1980). Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: Responses to nutrient limitation. *Journal of Bacteriology*, *143*(3), 1384-1394. https://doi.org/10.1128/jb.143.3.1384-1394.1980

Lin, Y.-H., Chien, W.-S., & Duan, K.-J. (2010). Correlations between reduction-oxidation potential profiles and growth patterns of *Saccharomyces cerevisiae* during very-high-gravity fermentation. *Process Biochemistry*, *45*(5), 765-770. https://doi.org/10.1016/j.procbio.2010.01.018

Lodolo, E. J., Kock, J. L. F., Axcell, B. C., & Brooks, M. (2008). The yeast *Saccharomyces cerevisiae*—The main character in beer brewing. *FEMS Yeast Research*, *8*(7), 1018-1036. https://doi.org/10.1111/j.1567-1364.2008.00433.x

Mansure, J. J. C., Panek, A. D., Crowe, L. M., & Crowe, J. H. (1994). Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochimica et Biophysica Acta (BBA)—Biomembranes*, *1191*(2), 309-316. https://doi.org/10.1016/0005-2736(94)90181-3

Marconi, O., Rossi, S., Galgano, F., Sileoni, V., & Perretti, G. (2016). Influence of yeast strain, priming solution and temperature on beer bottle conditioning. *Journal of the Science of Food and Agriculture*, *96*(12), 4106-4115. https://doi.org/10.1002/jsfa.7611

McGahon, A. J., Martin, S. J., Bissonnette, R. P., Mahboubi, A., Shi, Y., Mogil, R. J., & Green, D. R. (1995). The end of the (cell) line: Methods for the study of apoptosis in vitro. *Methods in Cell Biology*, *46*, 153-185. https://doi.org/10.1016/s0091-679x(08)61929-9

Michel, M., Meier-Dörnberg, T., Jacob, F., Schneiderbanger, H., & Hutzler, M. (2020). Optimisation of yeast vitality measurement to better predict fermentation performance. *Journal of the Institute of Brewing*, *126*(2), 161-167. https://doi.org/10.1002/jib.604

Moutsoglou, M. E., & Dearden, A. C. (2020). Effect of the respiro-fermentative balance during yeast propagation on fermentation and wort attenuation. *Journal of the Institute of Brewing*, *126*(3), 289-297. https://doi.org/10.1002/jib.621

Novo, M. T., Beltran, G., Rozès, N., Guillamón, M., & Mas, A. (2005). Effect of nitrogen limitation and surplus upon trehalose metabolism in wine yeast. *Applied Microbiology and Biotechnology*, *66*(5), 560-566. https://doi.org/10.1007/s00253-004-1676-5

O'Brien, J., Wilson, I., Orton, T., & Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity: Resazurin as a cytotoxicity assay. *European Journal of Biochemistry*, *267*(17), 5421-5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x

Oo, S. (2021). *Beer Manufacturing in Australia (C1212)*. https://my.ibisworld.com/au/en/industry/c1212/about

Ouain, D. E., Thurston, P. A., & Tubb, R. S. (1981). The structural and storage carbohydrates of *Saccharomyces cerevisiae*: Changes during fermentation of wort and a role for glycogen catabolism in lipid biosynthesis. *Journal of the Institute of Brewing*, *87*(2), 108-111. https://doi.org/10.1002/j.2050-0416.1981.tb03997.x

Painting, K., & Kirsop, B. (1990). A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining. *World Journal of Microbiology & Biotechnology*, *6*(3), 346-347. https://doi.org/10.1007/BF01201311

10970061, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/yea.3851 by University Of South Australia, Wiley Online Library on [15/05/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Pan, D., Wiedemann, N., & Kammerer, B. (2019). Heat stress-induced metabolic remodeling in *Saccharomyces cerevisiae*. *Metabolites*, *9*(11), 266. https://doi.org/10.3390/metabo9110266

Qin, H., & Lu, M. (2006). Natural variation in replicative and chronological life spans of *Saccharomyces cerevisiae*. *Experimental Gerontology*, *41*(4), 448-456. https://doi.org/10.1016/j.exger.2006.01.007

Quain, D. E. (1988). Cambridge prize lecture: Studies on yeast Physiology-Impact on fermentation performance and product quality. *Journal of the Institute of Brewing*, *94*(5), 315-323. https://doi.org/10.1002/j.2050-0416.1988.tb04589.x

Rogers, C. M., Veatch, D., Covey, A., Staton, C., & Bochman, M. L. (2016). Terminal acidic shock inhibits sour beer bottle conditioning by *Saccharomyces cerevisiae*. *Food Microbiology*, *57*, 151-158. https://doi.org/10.1016/j.fm.2016.02.012

Saison, D., De Schutter, D. P., Delvaux, F., & Delvaux, F. R. (2011). Improved flavor stability by aging beer in the presence of yeast. *Journal of the American Society of Brewing Chemists*, *69*(1), 50-56. https://doi.org/10.1094/ASBCJ-2011-0127-01

Saison, D., De Schutter, D. P., Vanbeneden, N., Daenen, L., Delvaux, F., & Delvaux, F. R. (2010). Decrease of aged beer aroma by the reducing activity of brewing yeast. *Journal of Agricultural and Food Chemistry*, *58*(5), 3107-3115. https://doi.org/10.1021/jf9037387

Sato, K., Yoshida, Y., Hirahara, T., & Ohba, T. (2000). On-line measurement of intracellular ATP of *Saccharomyces cerevisiae* and pyruvate during sake mashing. *Journal of Bioscience and Bioengineering*, *90*(3), 294-301. https://doi.org/10.1016/S1389-1723(00)80084-5

Shi, L., Sutter, B. M., Ye, X., & Tu, B. P. (2010). Trehalose is a key determinant of the quiescent metabolic state that fuels cell cycle progression upon return to growth. *Molecular Biology of the Cell*, *21*(12), 1982-1990. https://doi.org/10.1091/mbc.e10-01-0056

Tapia, H., & Koshland, D. E. (2014). Trehalose is a versatile and long-lived chaperone for desiccation tolerance. *Current Biology*, *24*(23), 2758-2766. https://doi.org/10.1016/j.cub.2014.10.005

Tapia, H., Young, L., Fox, D., Bertozzi, C. R., & Koshland, D. (2015). Increasing intracellular trehalose is sufficient to confer desiccation tolerance to *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(19), 6122-6127. https://doi.org/10.1073/pnas.1506415112

Uzarski, J. S., DiVito, M. D., Wertheim, J. A., & Miller, W. M. (2017). Essential design considerations for the resazurin reduction assay to noninvasively quantify cell expansion within perfused extracellular matrix scaffolds. *Biomaterials*, *129*, 163-175. https://doi.org/10.1016/j.biomaterials.2017.02.015

Vanbeneden, N., Vanderputten, D., Vanderhaegen, B., Derdelinckx, G., & Van Landschoot, A. (2006). Influence of the sugar composition of the added extract on the refermentation of beer in bottles. *Journal of the American Society of Brewing Chemists*, *64*(4), 206-213. https://doi.org/10.1094/ASBCJ-64-0206

Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K. J., Derdelinckx, G., & Verachtert, H. (2003). Bioflavoring and beer refermentation. *Applied Microbiology and Biotechnology*, *62*(2), 140-150. https://doi.org/10.1007/s00253-003-1340-5

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/10.1016/j.foodchem.2005.01.006

Wang, J., Ding, H., Zheng, F., Li, Y., Liu, C., Niu, C., & Li, Q. (2019). Physiological changes of beer brewer's yeast during serial beer fermentation. *Journal of the American Society of Brewing Chemists*, *77*(1), 10–20. https://doi.org/10.1080/03610470.2018.1546030

Wang, Z., Wang, J., Shan, W., Zheng, F., Niu, C., Liu, C., & Li, Q. (2019). Intracellular adenosine triphosphate (ATP) content sensitively reflects subtle differences in yeast physiology. *Journal of the American Society of Brewing Chemists*, *77*(2), 92-98. https://doi.org/10.1080/03610470.2019.1577706

Wauters, R., Britton, S. J., & Verstrepen, K. J. (2021). Old yeasts, young beer—The industrial relevance of yeast chronological life span. *Yeast*, *38*(6), 339-351. https://doi.org/10.1002/yea.3650

Werner-Washburne, M., Braun, E., Johnston, G. C., & Singer, R. A. (1993). Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiological Reviews*, *57*(2), 383-401. https://doi.org/10.1128/mr.57.2.383-401.1993

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3 Impact of propagation time on yeast physiology during bottle conditioning of beer at an industrial scale

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Summary

A previous study suggested that propagation time prior to transfer for primary fermentation can impact yeast quality. To gain a better understanding of the impact of propagation time prior to seeding for bottle conditioning, we propagated yeast for different times, corresponding to the logarithmic and post-diauxic shift growth phases, at an industrial scale (30 hL). The yeast was seeded into pale ale beer, bottle conditioned for five months, and yeast physiology and longevity monitored. We utilised proteomics to elucidate the underlying metabolic changes that allowed yeast grown to the post-diauxic shift to have better longevity in the bottle and ultimately improve the shelf-life characteristics of the product.

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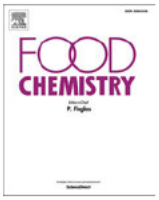
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Impact of propagation time on yeast physiology during bottle conditioning of beer on an industrial scale

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ABSTRACT

Bottle conditioning occurs when yeast and a fermentable extract are added to beer prior to packaging. Aside from ethanol and carbon dioxide production, this process can minimize the production of off-flavors and increase the shelf-life of beer. The advantages of bottle conditioning rely on the yeast being able to quickly referment the beer and maintain viability during storage. In this study, a commercial ale yeast was propagated in wort on a large scale (30 hL) for 24 h or 72 h and seeded into pale ale beer for bottle conditioning. We found that yeast propagated until the post-diauxic shift (72 h) provided better longevity in the bottle and improved foam stability compared to the 24 h propagated yeast. At the time of seeding, yeast propagated for 72 h showed an upregulation of proteins involved in cellular respiration and general stress pathways that may indicate responses toward mitigating cellular stress levels.

1. Introduction

Bottle conditioning (or bottle refermentation) is a secondary fermentation that occurs in the bottle. Typically, yeast is seeded between 0.5×10^6 and 1.0×10^6 cellsmL^{-1} into 'green' beer (young beer before the maturation period) that is primed with a fermentable extract, such as sugar (Marconi et al., 2016). During conditioning, the yeast metabolizes the extract to produce ethanol and carbon dioxide. The fermentation process during bottle conditioning is challenging for yeast, as beer provides a poor environment for cellular growth due to its: low pH, low oxygen content, high ethanol, and poor nutrient availability. However, bottle conditioning is beneficial because the yeast consumes excess oxygen, minimizes beer oxidation, and decreases the production of staling compounds (Saison et al., 2011). The delayed formation of aging compounds also prolongs the shelf-life of the beer.

Bottle conditioning relies on the yeast maintaining a good physiological condition for an extensive period of time. Yeast autolysis can lead to the secretion of proteolytic enzymes and unwanted fatty acids in the beer (Chen et al., 1980), which alters the sensory profile (Van Landschoot et al., 2004) and the foam stability of the beer (Brey et al., 2003). It has been shown that various yeast strains are capable of withstanding the harsh conditions of refermentation for different time spans (Van Landschoot et al., 2004), and, therefore, it would be favorable to select a yeast strain that exhibits superior survivability qualities. However, the selection of yeast for refermentation also depends on characteristics such as flocculation ability and aroma formation (Wauters et al., 2021). It is for these reasons that brewers do not typically change the refermentation yeast, which ensures that the characteristics of their beer brand are not modified. Alternative strategies that can be used to improve yeast survival during bottle conditioning include the selection of an appropriate fermentable extract (Marconi et al., 2016) and the preparation process of yeast (Van Landschoot et al., 2004).

The easiest method of yeast production for bottle conditioning is to use yeast obtained from the main fermentation. However, as the environment for bottle conditioning is dissimilar to that of the main fermentation (Van Landschoot et al., 2004), the use of alternative yeasts

Abbreviations: AE, apparent extract; LC-MS/MS, liquid chromatography tandem mass spectrometry; PBS, phosphate buffered saline; SWATH DIA, sequential window acquisition of all theoretical mass spectra data independent acquisition; SPEED, Sample Preparation by Easy Extraction and Digestion; TFA, trifluoroacetic acid.

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and production methods have been investigated. For example dried yeasts (Lodolo et al., 2008) have been utilized in many breweries for their ease of use. However, the propagation plant is the preferred method of yeast production for biomass, as it allows the brewer to expose the yeast to a mild stress that induces resistance against other stressors (Rogers et al., 2016; Wauters et al., 2021). Propagation for biomass is typically performed as a batch process and is comprised of a series of volumetric scale-ups of yeast from agar slants in aerobic conditions (Lodolo et al., 2008). Because it is cost-effective and easily accessible, wort, is usually utilized as the propagation medium. The batch culture of *Saccharomyces cerevisiae* in a rich medium under aerobic conditions usually results in a lag phase immediately after inoculation into a fresh medium, followed by a: logarithmic growth phase, diauxic shift phase, post-diauxic shift growth phase, and stationary phase. The logarithmic phase is when the yeast replicates rapidly and their metabolism is predominantly fermentative due to the high sugar content in the wort, whilst the oxygen supplied supports lipid synthesis (Moutsoglou & Dearden, 2020). As the glucose levels decline below a threshold, the yeast switches their metabolism from fermentative to respiratory and is known as the diauxic shift. During the post-diauxic shift, the yeast follows a slower period of growth and oxidizes non-fermentable carbon sources such as ethanol. This is followed by the stationary phase where the yeast cells enter a non-proliferative state (Werner-Washburne et al., 1993). It has been suggested that the optimal transfer time of yeast between propagations is during the late logarithmic phase prior to the onset of the stationary phase, as it can minimize or eliminate the possibility of a lag phase (Hulse, 2003). During bottle conditioning, a minimal lag phase is desirable to prevent the prolonged exposure of beer to oxygen, oxidative reactions, and the production of staling flavors (Derdelinckx et al., 1992).

It has been highlighted that the intracellular storage carbohydrates (glycogen and trehalose) play an important role in the ability of yeast to withstand stress and in cell survival (Cao et al., 2016). Glycogen is the main carbon source and energy reserve in yeast and is readily mobilized during adaption to new environments. It accumulates upon the limitation of: carbon, nitrogen, sulfur, or phosphorus (Lillie & Pringle, 1980). Trehalose has been shown to accumulate in response to different types of cellular stress, such as nutrient deprivation (Lillie & Pringle, 1980) and ethanol toxicity (Mansure et al., 1994). The monitoring of these intracellular storage carbohydrates may allow a better understanding of yeast metabolism and their roles in yeast survival during bottle conditioning.

The impact of the: fermentable extract (Vanbeneden et al., 2006), beer characteristics (Dekoninck et al., 2013), propagation medium (Van Landschoot et al., 2004), and yeast strain (Marconi et al., 2016) on bottle conditioning have been investigated. Despite some studies performing propagation at an industrial scale for the main fermentation (Hulse, 2003) and bottle conditioning (Van Landschoot et al., 2004; Vanbeneden et al., 2006), there have been no reports of the influence of propagation time on bottle conditioning. We hypothesized that propagation time could have an impact on product consistency and yeast longevity during bottle conditioning. We therefore performed yeast propagation at an industrial scale (30 hL) and evaluated their performance during bottle conditioning and the long-term storage of beer for 5 months duration. Yeast was propagated in wort for 24 h or 72 h, seeded into pale ale beer for bottle conditioning, and then monitored for several characteristics including alcohol content, apparent extract, pH, foam stability), cell viability, and storage carbohydrate ~~reserve~~ levels (glycogen and trehalose). Proteomics, which allows a relative quantitative comparison of protein abundances (Liu et al., 2022), was also determined on the two differently propagated yeast cell populations. We found that yeast propagated for 72 h afforded increased longevity, whilst proteomic analysis by Zeno sequential window acquisition of all theoretical mass spectra data independent acquisition (SWATH DIA) revealed an upregulation of pathways involved in cellular respiration and stress resistance in 72 h cells at the time of seeding. These results suggest that the induced stress adaptation responses may have enabled these cells to survive longer during bottle conditioning.

2. Materials and methods

2.1. Yeast propagation and bottle refermentation

Coopers commercial ale yeast (*Saccharomyces cerevisiae*) was obtained on an agar slant, streaked onto Wallerstein Laboratory Nutrient Agar (Thermo Fisher Scientific; Waltham, MA, USA), and incubated at 27 °C for 3 days. The wort was prepared from a concentrate (Coopers Brewery Ltd.) and sterilized at 121 °C for 20 min. A single colony was inoculated into 1 mL of sterile wort and incubated at room temperature for 24 h with shaking at 170 rpm. The yeast culture was serially subcultured in wort to obtain a final volume of 1 L. The culture was transferred to a 20 L Carlsberg Flask containing 9 L of sterile wort and incubated at room temperature for 24 h with continuous aeration (Fig. 1A). Wort was prepared and sterilized using hot water that was maintained at a temperature of 82.5 °C until 400 pressure units were obtained in the brewery. The yeast was then transferred to a 10 hL CONTI PROP tank (ESAU & HUEBER, Schrobenhausen, Germany) containing sterilized wort at 14°P and incubated at 14 °C for 24 h. Afterwards, yeast was transferred to a 60 hL CONTI PROP tank containing 40 hL of sterilized wort with an initial gravity of 14°P, with propagation performed at 14 °C with 13 min homogenization, 7 min aeration, and 20 min rest for a period of 96 h. The yeast (5 hL) was transferred to a 60 hL CONTI PROP tank containing 25 hL of sterilized wort with an initial gravity of 14°P. Propagation was performed at 14 °C with 13 min homogenization, 7 min aeration, and a 20 min rest for a period of 24 h or 72 h. The inoculum concentration at the start of propagation was $2.2 \pm 0.8 \times 10^7$ cellsmL^{-1} and $1.3 \pm 0.3 \times 10^7$ cellsmL^{-1} for 24 h and 72 h propagation, respectively.

Bottle refermentation was performed on an industrial filling line into pale ale beer. Yeast obtained from the propagator were seeded into pale ale beer that was pre-primed with sucrose (0.5°P) at a density of 0.5×10^6 cellsmL^{-1}. The bottles were capped, sealed, and then stored at 20 °C–22 °C for the entire experimental duration.

2.2. Yeast cell counts and viability determination

Yeast was obtained from the bottle by centrifugation at 4,000g (~6444 rpm using a HERAEUS Pico™ 17 Microcentrifuge fitted with an 8.6 cm rotor (Thermo Fisher Scientific)) for 5 min. The beer was removed, and the cells resuspended in phosphate buffered saline (PBS) (Sigma-Aldrich; St. Louis, MO, USA). Yeast counts were performed using a Neubauer improved hemocytometer in triplicate. Viability was determined using a Cellometer X2 automated cell counter (Nexcelom, Lawrence, MA, USA) with the ViaStain™ Yeast Kit for Live/Dead Concentration (Thermo Fisher Scientific). Briefly, 20 μL of yeast was mixed with 20 μL of yeast dilution buffer and 40 μL of ViaStain™ before incubation for 5 min at room temperature in the dark. Samples were placed onto a counting slide and the fluorescence measured using Cellometer Image Cytometer software (version 3.0.0.9). Acridine orange fluorescence was measured at an excitation and emission of 470 nm and 535 nm, respectively, with an adjustment threshold of 15. Fluorescence of propidium iodide was measured at an excitation and emission of 540 nm and 660 nm, respectively, with an adjustment threshold of 30. Samples were counted in triplicate.

2.3. Alcohol, apparent extract, and pH

Samples were filtered using a 0.25 μm filter and degassed for 10 min prior to apparent extract (fermentable sugars in the beer, AE) and alcohol measurements on a DMA 4500 M and Alcolyzer Beer ME (Anton Parr, Graz, Austria). The pH was determined using a pH meter from Mettler Toledo (Columbus, OH, USA).

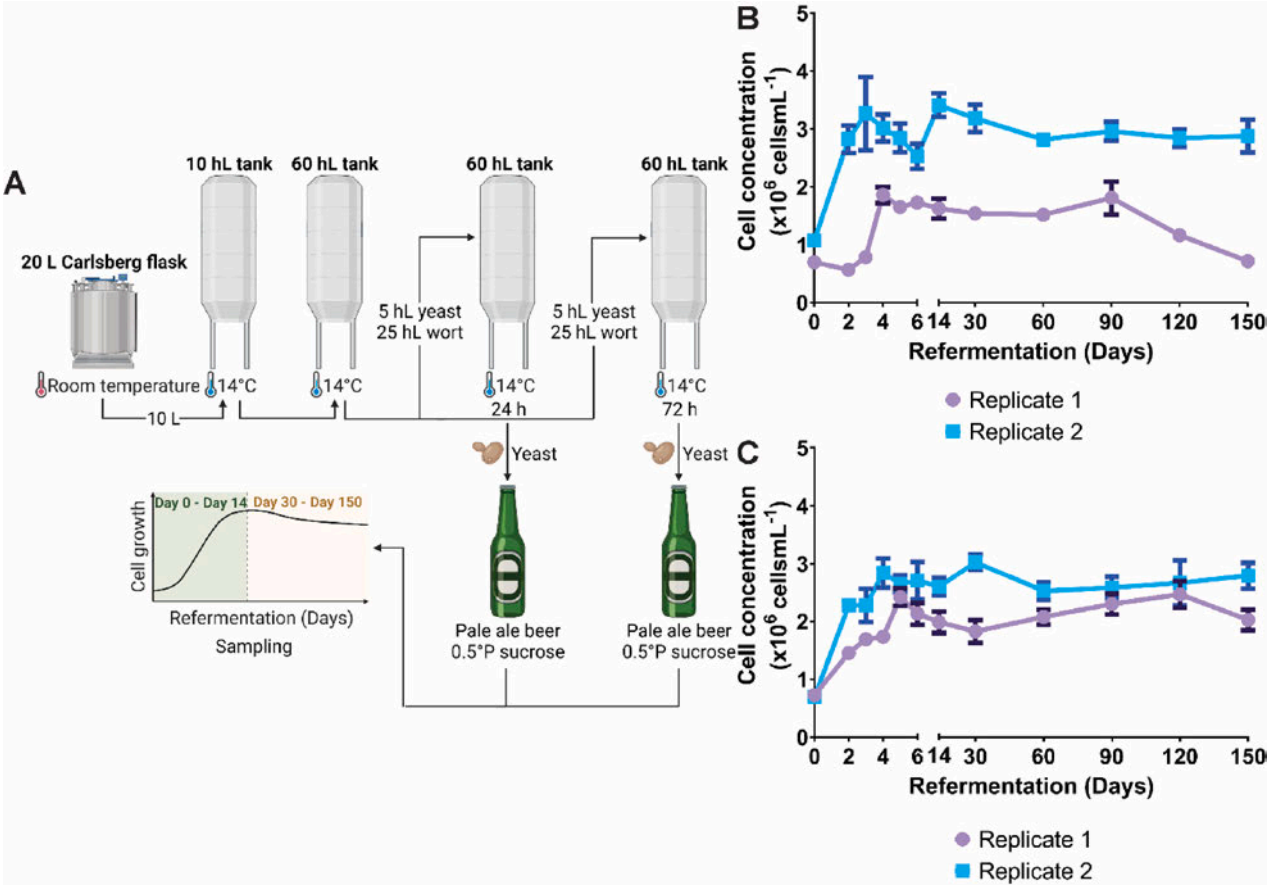


Fig. 1. Time-course of cell growth during bottle conditioning of pale ale beer by brewing yeast. A) Experimental design for the propagation of yeast cells. Yeast was propagated in wort for 24 h or 72 h, seeded into pale ale beer containing 0.5°P sucrose, sealed, and regularly sampled for a 5 month period. B) Y24 and C) Y72 cell concentration during bottle conditioning performed in biological duplicate. Cells were counted manually using a Neubauer cell counting chamber. Values expressed as mean ± standard deviation. Created with BioRender.com.

2.4. Foam stability

Foam stability was measured using a Haffmans NIBEM-TPH Foam Stability Tester (Pentair, London, UK). Beer bottles were immersed in a 20 °C water bath for a minimum of 1 h. The bottle was speared and injected with a combination of carbon dioxide and nitrogen in order to create large and small bubbles, respectively. The foam was forced into clean and dry 330 mL glass and a set of 5 prongs was inserted into the top of the foam head to permit an electrical circuit. When the circuit breaks due to foam collapse, the prongs move downward to restore connectivity. The speed of the foam collapse was measured in seconds over a 30 mm distance.

2.5. Glycogen and trehalose analysis

An analysis of glycogen and trehalose levels was performed as described previously (Shi et al., 2010) with minor modifications. Briefly, yeast cells (1.0×10^7) were obtained from the beer bottle by centrifugation at 4,000*g* for 5 min, washed with PBS, and immediately frozen at 80 °C. Glycogen and trehalose were extracted using 0.125 mL of 0.25 M sodium carbonate (BioExtra) (Sigma-Aldrich) and incubated at 95 °C for 2 h. Samples were acidified by the addition of 0.3 mL of 0.2 M anhydrous sodium acetate (Sigma-Aldrich) and 0.075 mL of 1 M acetic acid (Sigma-Aldrich). The samples were split and transferred to two fresh tubes for a control and digest samples. Amyloglucosidase from *Aspergillus niger* (lyophilized powder, ~70 Umg^{-1}) (Sigma-Aldrich) was added to obtain a final concentration of 1 UmL^{-1} or trehalase from porcine kidney (≥1.0 $unitsmg^{-1}$ protein) (Sigma-Aldrich) was added to the sample to obtain a final concentration of 0.025 UmL^{-1}. Samples for glycogen and trehalose analysis were incubated at 57 °C or 37 °C overnight, respectively. Centrifugation (20,000*g*, 10 min) was performed at room temperature and the supernatant transferred to a fresh tube. A calibration curve was prepared using glucose standard (0–0.08 mg/mL) (Sigma-Aldrich) and the supernatant was diluted to within this range. The standards and samples were added to a 96-well plate in triplicate and analyzed using the glucose (GO) assay kit (Sigma-Aldrich). The absorbance was measured at 540 nm using a Multiskan FC (Thermo Fisher Scientific) and SkanIt Software (ver 4.1).

2.6. Sample preparation for proteomic analysis

Samples were prepared for mass spectrometry analysis using the Sample Preparation by Easy Extraction and Digestion (SPEED) protocol, with some modifications (Doellinger et al., 2020). Yeast cell pellets (1.0×10^7 cells) were slowly defrosted on ice, lysed by trifluoroacetic acid (TFA) (Sigma-Aldrich) at a 1:4 (cell volume: TFA) ratio, and incubated for 10 min at room temperature. Samples were neutralized by the addition of 2 M Tris-base (10X volume of TFA) (Sigma-Aldrich). Proteins were reduced by the addition of 10 Mm tris(2-carboxyethyl)phosphine (Sigma-Aldrich) and incubated at 95 °C for 5 min. 2-Chloroacetamide (Sigma-Aldrich) was added to a final concentration of 15 mM and incubated at 95 °C for 5 min. Samples were cooled to room temperature prior to the addition of Trypsin Gold (Promega (Madison, WI, USA) at a 1:50 (enzyme: protein) ratio and incubated overnight at 37 °C. Samples were acidified to pH 2 using TFA and centrifuged at 20,000*g* for 10 min. Peptides were cleaned using in-house packed C18 (3 M; St. Paul, MN,

USA) StageTips dried to completion under vacuum at 50 °C and stored at 20 °C.

2.7. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

Samples were analyzed by LC-MS/MS using an ACQUITY UPLC M-Class system (Waters Corporation, Milford, MA, USA) connected to a ZenoTOF 7600 mass spectrometer with an OptiFlow Turbo V ion source (SCIEX, Singapore, Singapore). Approximately 1 μg of peptide was resuspended in 99.9% water/ 0.1% formic acid (Thermo Fisher Scientific) (Buffer A) and loaded at 6 μLmin^{-1} onto a nanoEase M/Z HSS T3 reversed phase C18 column (100 Å pore size, 1.8 μm particle size, 300 μm i.d. × 150 mm, Waters) heated to 40 °C. Peptides were separated with a gradient of 3–35% buffer B (99.9% acetonitrile (Merck), 0.1% formic acid) over 45 min, followed by 90% buffer B for 3 min and an equilibration of 3% buffer B for 5 min. Additional settings included: ion source gas 1 and 2 set to 20 and 15 psi, respectively; curtain gas 35 psi, collision gas (CAD) 7, source temperature 150 °C, and the spray voltage set to 5000 V. A Zeno SWATH-DIA scheme (64 variable-size windows covering a 400–1500 *m/z* precursor mass range) was performed with an 0.1 s accumulation time, 80 V declustering potential and 10 V collision energy. Raw data was acquired using SCIEX OS (v3.1.0).

2.8. Data processing and analysis

The mass spectrometry data was processed with Spectronaut 17 (v17.4) (Biognosys, Schlieren, Switzerland) (Bruderer et al., 2015) using the directDIA+ workflow with factory settings, which included enzyme: Trypsin/P, fixed modification: carbamidomethyl (C), variable modifications: acetyl (Protein *N*-terminus) and oxidation (M), false discovery rates at 1%. Mass spectra were searched against the *Saccharomyces cerevisiae* FASTA database from UniProt (yeast: UP000002311_559292, 6060 entries, 15 March 2023). The mass spectrometry proteomics data is available upon request.

3. Results and discussion

3.1. Yeast growth and beer characteristics during bottle refermentation of pale ale beer

Yeast was serially propagated in the laboratory to obtain an initial 20 L culture before pitching into a 10 hL propagator for 24 h and then transferred into a 30 hL propagator (Fig. 1A). Yeast samples were grown for 24 h (Y24) or 72 h (Y72) and then seeded into pale ale beer for bottle conditioning. At their respective seeding time points, Y24 yeast were in the logarithmic phase based upon their continued growth (Fig. S1A), but Y72 cells were in the post-diauxic shift as their concentration plateaued at 32 h–48 h (Fig. S1A). By 48 h, the AE level decrease had plateaued (Fig. S1B) and the maximum ethanol concentration was reached (Fig. S1C), confirming the Y72 cells had entered the post-diauxic shift phase.

It is well known that yeast cells in different growth phases exhibit a different physiology (Gasch & Werner-Washburne, 2002). We were interested in evaluating how this affected yeast metabolism, their survivability, and the final characteristics of the beer. Y24 and Y72 were seeded into 'green' beer at a concentration of 0.5–1.0 × 10^6 cellsmL^{-1} in biological duplicates. Interestingly, Y24 showed different patterns of growth (Fig. 1B.), with replicate 1 having a lag phase at the start of bottle conditioning compared to replicate 2, which entered straight into logarithmic growth. Furthermore, the maximum cell concentration was different between replicates, reaching 1.86 ± 0.1 × 10^6 cellsmL^{-1} and 3.27 ± 0.6 × 10^6 cellsmL^{-1} for replicate 1 and replicate 2, respectively. The growth rate plateaued after 4 days for replicate 1 and 3 days for replicate 2 for Y24. However, both Y72 replicates entered logarithmic growth when seeded into beer and had a more consistent growth pattern compared to Y24 (Fig. 1C.). The maximum cell concentration was also more consistent between replicates, reaching 2.42 ± 0.2 × 10^6 cellsmL^{-1} and 2.84 ± 0.3 × 10^6 cellsmL^{-1} at day 5 for replicate 1 and replicate 2, respectively. The growth rate plateaued after 4 days and 5 days for replicate 1 and replicate 2, respectively, by Y72. Despite both Y24 and Y72 cells performing the active phase of refermentation within a short duration, Y24 cells produced a more variable initial growth profile, suggesting that it was less robust and not particularly well adapted to the beer environment. Furthermore, this may indicate that Y24 cells could result in a less consistent product. We therefore performed experiments to measure the effect of different Y24 and Y72 growth profiles on several beer characteristics. Surprisingly, there were no obvious differences in the AE percentage (Fig. S2A), alcohol content percentage (Fig. S2B), and the pH (Fig. S2C) of the final product prior to distribution (Day 14), indicating that the ability of the yeasts to referment the extract during bottle conditioning was not affected.

3.2. Yeast viability during bottle refermentation over a 5-month period

Although our observations revealed no differences in the refermentation capabilities of Y24 and Y72, we investigated if their survivability was affected. This is important as yeast viability is associated with yeast autolysis, the secretion of unwanted compounds into beer, and a reduction in foam stability (Brey et al., 2003; Chen et al., 1980). Although the viability of the yeast was above 80% for Y24 (Fig. 2A) and Y72 (Fig. 2B) at the start of bottle conditioning, the viability of Y24 started to decrease at 30 days post-seeding for one replicate (Fig. 2A), with both replicates obtaining viabilities below 50% at day 90. However, Y72 viability for both replicates started to decrease at day 60 post-seeding for refermentation (Fig. 2B), with lower than 50% viabilities only occurring at day 120 (replicate 1) and day 150 (replicate 2). This demonstrates that Y24 cells had a more rapid decrease in viability compared to Y72 over a period of 5 months and therefore potentially resulting in poorer shelf-life as the onset of off-flavors would occur earlier (Derdelinckx et al., 1992). Our results agree with the study that stated that the optimal time to transfer yeast from one propagation to the next was during the late logarithmic phase (Hulse, 2003). Additionally, the growth profile of the yeast during bottle conditioning does not correlate with yeast survival.

Foam stability can be affected by yeast viability, since yeast autolysis results in leakage of intracellular compounds into the beer (Song et al., 2019). Specifically, proteinase A (an aspartic acid protease) is secreted into the beer during yeast autolysis. Proteinase A degrades the foam stabilizing protein, Lipid Transfer Protein 1, resulting in a concomitant decrease in foam stability (Leisegang & Stahl, 2005). We observed that as the viability decreased with both yeasts, there was a subsequent significant decrease overall in foam stability (Fig. 2C-D). However, the shorter propagation time contributed to an earlier onset and to a more rapid decline in Y24 cell viability that reduced foam stability, a critical quality metric that is often perceived by a consumer (Aquilani et al., 2015).

3.3. Storage carbohydrates in yeast during the refermentation of beer

It was shown that, despite the propagation time of yeast having a minimal influence on beer characteristics, such as AE, refermentation time, and alcohol content, the viability of Y24 decreased at a faster rate than Y72. Glycogen has been linked with yeast viability (Silljé et al., 1999) and therefore may play a role in yeast survival, whilst trehalose has been shown to have a direct role in stress tolerance (Mansure et al., 1994). We monitored the concentration of glycogen and trehalose in yeast during bottle conditioning to ascertain whether the propagation time influenced their levels and possibly conferred an advantage in terms of survivability (Fig. 3A-D).

The normalized glycogen concentration of Y24 at day 0 was 62 μgmL^{-1} and 11 μgmL^{-1} for replicate 1 and replicate 2, respectively

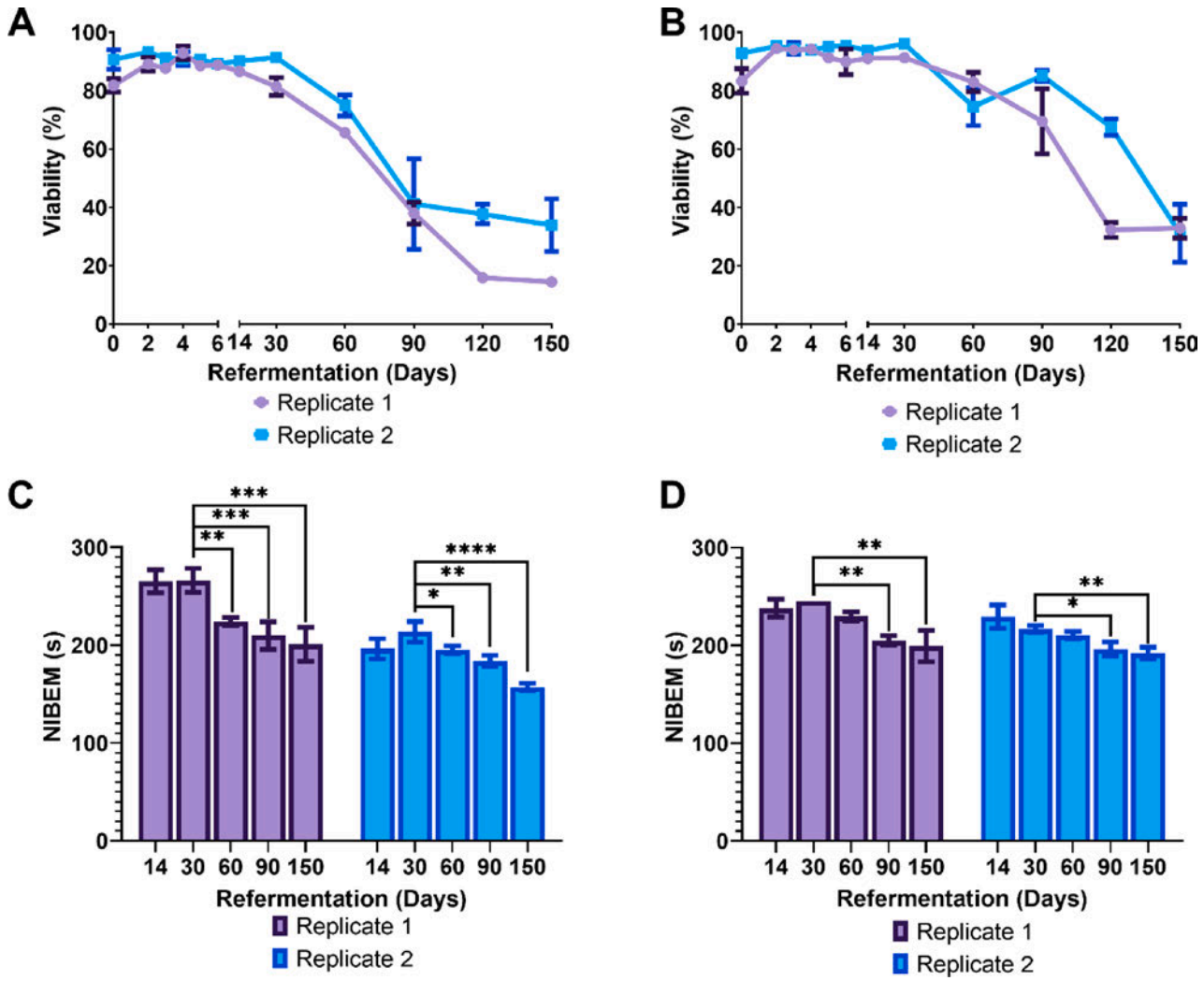


Fig. 2. Influence of propagation time on yeast survival and foam stability during bottle conditioning over 5-months. Viability of A) Y24 and B) Y72 using propidium iodide and acridine orange on a Cellometer X2 automated cell counter. Foam stability of C) Y24 and D) Y72 measured using the NIBEM Foam Stability Tester and values were expressed as mean ± standard deviation and were performed in biological duplicate. Statistical analyses were performed using Ordinary one-way ANOVA: *$p \leq 0.05$, **$p \leq 0.005$, *** $p \leq 0.0005$, **** $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3A). The difference in the glycogen concentration at day 0 was most likely from the variation in sampling time with respect to post-seeding; the collection time of the bottles was different due to changes in the production schedule. As the yeast was collected later for replicate 2, some of the available glycogen may have been consumed. Interestingly, the pattern of glycogen accumulation and degradation at the start of bottle conditioning was different between biological replicates for Y24 and related to the differences in their growth profiles (Fig. 1B). The slower metabolism of glycogen in replicate 1 of Y24 was associated with the length of the lag phase during bottle conditioning. The decline in glycogen metabolism during the early stages of refermentation could be for *de novo* lipogenesis in the presence of oxygen, which is essential for growth during anaerobic conditions (Sall et al., 1988). Afterwards, the glycogen concentration achieved a maximum concentration of 36 μgmL^{-1} and 41 μgmL^{-1} at day 6 for replicate 1 and replicate 2, respectively, in Y24.

Similarly, the concentration of glycogen at day 0 for Y72 was 74 μgmL^{-1} and 10 μgmL^{-1} for replicate 1 and replicate 2, respectively (Fig. 3B). Additionally, Y72 reached a maximum concentration of glycogen of 36 μgmL^{-1} (Day 5) and 42 μgmL^{-1} (Day 6) for replicate 1 and replicate 2, respectively. The accumulation of glycogen during refermentation was expected as it is known that glycogen synthesis occurs under carbon and energy limitation (Lillie & Pringle, 1980). The plateauing of glycogen accumulation occurred with the ceasing of yeast cell growth (Fig. 1B-C) and the consumption of AE in Y24 (Fig. S3A) and Y72 (Fig. S3B). A decline in glycogen concentration occurred after the maximum concentration was reached and was minimal at day 60 for all samples. As the glycogen was similarly metabolized for Y24 and Y72, it is suggested that the glycogen concentration did not directly confer an advantage in yeast longevity.

Trehalose was degraded in a similar pattern to glycogen when seeded into beer for Y24 (Fig. 3C). This was previously shown in yeast cells inoculated into synthetic grape must and the metabolism of trehalose was suggested to enable cells to adapt their metabolic machinery to the new medium (Novo et al., 2003). For Y24 (replicate 1), trehalose levels decreased from day 0 to day 4 during the lag phase, but rapidly increased by day 5 (onset of the stationary phase), and then continued to slowly increase until day 14, reaching a maximum level of 18 μgmL^{-1}. The slow decrease in trehalose concentration from day 0 to day 4 by Y24 (replicate 1) was associated with an increased lag phase. Although the reason for this occurring is unclear, this has been reported in wine yeast (Novo et al., 2003). Like glycogen, the low concentration of trehalose at day 0 for replicate 2 in Y24 was most likely due to the sampling time of the bottles from the production line as discussed previously, which was

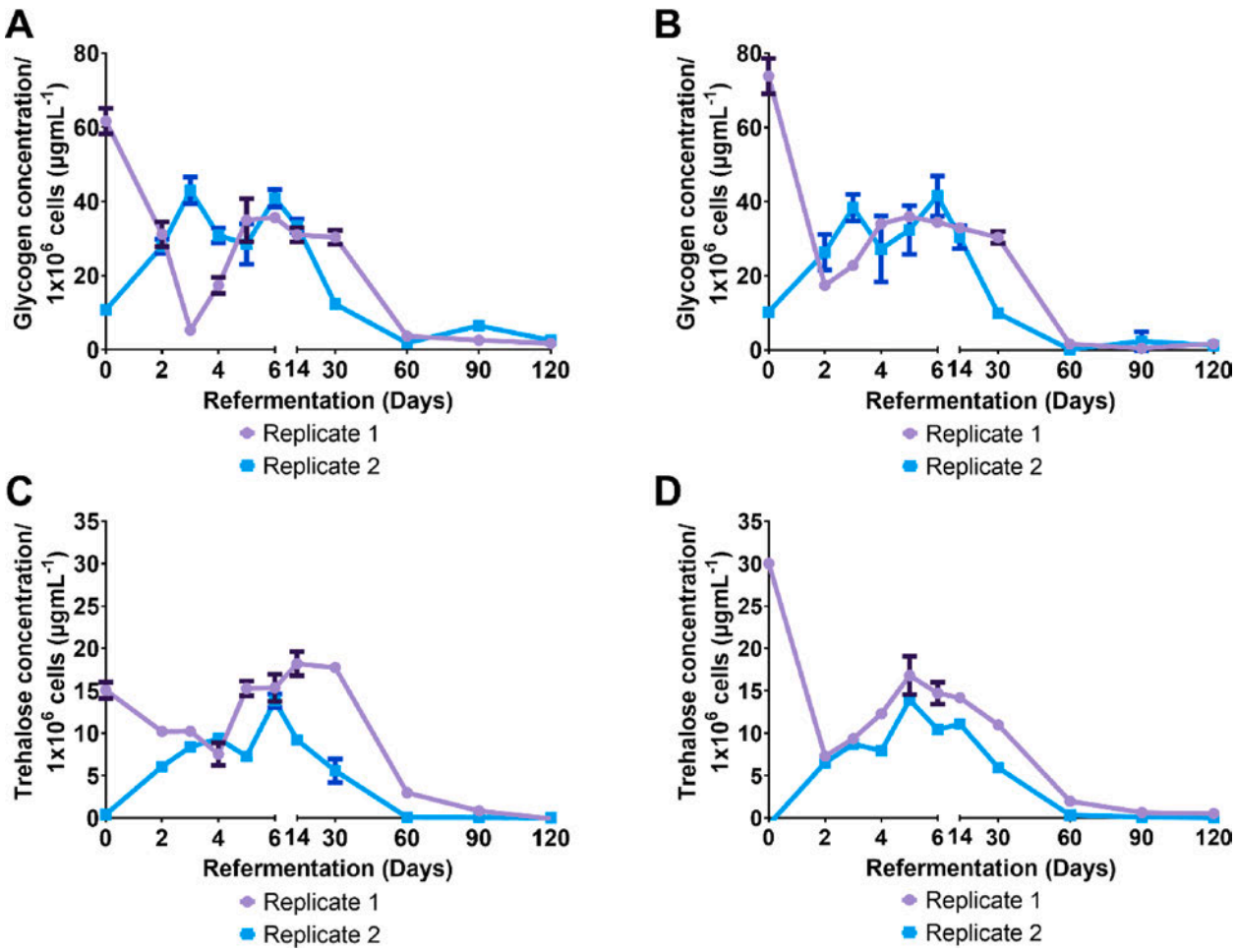


Fig. 3. Changes in storage carbohydrates in yeast during bottle conditioning. Glycogen concentration normalized to extracted cell number for A) Y24 and B) Y72. Trehalose concentration normalized to extracted cell number for C) Y24 and D) Y72. Values expressed as mean ± standard deviation and was performed in biological duplicate.

difficult to control at an industrial scale. In contrast to replicate 1 for Y24, trehalose began to accumulate immediately after day 0 for Y24 (replicate 2) and reached a maximum level of 14 µgmL^{-1} at day 6.

Similarly, to Y24 and glycogen concentrations, there was a disparity in the concentration of trehalose at day 0 for Y72 (Fig. 3D). However, the concentration of trehalose gradually increased after day 2 (replicate 1) and day 0 (replicate 2) and reached a maximum concentration of 17 µgmL^{-1} and 14 µgmL^{-1} at day 5 for replicate 1 and replicate 2, respectively, in Y72. Irrespective of the propagation time, the concentration of trehalose decreased after reaching a maximum concentration. The concentration of trehalose at day 60 was minuscule and most likely the result of a higher number of dead cells in the samples.

Although the concentration of glycogen and trehalose did not appear to confer an advantage in yeast longevity, the profiles of their accumulation and degradation in Y72 cells were more consistent between the replicates. This is associated with the more consistent growth profile observed for Y72 and demonstrates that propagating for an increased time improved the consistency of refermentation.

3.4. Proteomic analysis of propagated yeast prior to bottle conditioning

It is clear from our analysis that propagation time does play a role in the ability of yeast to survive longer during bottle conditioning. To obtain a better understanding of the underlying causes of the physiological differences between Y24 and Y72, we conducted a Zeno SWATH DIA analysis of Y24 and Y72 cells at the time of seeding for bottle conditioning. Data analysis was performed using Spectronaut 17 (Bruderer et al., 2015) and resulted in the identification of 3,291 proteins across all the samples (Table S1). We observed 625 upregulated and 415 downregulated proteins to be significantly (adj. p-value < 0.05) changed in abundance by at least 1.5-fold ($\log_2 \geq 0.58$) in Y72 compared to Y24 (Fig. 4A, Table S2-S4).

To investigate the major metabolic processes that were differentially regulated between Y24 and Y72, we used the DAVID functional annotation tool to assign over-represented gene ontology terms (Benjamini-Hochberg corrected p-value < 0.05) (Table 1) (Huang et al., 2009). Overall, there were 13 biological processes upregulated and 2 biological processes downregulated.

We observed an increase in proteins involved in the electron transport chain, including those of complex III or Cytochrome *bc*1 (Cyc1, Qcr6-10p, and Rip1p), complex IV or Cytochrome *c* oxidase (Cox4-8p, Cox12-13p), and complex V or ATP synthase (Atp5p, Atp7p, Atp14-16p, Atp18-19p, and Tim11p). The increase in the abundance of these proteins suggested that metabolism in Y72 had switched from glucose fermentation to ethanol respiration. This is largely in agreement with the study that investigated the mitochondrial protein changes in yeast during the diauxic shift that found proteins involved in the tricarboxylic acid cycle, the respiratory chain, and ATP synthase were upregulated in the diauxic shift (Di Bartolomeo et al., 2020). This is consistent with the decrease in alcohol concentration occurring at the later stages of propagation (48 h–72 h) in Y72 cells (Fig. S1C). Furthermore, a concomitant increase in the abundance of mitochondrial ribosomal proteins for the

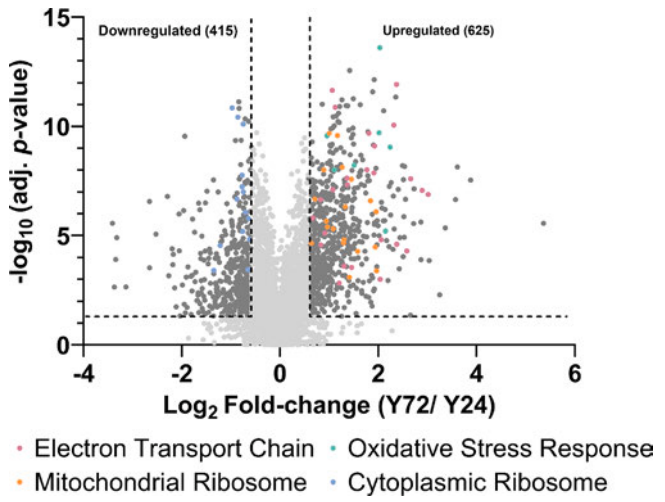


Fig. 4. Proteomic analysis of yeast cells at the time of seeding for bottle conditioning. Volcano plot displaying the -log10 adj. *p*-value and the log2 fold-change between Y72 and Y24. Highlighted are proteins that were differentially regulated and over-represented using functional annotation. Electron transport chain (pink); Cytoplasmic ribosome (blue); Mitochondrial ribosome (orange); oxidative stress response (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Gene ontology of biological processes for proteins with increased or decrease abundance in Y72 compared to Y24.

	Biological Process	Number of Proteins	Fold Enrichment	-log10 adj. *p*-value
UP	Cellular respiration	17	4.89	4.31
	Hydrogen ion transmembrane transport	25	2.68	2.50
	Mitochondrial translation	29	2.45	2.50
	Protein folding	24	2.52	2.02
	Positive regulation of transcription initiation from RNA polymerase II promoter	12	4.16	2.02
	Mitochondrial electron transport, ubiquinol to cytochrome *c*	9	5.66	2.02
	RNA polymerase II transcriptional preinitiation complex assembly	15	3.21	1.79
	Oxidative phosphorylation	7	7.04	1.79
	Positive regulation of transcription from RNA polymerase II promoter	46	1.76	1.79
	Mitochondrial electron transport, cytochrome *c* to oxygen	9	5.03	1.79
	Cellular response to oxidative stress	22	2.38	1.67
	ATP synthesis coupled proton transport	9	4.77	1.67
	ATP biosynthetic process	8	5.37	1.64
DOWN	Cytoplasmic translation	28	2.34	1.49
	Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	17	3.14	1.49

small complex (37S) and large complex (54S) was observed, which was expected as they are responsible for the translation of necessary polypeptides in the electron transport chain complexes (Derbikova et al., 2018). It has been well established that mitochondria play an important role in the extension of the yeast's chronological lifespan (Pan et al., 2011). Specifically, activation of mitochondrial respiration above a certain threshold leads to the sufficient storage of nutrients, induces stress resistance, and results in the prolongation of the yeast's chronological lifespan (Ocampo et al., 2012). The upregulation of cellular respiration may have led to increased stress resistance in Y72 and thus their ability to survive longer during bottle conditioning.

There was also an increased abundance of different stress-related proteins in Y72 including those associated with general stress (Hsp26p, Hsp42p, and Ssa1p) and oxidative stress (Cta1p, Grx1p, Grx2p, Sod1p, and Trx1-3p) responses. This is consistent with a study that investigated temporal proteome dynamics during the diauxic shift and showed that proteins related to the stress response (Hsp26p, Hsp42p, and Ssa1p) and oxidative stress (Grx1p, Grx2p, Sod1p, and Trx3p) were induced early during the metabolic shift (Murphy et al., 2015). The observed increases in oxidative stress proteins may have occurred due to elevated levels of reactive oxygen species as a result of leakage from the respiratory chain and damaging proteins (Baccolo et al., 2018). Alternatively, a previous genomic study that investigated the oxidative stress response during a brewery propagation showed that antioxidant genes are upregulated as a general stress response to glucose limitation (Gibson et al., 2008). The induction of oxidative and general stress pathways could potentially have a hormetic effect on Y72 longevity during bottle conditioning. Hormesis is a phenomenon whereby exposure to mild stress can induce resistance against another stress (Wauters et al., 2021) and there is support for the role of reactive oxygen species having a hormetic effect on longevity (Pan et al., 2011). This has been demonstrated within the brewing context, where yeast that was adapted to stress (compared to yeast that was grown in rich nutrients) during propagation were able to referment the beer better (Rogers et al., 2016). Irrespective of the nature of the stressors that resulted in the increased abundance of stress-related proteins, our results indicate that respiration and the cellular stress responses were likely contributors to increased stress resistance in Y72 cells and therefore their ability to survive longer during bottle conditioning.

Proteins that play a role in cell wall remodeling and binding were significantly upregulated in Y72 cells, such as the cell wall mannoproteins Cwp1p, Ccw14p, Cis3p, and Pir1p. It has been shown that mannoproteins are an important determinant of cell wall porosity and that their disruption can lead to higher susceptibility to cell wall perturbations (De Nobel et al., 1990). Furthermore, Pir1p, a protein directly linked to the 1,3-β-glucan network was reported to increase in response to cell wall stress, suggesting a role for this protein in cell wall reinforcement (Boorsma et al., 2004). Proteins involved in cell wall biosynthesis (Chs2p, Fks1p, and Kre6p) were downregulated in Y72. Chs2p is involved in the synthesis of chitin during cytokinesis (Klis et al., 2006), whilst Fks1p expression is higher under optimal growth conditions (Mazur et al., 1995). Alternatively, this may explain the higher abundance of Chs2p and Fks1p in Y24 cells as they were in logarithmic phase.

The downregulation of ribosomal proteins in Y72 was anticipated, as cellular growth had slowed considerably due to the prolonged propagation time compared to Y24 cells (Fig. S1A). The repression of ribosomal proteins was consistent with earlier genomic (Derisi, Vishwanath, & Brown, 1997) and proteomic (Murphy et al., 2015) studies, where ribosomal biogenesis was decreased as cellular growth decreased.

4. Conclusion

In this study, we examined whether the propagation time of yeast prior to seeding into beer influences their longevity during bottle conditioning. We discovered that the longevity of yeast seeded after reaching the post-diauxic shift (Y72) was increased compared to yeast seeded in the logarithmic phase (Y24). Interestingly, several beer characteristics (AE, alcohol, and pH) during bottle conditioning were similar regardless of propagation time. However, the foam stability of the product decreased concomitantly with cell viability. The glycogen and trehalose levels of Y24 and Y72 did not seem to provide any

advantage in terms of longevity, with Y24 and Y72 reaching comparable maximum concentrations after one week of bottle conditioning and were similarly consumed afterwards. Proteomic analysis of Y24 and Y72 at the point of seeding showed that there was an upregulation in cellular respiration, several stress response pathways and cell wall mannoproteins in Y72. These responses may have increased the stress resistance properties of Y72 cells and therefore contributed to their longevity during bottle conditioning.

Future work could investigate the influence of changing other propagation parameters, such as aeration times and initial AE levels, to influence yeast metabolism and reduce propagation time. Additionally, the influence of seeding yeast later in the logarithmic phase may provide brewers with a time span where yeast can reach optimal performance for bottle conditioning. Overall, the results of this study show that the propagation time of yeast did not severely impact the ability of yeast to referment during bottle conditioning, but it does impact on their longevity. Although these differences might be rather subtle, they can impact other quality metrics that are perceived to be important by the consumer, such as the beer's foam stability. These results indicate that propagating yeast cells after they reached the diauxic shift or the stationary phase improves the batch-to-batch consistency of the beer, foam stability, and yeast longevity.

CRediT authorship contribution statement

Brooke A. Dilmetz: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Christopher T. Desire:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Jon Meneses:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration. **Manuela Klingler-Hoffmann:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Clifford Young:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Peter Hoffmann:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137655.

References

Aquilani, B., Laureti, T., Poponi, S., & Secondi, L. (2015). Beer choice and consumption determinants when craft beers are tasted: An exploratory study of consumer preferences. *Food Quality and Preference, 41*, 214–224. https://doi.org/10.1016/j.foodqual.2014.12.005

Baccolo, G., Stamerra, G., Coppola, D. P., Orlandi, I., & Vai, M. (2018). Chapter One - Mitochondrial Metabolism and Aging in Yeast. In C. López-Otín, & L. Galluzzi (Eds.), *International Review of Cell and Molecular Biology* (pp. 1–33). Academic Press.

Boorsma, A., Nobel, H. D., Riet, B. T., Bargmann, B., Brul, S., Hellingwerf, K. J., & Klis, F. M. (2004). Characterization of the transcriptional response to cell wall stress in Saccharomyces cerevisiae. *Yeast, 21*(5), 413–427. https://doi.org/10.1002/yea.1109

Brey, S. E., de Costa, S., Rogers, P. J., Bryce, J. H., Morris, P. C., Mitchell, W. J., & Stewart, G. G. (2003). The effect of proteinase a on foam-active polypeptides during high and low gravity fermentation. *Journal of the Institute of Brewing, 109*(3), 194–202. https://doi.org/10.1002/j.2050-0416.2003.tb00159.x

Bruderer, R., Bernhardt, O. M., Gandhi, T., Miladinović, S. M., Cheng, L.-Y., Messner, S., … Reiter, L. (2015). Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues. *Molecular & Cellular Proteomics, 14*(5), 1400–1410. https://doi.org/10.1074/mcp.M114.044305

Cao, L., Tang, Y., Quan, Z., Zhang, Z., Oliver, S. G., & Zhang, N. (2016). Chronological lifespan in yeast is dependent on the accumulation of storage carbohydrates mediated by Yak1, Mck1 and Rim15 Kinases. *PLOS Genetics, 12*(12), e1006458.

Chen, E. C. H., Jamieson, A. M., & Van Gheluwe, G. (1980). The release of fatty acids as a consequence of yeast autolysis. *Journal of the American Society of Brewing Chemists, 38*(1), 13–18. https://doi.org/10.1094/ASBCJ-38-0013

De Nobel, J. G., Klis, F. M., Priem, J., Munnik, T., & Van Den Ende, H. (1990). The glucanase-soluble mannoproteins limit cell wall porosity in Saccharomyces cerevisiae. *Yeast, 6*(6), 491–499. https://doi.org/10.1002/yea.320060606

Dekoninck, T. M. L., Mertens, T., Delvaux, F., & Delvaux, F. R. (2013). Influence of beer characteristics on yeast refermentation performance during bottle conditioning of Belgian beers. *Journal of the American Society of Brewing Chemists, 71*(1), 23–34. https://doi.org/10.1094/ASBCJ-2013-0118-01

Derbikova, K. S., Levitsky, S. A., Chicherin, I. V., Vinogradova, E. N., & Kamenski, P. A. (2018). Activation of yeast mitochondrial translation: Who is in charge? *Biochemistry (Moscow), 83*(2), 87–97. https://doi.org/10.1134/S0006297918020013

Derdelinckx, G., Vanderhasselt, B., Maudoux, M., & Dufour, J. (1992). Refermentation in bottles and kegs. A rigorous approach. *Brauwelt International, 2*, 156–164.

Derisi, J. L., Vishwanath, R. I., & Brown, P. O. (1997). Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale. *Science, 278*, 680–686. https://doi.org/10.1126/science.278.5338.680

Di Bartolomeo, F., Malina, C., Campbell, K., Mormino, M., Fuchs, J., Vorontsov, E., … Nielsen, J. (2020). Absolute yeast mitochondrial proteome quantification reveals trade-off between biosynthesis and energy generation during diauxic shift. *Proceedings of the National Academy of Sciences, 117*(13), 7524–7535. https://doi.org/10.1073/pnas.1918216117

Doellinger, J., Schneider, A., Hoeller, M., & Lasch, P. (2020). Sample preparation by easy extraction and digestion (SPEED) - A universal, rapid, and detergent-free protocol for proteomics based on acid extraction. *Molecular & Cellular Proteomics, 19*(1), 209–222. https://doi.org/10.1074/mcp.TIR119.001616

Gasch, A. P., & Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. *Functional & Integrative Genomics, 2*(4), 181–192. https://doi.org/10.1007/s10142-002-0058-2

Gibson, B. R., Lawrence, S. J., Boulton, C. A., Box, W. G., Graham, N. S., Linforth, R. S. T., & Smart, K. A. (2008). The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation. *FEMS Yeast Research, 8*(4), 574–585. https://doi.org/10.1111/j.1567-1364.2008.00371.x

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols, 4*(1), 44–57. https://doi.org/10.1038/nprot.2008.211

Hulse, G. A. (2003). Yeast Propagation. In K. Smart (Ed.), *Brewing Yeast Fermentation Performance* (pp. 249–256). Blackwell Science.

Klis, F. M., Boorsma, A., & De Groot, P. W. J. (2006). Cell wall construction in Saccharomyces cerevisiae. *Yeast, 23*(3), 185–202. https://doi.org/10.1002/yea.1349

Leisegang, R., & Stahl, U. (2005). Degradation of a Foam-Promoting Barley Protein by a Proteinase from Brewing Yeast. *Journal of the Institute of Brewing, 111*(2), 112–117. https://doi.org/10.1002/j.2050-0416.2005.tb00656.x

Lillie, S. H., & Pringle, J. R. (1980). Reserve carbohydrate metabolism in Saccharomyces cerevisiae: Responses to nutrient limitation. *Journal of Bacteriology, 143*(3), 1384–1394. https://doi.org/10.1128/jb.143.3.1384-1394.1980

Liu, S., Kerr, E. D., Pegg, C. L., & Schulz, B. L. (2022). Proteomics and glycoproteomics of beer and wine. *Proteomics, 22*(15–16), 2100329. https://doi.org/10.1002/pmic.202100329

Lodolo, E., Kock, J. L. F., Axcell, B. C., & Brooks, M. (2008). The yeast Saccharomyces cerevisiae– the main character in beer brewing. *FEMS Yeast Research, 8*(7), 1018–1036. https://doi.org/10.1111/j.1567-1364.2008.00433.x

Mansure, J. J. C., Panek, A. D., Crowe, L. M., & Crowe, J. H. (1994). Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochimica et Biophysica Acta (BBA) - Biomembranes, 1191*(2), 309–316. https://doi.org/10.1016/0005-2736(94)90181-3

Marconi, O., Rossi, S., Galgano, F., Sileoni, V., & Perretti, G. (2016). Influence of yeast strain, priming solution and temperature on beer bottle conditioning. *Journal of the*

Science of Food and Agriculture, 96(12), 4106–4115. https://doi.org/10.1002/jsfa.7611

Mazur, P., Morin, N., Baginsky, W., El-Sherbeini, M., Clemas, J. A., Nielsen, J. B., & Foor, F. (1995). Differential expression and function of two homologous subunits of yeast 1,3-beta-D-glucan synthase. *Molecular and Cellular Biology, 15*(10), 5671–5681. https://doi.org/10.1128/MCB.15.10.5671

Moutsoglou, M. E., & Dearden, A. C. (2020). Effect of the respiro-fermentative balance during yeast propagation on fermentation and wort attenuation. *Journal of the Institute of Brewing, 126*(3), 289–297. https://doi.org/10.1002/jib.621

Murphy, J. P., Stepanova, E., Everley, R. A., Paulo, J. A., & Gygi, S. P. (2015). Comprehensive temporal protein dynamics during the diauxic shift in saccharomyces cerevisiae. *Molecular & Cellular Proteomics, 14*(9), 2454–2465. https://doi.org/10.1074/mcp.M114.045849

Novo, M. T., Beltran, G., Torija, M. J., Poblet, M., Rozès, N., Guillamón, J. M., & Mas, A. (2003). Changes in wine yeast storage carbohydrate levels during preadaptation, rehydration and low temperature fermentations. *International Journal of Food Microbiology, 86*(1), 153–161. https://doi.org/10.1016/S0168-1605(03)00253-8

Ocampo, A., Liu, J., Schroeder, E. A., Shadel, G. S., & Barrientos, A. (2012). Mitochondrial respiratory thresholds regulate yeast chronological life span and its extension by caloric restriction. *Cell Metabolism, 16*(1), 55–67. https://doi.org/10.1016/j.cmet.2012.05.013

Pan, Y., Schroeder, E. A., Ocampo, A., Barrientos, A., & Shadel, G. S. (2011). Regulation of yeast chronological life span by TORC1 via adaptive mitochondrial ROS signaling. *Cell Metabolism, 13*(6), 668–678. https://doi.org/10.1016/j.cmet.2011.03.018

Rogers, C. M., Veatch, D., Covey, A., Staton, C., & Bochman, M. L. (2016). Terminal acidic shock inhibits sour beer bottle conditioning by Saccharomyces cerevisiae. *Food Microbiology, 57*, 151–158. https://doi.org/10.1016/j.fm.2016.02.012

Saison, D., De Schutter, D. P., Delvaux, F., & Delvaux, F. R. (2011). Improved flavor stability by aging beer in the presence of yeast. *Journal of the American Society of Brewing Chemists, 69*(1), 50–56. https://doi.org/10.1094/ASBCJ-2011-0127-01

Sall, C. J., Seipp, J. F., & Pringle, A. T. (1988). Changes in Brewer's yeast during storage and the effect of these changes on subsequent fermentation performance. *Journal of the American Society of Brewing Chemists, 46*(2), 23–25. https://doi.org/10.1094/ASBCJ-46-0023

Shi, L., Sutter, B. M., Ye, X., & Tu, B. P. (2010). Trehalose is a key determinant of the quiescent metabolic state that fuels cell cycle progression upon return to growth. *Molecular Biology of the Cell, 21*(12), 1982–1990. https://doi.org/10.1091/mbc.e10-01-0056

Silljé, H. H. W., Paalman, J. W. G., ter Schure, E. G., Olsthoorn, S. Q. B., Verkleij, A. J., Boonstra, J., & Verrips, C. T. (1999). Function of trehalose and glycogen in cell cycle progression and cell viability in *Saccharomyces cerevisiae*. *Journal of Bacteriology, 181*(2), 396–400. https://doi.org/10.1128/JB.181.2.396-400.1999

Song, L., Chen, Y., Guo, Q., Huang, S., Guo, X., & Xiao, D. (2019). Regulating the Golgi apparatus sorting of proteinase A to decrease its excretion in Saccharomyces cerevisiae. *Journal of Industrial Microbiology and Biotechnology, 46*(5), 601–612. https://doi.org/10.1007/s10295-019-02147-9

Van Landschoot, A., Vanbeneden, N., Vanderputten, D., & Derdelinckx, G. (2004). Effect of pitching yeast preparation on the refermentation of beer in bottles. *Cerevisia, 29*(3), 140–145.

Vanbeneden, N., Vanderputten, D., Vanderhaegen, B., Derdelinckx, G., & Van Landschoot, A. (2006). Influence of the sugar composition of the added extract on the refermentation of beer in bottles. *Journal of the American Society of Brewing Chemists, 64*(4), 206–213. https://doi.org/10.1094/ASBCJ-64-0206

Wauters, R., Britton, S. J., & Verstrepen, K. J. (2021). Old yeasts, young beer—The industrial relevance of yeast chronological life span. *Yeast, 38*(6), 339–351. https://doi.org/10.1002/yea.3650

Werner-Washburne, M., Braun, E., Johnston, G. C., & Singer, R. A. (1993). Stationary phase in the yeast Saccharomyces cerevisiae. *Microbiological Reviews, 57*(2), 383–401. https://doi.org/10.1128/mr.57.2.383-401.1993

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4 Performance of Different *Saccharomyces* Strains on Secondary Fermentation during the Production of Beer

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All **Tables** and **Figures** that correspond to the supplementary documentation within this chapter can be accessed via the appropriate Digital Object Identifier (DOI) listed above.

Summary

Selection of a yeast strain for bottle conditioning is important. It has been shown that different yeast strains have different propensities to survive. To determine whether an alternative yeast could have better longevity in bottle conditioning, we compared six yeast strains from the brewing, sparkling wine, and distilling industries. We propagated the yeast in the laboratory until the stationary phase and then seeded the yeast into pale ale beer that was primed with fermentable extract. Beer quality attributes such as apparent extract, alcohol concentration, foam stability, and sensory profiles were investigated over four months. Additionally, yeast physiology was evaluated using cell counts and viability. Proteomic analysis was performed to gain an improved understanding of the biological processes that enabled certain yeast strains to perform better during bottle conditioning.

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Performance of Different Saccharomyces Strains on Secondary Fermentation during the Production of Beer

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Abstract: Bottle conditioning of beer is an additional fermentation step where yeast and fermentable extract are added to the beer for carbonation. During this process, yeast must overcome environmental stresses to ensure sufficient fermentation in the bottle. Additionally, the yeast must be able to survive for a prolonged time, as a decline in viability will lead to alterations in the product. Here, we investigated the effects of bottle conditioning on beer using six different yeast strains from the brewing, wine making, and distilling industries over 120 days. The ale and lager strains resulted in a beer possessing typical characteristics of a pale ale-style beer, whereas sparkling wine and distilling yeast strains resulted in aromas that were uncharacteristic, which was expected. In addition, we observed that the various strains had different propensities to survive during bottle conditioning. Proteomic analysis was performed to ascertain protein abundance changes and reveal biological processes that potentially enabled specific yeast strains to survive longer during secondary fermentation. Our results showed that proteins associated with oxidoreductase activity and mitochondrial ribosomes were increased in the yeast strain with superior survival and were able to respond to cellular stress more effectively, whereas proteins associated with cell wall modulation were increased in the strain with poor survival characteristics. Overall, we demonstrated the impact of yeast selection on bottle conditioning and the biological processes involved in yeast physiology under these conditions.

Keywords: *Saccharomyces cerevisiae*; bottle conditioning; beer; proteomics; viability; secondary fermentation

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1. Introduction

Bottle conditioning, which is also known as beer refermentation, is a commonly used process among breweries in Belgium, the UK, and the USA [1]. This process involves the inoculation of yeast into mature beer that has been primed with a fermentable extract such as sugar, where the yeast performs a secondary fermentation for one to two weeks [2]. During bottle conditioning the yeast naturally carbonates the beer and improves flavour stability [3]. Flavour stability is mainly improved as the yeast reduces the formation of ageing compounds such as (E)-2-nonenal and diacetyl [4,5].

Yeast has four distinct growth stages during secondary fermentation: a lag phase, a growth phase, a stationary phase, and a death phase [3]. Ideally, the lag phase should be non-existent or minimal during bottle conditioning as it is at this time that the risk of microbial contamination is highest [3]. The yeast subsequently enters the growth phase where they rapidly divide, consume the available fermentable extract, and produce ethanol, carbon dioxide, and other aromatic compounds [6]. Yeast growth slows as the concentration of fermentable extract declines and the yeast enter the stationary phase. After a prolonged period in the stationary phase, the viability of the yeast declines.

The positive influence of yeast on flavour stability during bottle conditioning relies on the yeast maintaining viability. As viability declines, the yeast undergoes autolysis,

which results in the accumulation of intracellular compounds such as fatty acids and proteases in the beer, ultimately impacting the flavour and foam stability [7,8]. Beer is a sub-optimal environment for yeast growth as it is nutrient poor, has low pH, a high ethanol concentration, carbon dioxide pressure, and experiences changes in temperature during transport and storage [6]. It is likely that the ability of the yeast to withstand these conditions is imperative to maintaining beer quality and improving shelf-life characteristics. Therefore, it is crucial to select a yeast strain that has an increased lifespan during these conditions and does not compromise the sensory aroma of the beer.

Bottle conditioning is also used to produce sparkling wines [9]. The capacity of the yeasts to survive in this environment have also been considered important to ensure successful bottle conditioning in sparkling wine production [10,11]. Additionally, a transcriptomic study reported that despite some similarities to primary fermentation, metabolic processes in yeast such as low temperature and carbon dioxide pressure were specific to secondary fermentation [9].

The aim of this study was to compare the viability and impact of different yeast strains on bottle-conditioned beer and to investigate the biological processes that underpin their ability to survive. Here, we analysed the impact of yeast strains utilised in the brewing, wine, and distilling industries, and their influence on different attributes of the beer, such as alcohol concentration, apparent extract, foam stability, and aroma during bottle conditioning over four months. Volatile and sensory analysis for the different yeast strains is important as changes to the aroma can impact the product and consumer preference. The viability of the yeast during prolonged bottle conditioning was also evaluated. Proteomic analysis was performed to gain a better understanding of the underlying biological process that enabled certain yeast strains to survive longer during bottle conditioning. Biological processes associated with oxidoreductase metabolism were upregulated in the strain with good survival characteristics, whereas processes associated with cell wall organisation were downregulated in yeast that had poor survival during bottle conditioning.

2. Materials and Methods

2.1. Yeast Strains, Media, and Culture Conditions

The six Saccharomyces strains were obtained from different fermentation industries such as brewing, wine making, and distilling (Table 1).

Ale brewing yeast 1 and Lager brewing yeast 1 were streaked onto Wallerstein laboratory nutrient agar (WLN-A) (Part number: PP2137, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 25 °C for 5 days. Ale brewing yeast 2 was isolated by filtration of a commercial beer with a 0.2 µm filter and incubated at 25 °C for 5 days on WLN-A. Colonies were re-streaked onto WLN-A and incubated at 25 °C for 5 days. Approximately 1 g of the dried yeasts (Lager brewing yeast 2, Sparkling wine yeast, and Distilling yeast) were added to 10 mL of sterile water and incubated at 30 °C for 30 min. The yeast was diluted, spread onto WLN-A, and incubated at 25 °C for 5 days. All yeast strains were maintained at 4 °C.

Wort was prepared from a concentrate (Coopers Brewery Ltd., Regency Park, Australia) and made up to a gravity of 12.35 ± 0.04 °P before autoclaving. Two biological trials for each yeast strain were carried out by inoculating colonies of each yeast strain into 15 mL of wort in disposable 50 mL sterile screw cap containers. The yeast cultures were incubated at 20 °C with shaking at 170 rpm for 24 h. The cultures were transferred to 150 mL of wort in 250 mL glass bottles and incubated at 20 °C for 24 h. Afterwards, the 150 mL cultures were transferred to 1500 mL of wort in 2000 mL glass shake flasks that were plugged with cotton wool and incubated at 20 °C for 72 h.

Pale ale beer was primed with 0.5 °P sucrose and then capped in the brewery. The beer was uncapped, inoculated with yeast at approximately 0.2×10^6–1.0×10^6 cells/mL, and then recapped. Beer was stored at 20–22 °C for 1 day, 14 days, 30 days, 60 days, 90 days, and 120 days before opening.

Table 1. Commercial Saccharomyces strains used in this study.

Name	Strain	Taxonomic Name	Commercial Use	Supplied Format	Source
Ale brewing yeast 1	NA	*S. cerevisiae*	Primary ale fermentation and secondary fermentation of beer	Provided by agar slant	Coopers Brewery Ltd. (Regency Park, Australia)
Ale brewing yeast 2	NA	*S. cerevisiae*	Secondary fermentation of beer	Isolated by filtration from a commercial beer	Dan Murphy's (Adelaide, Australia)
Lager brewing yeast 1	Nebulosa-TUM 66/70	*S. pastorianus*	Primary and secondary fermentation of beer	Provided by agar slant	Weihenstephan for Brewing and Food Quality, (Munich, Germany)
Lager brewing yeast 2	SafLager 34/70	*S. pastorianus*	Primary lager fermentation	Dried yeast	Coopers Brewery Ltd., (Regency Park, Australia)
Sparkling wine yeast	Lalvin EC-1118™	*Saccharomyces cerevisiae bayanus*	Secondary fermentation of sparkling wine	Dried yeast	Wine Quip, (Reservoir, Australia)
Distilling yeast	DistilaMax® HT	*S. cerevisiae*	Neutral spirit fermentation	Dried yeast	Wine Quip, (Reservoir, Australia)

2.2. Cell Concentration and Viability Determination

Cell concentration and viability was determined using a Cellometer X2 Fluorescent Viability Counter (Nexcelom Bioscience LLC, Lawrence, MA, USA) and the ViaStain™ Yeast Kit for Live/Dead Concentration (PerkinElmer, Mulgrave, Victoria) as previously described [12]. Briefly, beer samples were tossed 20× to remove gas and then 50 mL of beer was centrifuged at 3000× *g* for 5 min. The supernatant was removed, the cell pellet resuspended in yeast dilution buffer and then an equal volume of ViaStain™ was added. Samples were counted in triplicate.

2.3. Vitality Determination

The vitality of the different yeast strains during propagation in wort was performed using resazurin as previously described [13].

2.4. Foam Stability

Foam stability was determined using a Haffmans NIBEM-TPH Foam Stability Tester (Pentair, London, UK) as previously described [12].

2.5. Apparent Extract (AE), Alcohol Concentration, and pH Measurement

AE, alcohol concentration, and pH measurement were performed as previously described [12].

2.6. Volatile Analysis

Standard solutions were prepared by accurately weighing amounts (± 0.001 g) of chemical standard (Merck, Darmstadt, Germany) and dissolving compounds in ethanol (Wilmar, Yaraville, Victoria). Standard solution A was prepared by the addition of 2-methyl-1-butanol (2.50 g), 3-methyl-1-butanol (2.50 g), isobutanol (1.25 g), propanol (1.25 g), and acetaldehyde (2.5 g) in 50 mL of ethanol. Standard solution B was prepared by the addition of ethyl hexanoate (0.5 g) and dimethyl sulphide (DMS) (0.1 g) in 50 mL of ethanol. Standard solution C was prepared by the addition of isoamyl acetate (0.25 g) and ethyl acetate (5.00 g) in 50 mL of ethanol. Standard solution D was prepared by the addition

of 10 mL of standard solution A, 10 mL of standard solution B, and 5 mL of standard solution C and made up to 200 mL with ethanol. Calibration standards were prepared by diluting 1–10 mL of standard solution D in 0–9 mL of ethanol. Four mL of each calibration standard was made up to one hundred mL with RO water. The internal standard was a solution of ethyl methyl sulphide (100 µg/L) for DMS and 3-heptanone (1.6 mg/mL) in ethanol. Both samples and standard solutions were stored refrigerated at 4 °C.

Beer samples were stored at 4 °C for at least 12 h. Autosampler vials containing sodium chloride (2 ± 0.1 g) (Chem-Supply, Gillman, SA, Australia) were cooled to 0–4 °C. Five mL of cooled beer was added to the autosampler vial, followed by 50 µL of each of the two internal standards. Autosampler vials were capped immediately, agitated for 30 s, and then equilibrated in the autosampler for at least 30 min prior to injection.

An Agilent 6890 N gas chromatograph (GC) (Agilent Technologies, Waldbronn, Germany) with a Zebron ZB-Wax_{Plus} column (30 m, 320 µm i.d., 0.5 µm film thickness) (Phenomenex, Torrance, CA, USA) was connected to an Agilent G1888 Headspace sampler (Agilent Technologies) and a Flame Ionisation Detector (FID). The FID temperature was 200 °C with a hydrogen and air flow of 30 mL/min and 300 mL/min, respectively. An inlet split mode (ratio 30:1) and injection temperature of 200 °C was utilised. The flow rate of the carrier gas (nitrogen) was 30 mL/min. The temperature program was as follows: 43 °C for 4 min, raised to 160 °C at 10 °C/min for 12 min, and then reduced to 43 °C at 40 °C/min for 3 min. The ChemStation OpenLabs CDS (Rev C.01.07 (27)) software package for GC control and data integration (Agilent Technologies) was used.

2.7. Sensory Evaluation of Beer

The aroma of bottle-conditioned beers (14 days, 30 days, 90 days and 120 days) was evaluated by a trained panel comprised of a minimum of five tasters. All tasters were employees of Coopers Brewery Ltd. who possess extensive experience in sensory evaluation. Blind tasting was conducted on all experimental samples, with the trained panel asked to comment on the sensory aroma of the presented sample in comparison to a typical pale ale profile. Ethical review and approval were waived for this study due to the evaluation being performed using standard practices at a commercial brewery.

2.8. Sample Preparation for Proteomic Analysis

Yeast cells were collected from beer by centrifugation at 3000× *g* for 5 min. Pellets were washed with ultrapure water, centrifuged at 3000× *g* for 5 min, and then immediately frozen on dry ice. When required, cell pellets (1×10^7 cells) were defrosted on ice. Cells were lysed in 4% *w/v* sodium dodecyl sulfate (Fisher BioReagents, Fair Lawn, NJ, USA)/50 mM Tris-HCl (pH 7.4) (Sigma-Aldrich, St. Louis, USA) buffer. Cells were added to 50 ± 5 mg of zirconium oxide beads (BioTools Pty Ltd, Brendale, Australia) and homogenised using a Next Advance Bullet Blender Storm 24 (BioTools Pty Ltd.) on level 10 for 3 min. Sample debris was removed by centrifugation at 20,000× *g* for 5 min at room temperature and the supernatant transferred to a fresh eppendorf. Proteins were precipitated using 4× ice-cold (−20 °C) acetone (Chem-Supply) overnight. Proteins were isolated by centrifugation at 20,000× *g* for 10 min at −9 °C, washed using ice-cold acetone and centrifuged at 20,000× *g* for 10 min at −9 °C, and then dried on ice for 5 min. Proteins were resuspended in 8 M urea (Sigma-Aldrich)/50 mM ammonium bicarbonate (Sigma-Aldrich) and then quantified using tryptophan fluorescence [14]. Proteins were reduced using dithiothreitol (final concentration 10 mM) (Sigma-Aldrich) and incubated at room temperature for 60 min with shaking at 300 rpm. Proteins were alkylated using 2-chloroacetamide (final concentration 15 mM) (Sigma-Aldrich) and incubated in the dark at room temperature for 30 min with shaking at 300 rpm. Samples were diluted with 50 mM ammonium bicarbonate to achieve a urea concentration of ≤0.8 M. Trypsin Gold (Promega Corporation, Madison, WI, USA) was added to achieve a 1:50 (enzyme/protein) ratio. Samples were incubated at 37 °C overnight with shaking at 500 rpm. Samples were acidified using 100% formic acid (Fisher Scientific, Pardubice, Czech Republic) to obtain a pH of approximately 2–3 and then

desalted using Sep-Pak® Vac 1cc (50 mg) C18 cartridges (Waters Corporation, Milford, MA, USA). Samples were subsequently dried to completion under vacuum at 50 °C. Peptides were resuspended in 0.1% formic acid and quantified using tryptophan fluorescence prior to liquid chromatography tandem mass spectrometry (LC-MS/MS).

2.9. LC-MS/MS

Samples were analysed using an ACQUITY UPLC M-Class system (Waters Corporation, Milford, USA) connected to a ZenoTOF 7600 mass spectrometer with an OptiFlow Turbo V ion source (SCIEX, Singapore, Singapore). Approximately 200 ng of peptides were loaded onto a nanoEase M/Z HSS T3 reversed phase C18 column (100 Å pore size, 1.8 µm particle size, 300 µm i.d. × 150 mm) (Waters Corporation) heated to 40 °C at a flow rate of 5 µL/min. Buffer A was 99.9% water and 0.1% formic acid and Buffer B was 99.9% acetonitrile (Merck, Burlington, MA, USA) and 0.1% formic acid. Peptides were separated with a gradient of 3–35% buffer B over 45 min. Other MS settings include ion source gas 1 and 2 set to 20 and 15 psi, respectively; curtain gas 35 psi; collision gas (CAD) 7; source temperature 150 °C; and the spray voltage set to 5000 V. A Zeno Sequential Window Acquisition of All Theoretical Mass Spectra data independent acquisition (Zeno SWATH-DIA) scheme (64 variable-size windows covering a 400–1500 m/z precursor mass range) was performed with an 0.1 s accumulation time, 80 V declustering potential, and 10 V collision energy. Raw data were acquired using SCIEX OS (v3.1.0).

2.10. Data Processing and Analysis

Proteomic data were analysed using Spectronaut 18 (Biognosys, Schlieren, Switzerland) [15] using the directDIA+™ workflow. Standard settings included enzyme, Trypsin/P; fixed modifications, carbamidomethyl (C); variable modifications, acetyl (Protein N-term) and oxidation (M); false discovery rate, 1%. Mass spectra were searched against the *Saccharomyces cerevisiae* FASTA database from UniProt (yeast: UP000002311_559292, 6060 entries, 15 March 2023). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [16] via the PRIDE [17] partner repository with the dataset identifier PXD053930. GO terms were assessed using the tool "GO Term finder" from SGD database [18] and GO terms with an adj. p-value ≤ 0.01 were selected. Redundant terms were removed.

Data visualisation and statistical analysis for non-proteomic data was performed in GraphPad Prism 10 (v10.0.2) using an ordinary, one-way ANOVA.

3. Results and Discussion

In this study we evaluated six yeast strains that are used in the commercial brewing, sparkling wine, and distilling industries on their ability to perform secondary fermentation in beer, their long-term viability, and impact on the final product. The yeast strains were grown in wort until they reached post-diauxic/stationary phase, prior to seeding for bottle conditioning. The metabolic activity of the different yeasts during propagation was assessed using resazurin, where they had entered the post-diauxic or stationary phase after 72 h (Supplementary Figure S1). It has been previously shown that as the yeast enters the stationary phase, there is minimal resazurin production [13]. Yeast strains were grown to the post-diauxic or stationary phase to ensure they were seeded at a similar growth period and to potentially improve their stress tolerance [12]. We observed an increase in fluorescence at 24 h compared to 4 h and 72 h for Ale brewing yeast 2, which was due to a large outlier and not due to biological changes in the yeast. Compared to the brewing strains, the Sparkling wine and Distilling yeast strains did not readily ferment the wort and produced a higher apparent extract (AE), as well as a lower alcohol concentration after 72 h propagation (Supplementary Table S1). As maltose and maltotriose are the most abundant sugars in the wort, the Sparkling wine and Distilling yeasts may be less efficient in metabolising this sugar [19]. Additionally, the viability of the different yeast strains was between 88% and 99% at the time of seeding for bottle conditioning (Supplementary

Figure S2). The different yeast strains had good viability for bottle conditioning despite their differences in fermentation ability.

3.1. Growth of Yeast Strains during Bottle Conditioning

The growth rate of the different yeast strains during bottle conditioning of beer were compared using cell counts (Figure 1 and Supplementary Figure S3).

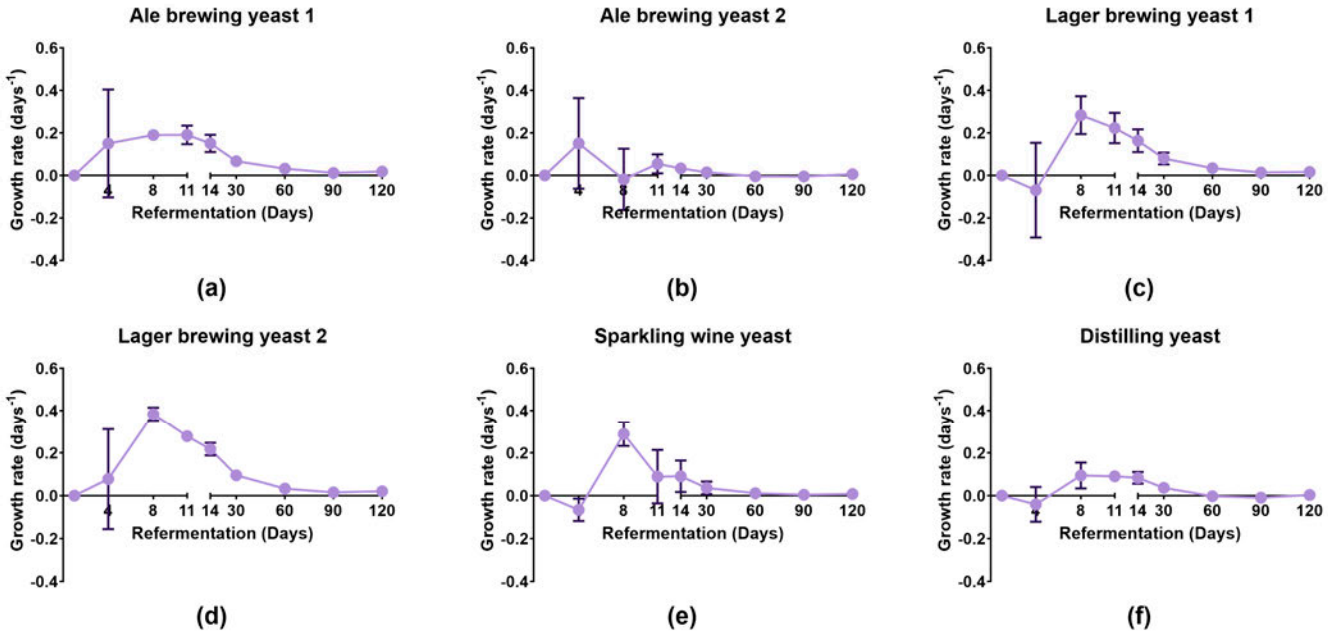


Figure 1. Growth rate of different yeast strains during bottle conditioning. Yeast strains were seeded into pale ale beer that was primed with 0.5 °P fermentable extract and incubated at 20–22 °C for 120 days. Growth rates were determined for (**a**) Ale brewing yeast 1, (**b**) Ale brewing yeast 2, (**c**) Lager brewing yeast 1, (**d**) Lager brewing yeast 2, (**e**) Sparkling wine yeast, and (**f**) Distilling yeast. Data shown as the mean growth rate and error bars represent standard deviation of biological replicates.

Lager brewing yeast 1, Lager brewing yeast 2, and Sparkling wine yeast had the fastest growth rate which reached a maximum of 0.28 ± 0.09 days^{-1}, 0.38 ± 0.03 days^{-1}, and 0.29 ± 0.06 days^{-1}, respectively, on day 8. Lager brewing yeast 1, Lager brewing yeast 2, and Sparkling wine yeast strains had a decline in growth rate after day 8 of secondary fermentation. In contrast, all other strains had a decline in growth rate after 14 days. This indicated that Lager brewing yeast 1, Lager brewing yeast 2, and Sparkling wine yeast completed secondary fermentation faster than the other strains. The improved growth of the Sparkling wine and Distilling yeast strains during bottle conditioning compared to propagation was most likely due to the main fermentable extract being sucrose instead of maltose. It has been previously shown that glucose, fructose, and sucrose were easily fermented compared to maltose and maltotriose during bottle conditioning [20]. The slower decline in growth rate for the other yeast strains suggested that these strains may utilise other sugars to continue their growth compared to the Sparkling wine yeast. Ale brewing yeast 2 had a low growth rate throughout bottle conditioning. Despite this, it seemed that Ale brewing yeast 2 was able to successfully complete bottle conditioning within 30 days, as discussed in the following sections.

3.2. Impact of Yeast Strain on Beer Characteristics

To obtain a better understanding of the influence of the different yeast strains on the final beer product, characteristics such as AE, alcohol concentration, and pH were evaluated for up to 120 days. We found that while the AE decreased, the alcohol concentration increased until day 14 of bottle conditioning (Supplementary Figure S4a,b). This was

expected as the yeast utilises the fermentable extract and produces ethanol through fermentative metabolism. After the yeast reached the stationary phase, the rate of AE consumption and alcohol production were minimal as the yeast cells entered a non-proliferative state and downregulated processes for active growth [21]. The pH of the beer decreased for all samples (Supplementary Figure S4c), which was expected because of the excretion of organic acids that typically occurs during fermentation [22]. Beyond 14 days, the AE of Lager brewing yeast 2 was lower than all other strains at all time points (Figure 2a). A similar trend was observed for the alcohol concentration, which was higher in beer conditioned with Lager brewing yeast 2 until day 60 (Figure 2b). This suggested that the Lager brewing yeast 2 had improved fermentation performance compared to the other strains. Compared to days 14 and 30, Ale brewing yeast 2 and Lager brewing yeast 2 showed a lower AE and higher alcohol concentration at day 60 of bottle conditioning. This suggests that these strains required more time to fully referment the beer. The ability of Ale brewing yeast 2 to referment the beer at a low cell concentration could indicate that successful bottle conditioning does not require high cell concentrations. Generally, the pH of beer increased from day 60 for all yeast strains during bottle conditioning (Figure 2c). Overall, all strains were able to perform secondary fermentation of the beer.

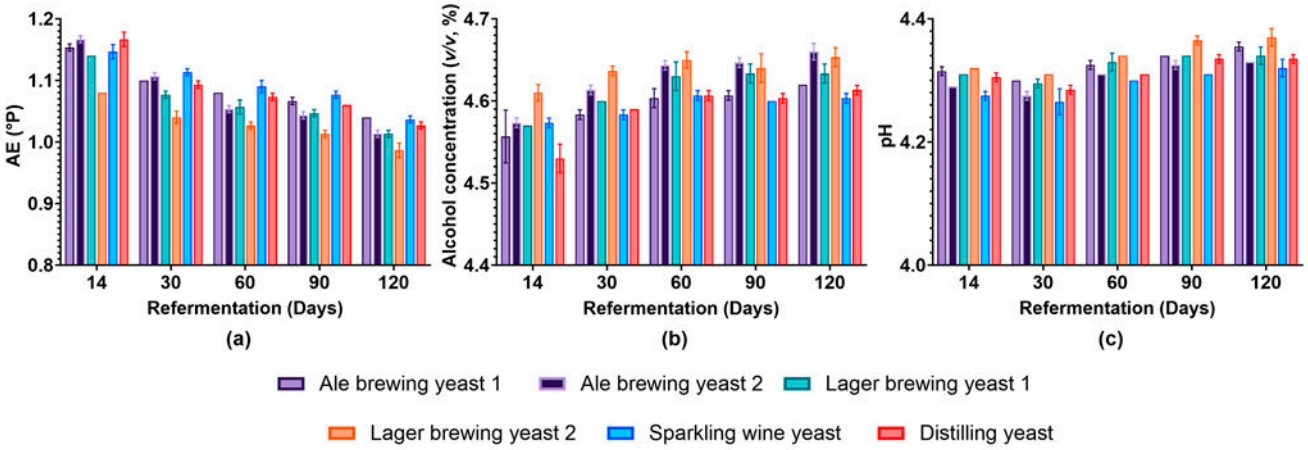


Figure 2. Evaluation of beer characteristics for the different yeast strains during bottle conditioning. (**a**) AE (°P), (**b**) alcohol concentration (*v*/*v*), and (**c**) pH were determined at different time points during bottle conditioning for the different yeast strains. Light purple: Ale brewing yeast 1; dark purple: Ale brewing yeast 2; green: Lager brewing yeast 1; orange: Lager brewing yeast 2; blue: Sparkling wine yeast; red: Distilling yeast. Data shown as the mean and error bars represent standard deviation of biological replicates.

3.3. Yeast Viability and Its Impact on Foam Stability during Bottle Conditioning

The viability of the different yeast strains was determined over four months of bottle conditioning (Figure 3a). At day 30 of bottle conditioning, the Sparkling wine strain showed the largest decline in viability amongst all the strains (89 ± 4%) when compared to day 14. The Ale brewing yeast 1 (99 ± 2%), Ale brewing yeast 2 (106 ± 14%), Lager brewing yeast 1 (95 ± 10%), Lager brewing yeast 2 (91 ± 5%), and Distilling yeast (98 ± 1%) did not show a large decrease in viability. At day 60, all yeast strains (except for Ale brewing yeasts 1 and 2) began to decline in viability. The viability of Lager brewing yeast 2 and Sparkling wine yeast decreased considerably, obtaining viabilities of 66 ± 5% and 57 ± 6%, respectively. Interestingly, the viability of Ale brewing yeast 2 did not demonstrate any decline until the day 120 measurement. All yeast strains by day 120 possessed a low viability (below 30%), with a significant proportion of the yeast cells expected to have autolysed [23]. Overall, we observed that the Ale brewing yeast strains and Lager brewing yeast 1 were able to maintain their viability for longer compared to the Lager yeast strain 2, Sparkling wine, and Distilling yeast strains, with Ale brewing yeast 2 outperforming all yeast strains.

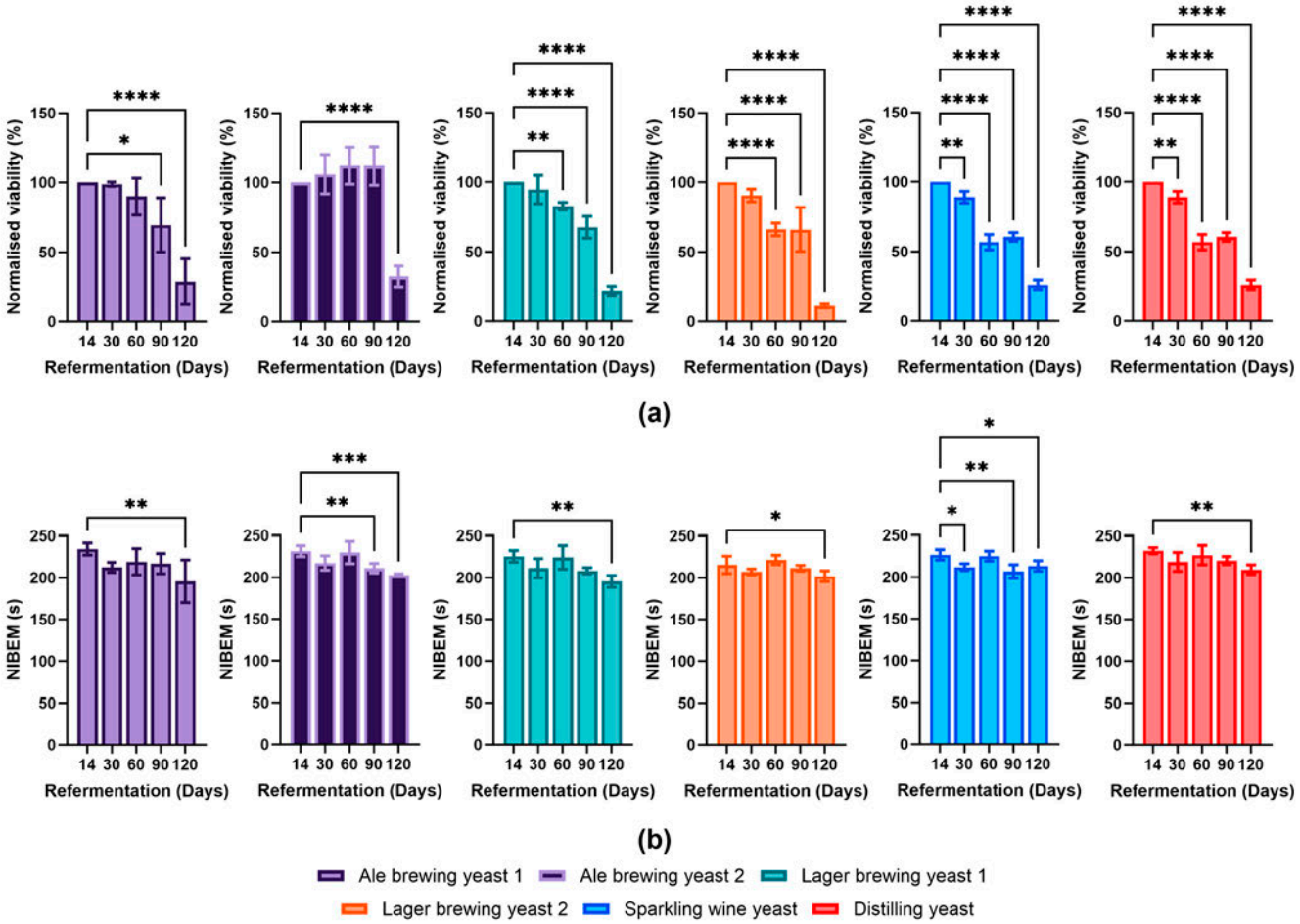


Figure 3. Impact of bottle conditioning on yeast survival and foam stability over 120 days. The (**a**) viability (normalised to day 14) and (**b**) foam stability of the different yeast strains was determined at day 14–day 120 of bottle conditioning. Data shown as the mean value and error bars represent standard deviation. Light purple: Ale brewing yeast 1; dark purple: Ale brewing yeast 2; green: Lager brewing yeast 1; orange: Lager brewing yeast 2; blue: Sparkling wine yeast; red: Distilling yeast. Statistical tests were performed for each strain using an ordinary, one-way ANOVA. * p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001, **** p-value < 0.0001.

The foam stability of beer can be impacted by a decrease in yeast viability. For example, the enzyme Proteinase A can be secreted into the beer during yeast autolysis, resulting in cleavage of foam stabilising compounds and a decrease in foam stability [24,25]. The viability of the Sparkling wine yeast significantly decreased from day 30. This corresponded with a faster decline in viability. Interestingly, Ale brewing yeast 2 showed a decline in foam stability after day 60 despite no decline in viability. There were decreases in foam stability observed for all yeast strains at day 120 of bottle conditioning, some of which were statistically significant compared to day 14 (Figure 3b). As the viability of all yeast strains at day 120 was below 30%, it is likely that yeast autolysis contributed to the decreased foam stability [23]. It seems that for most of the strains utilised in this study, the foam stability was not impacted until there was a severe decline in viability. As a decrease in foam stability did not correspond with a decrease in viability for all yeast strains, it indicated that the secretion of proteolytic enzymes into the beer was strain-specific. Therefore, consideration of the yeast strain for bottle conditioning is important as some strains are more likely to impact foam stability than others.

3.4. Impact of Yeast Strain on Volatile Profile

The aim of this evaluation was to produce a beer with a yeast that had enhanced viability but did not alter the profile of the beer to preserve product identity. The concentration of eight volatile compounds was assessed at day 14 to determine whether the different yeast species changed the volatile distribution compared to that expected for a pale ale. This was performed at day 14 to align with the standard quality control practices within the brewery. The concentration of acetaldehyde, propanol, isobutanol, and 2-methyl-1-butanol/3-methyl-1-butanol (2+3-MB) were similar at day 14 between yeast strains (Figure 4a–d). Generally, the concentration of dimethyl sulfide (DMS), ethyl acetate, and isoamyl acetate remained similar between yeast strains at the different time points analysed (Figure 4f,g).

The acetaldehyde concentration decreased during secondary fermentation. This was expected as acetaldehyde is a precursor for ethanol formation [26]. The concentration of acetaldehyde was highest in Lager brewing yeast 2 at day 30; however, on day 120, it was at a similar concentration to all the other strains except Ale brewing yeast 1. This reflected the ability of different strains to metabolise the acetaldehyde [27]. Propanol, isobutanol, and 2+3-MB were consistently lower in Ale brewing yeast 1 at day 30 and day 120 and Distilling yeast at day 30 compared to the other yeast strains. Higher alcohols such as propanol, isobutanol, and 2+3-MB are formed as a by-product of yeast amino acid metabolism from keto-acids via the Ehrlich pathway [26]. The lower concentration in Ale brewing yeast 1 may indicate that this strain was more efficient at utilising residual amino acids present in the beer [27]. Hexanol concentrations were higher in Sparkling wine yeast and Distilling yeast at day 14 and day 30 and was most likely strain-dependent [27]. However, the reduction in the content of hexanol during bottle conditioning most likely occurred due to the formation of the corresponding ester, hexyl acetate, as a product of yeast metabolism [28]. Isoamyl acetate concentration decreased in concentration by day 120 of bottle conditioning for all yeast strains. A decrease in isoamyl acetate also occurred during beer ageing and was associated with a decrease in fruity flavour and increasing the perception of eventual stale flavours [29].

Overall, the concentration of the main volatile compounds was relatively similar between yeast strains in this study. We performed a sensory evaluation to determine whether the different yeast species resulted in an aroma typical for a pale ale (Supplementary Table S2). The panel was asked to comment on the sensory aroma of the beer compared to a typical pale ale profile and to select whether the product was typical (or atypical) for a pale ale beer. The use of Sparkling wine yeast and Distilling yeast resulted in a different aroma profile to the standard profile expected, so they were categorised as atypical. The panel commented that this was mainly due to the presence of 4-vinyl guaiacol. Ale brewing yeast 1 (the yeast normally used for bottle conditioning at the brewery) was consistently selected as typical, with at least 60% of the panellists stating it possessed typical characteristics across all time points. Interestingly, Ale brewing yeast 2 was initially classified as atypical by the panellists but subsequently found to be unanimously typical from day 30 to 90. This indicated that Ale brewing yeast 2 required a longer bottle conditioning time to obtain similar characteristics as the control beer. In addition, 60% of the panellists found the product using Ale brewing yeast 2 was atypical at day 120. Lager brewing yeast 1 and Lager brewing yeast 2 had similar typical rankings throughout bottle conditioning. Interestingly, Ale brewing yeast 1, Lager brewing yeast 1, and Lager brewing yeast 2 were ranked as typical by 100% of the panellists at day 120 despite a decline in viability. These results show that a similar sensory profile was obtained when any of the brewing yeast strains were utilised for bottle conditioning.

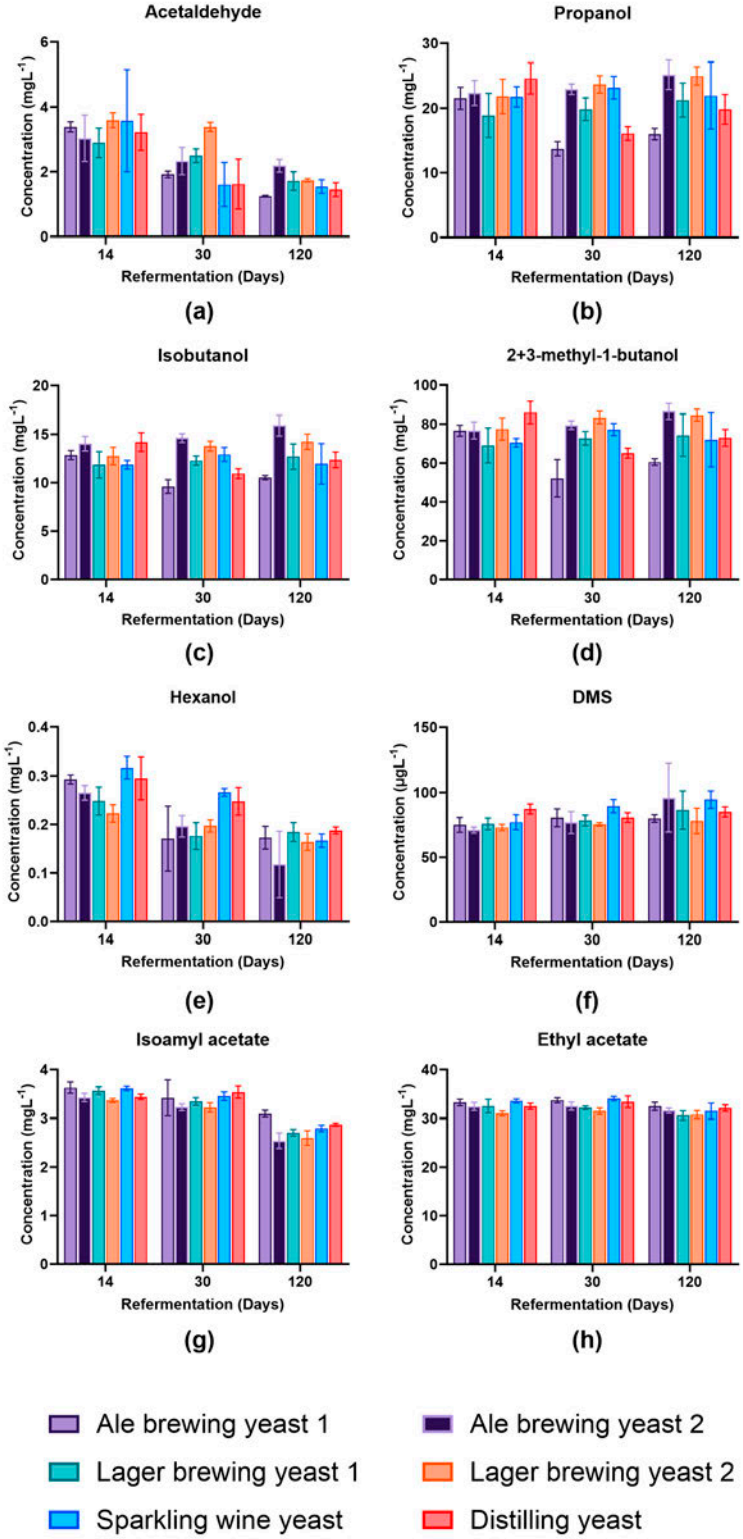


Figure 4. Concentration of volatile compounds produced by different yeast strains at various stages of secondary fermentation in beer. Concentration (ppm) of (**a**) acetaldehyde, (**b**) propanol, (**c**) isobutyl alcohol, (**d**) 2-methyl-1-butanol and 3-methyl-1-butanol, (**e**) hexanol, (**f**) dimethyl sulfide (DMS) in μgL^{-1} (ppb), (**g**) isoamyl acetate, and (**h**) ethyl acetate. Data shown as the mean value and error bars represent standard deviation. Light purple: Ale brewing yeast 1; dark purple: Ale brewing yeast 2; green: Lager brewing yeast 1; orange: Lager brewing yeast 2; blue: Sparkling wine yeast; red: Distilling yeast.

3.5. Proteomic Profiling to Investigate Key Protein Changes That Are Associated with Decreased Viability

In order to determine the biological changes that allow specific yeast strains to maintain their viability, we performed proteomic analysis of Ale brewing yeast 2 (superior viability) compared to Sparkling wine yeast (poor viability) from day 14 to day 120 using Zeno Sequential Window Acquisition of All Theoretical Mass Spectra (Zeno SWATH-MS). Day 14 was chosen as the control in this study as the yeast strains were all in stationary phase at this time point and it was prior to the onset of a decline in viability. Our proteomic analysis resulted in the quantification of 2383 ± 219 proteins across all samples (Supplementary Table S3).

Principal component analysis (PCA) was performed to evaluate the major principal components of variance between the different days of late-stage secondary fermentation for Ale brewing yeast 2 (Figure 5a) and Sparkling wine yeast (Figure 5b).

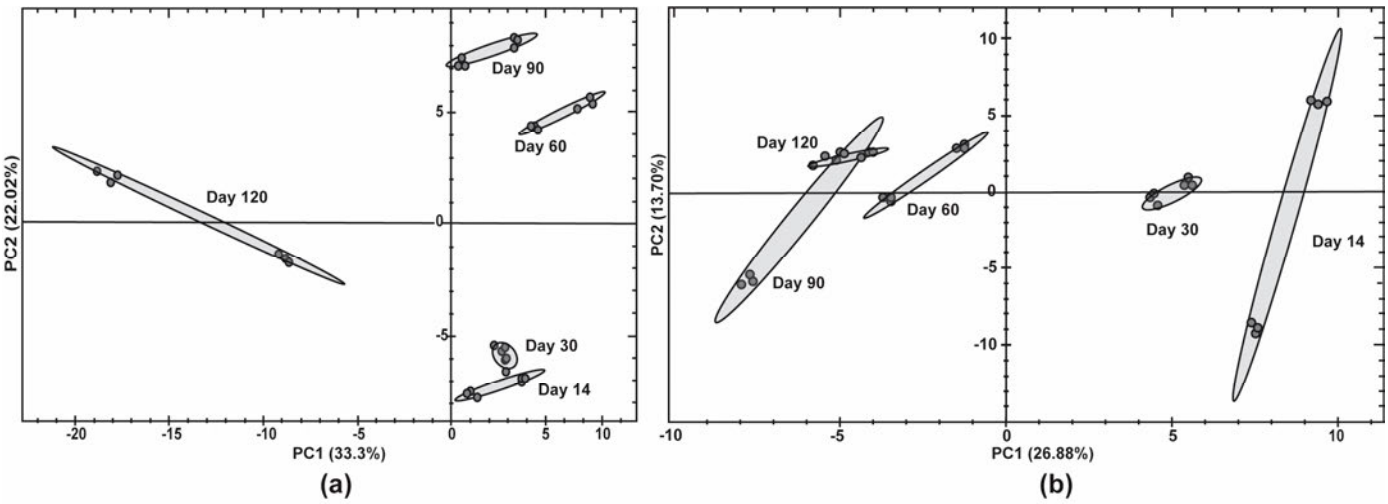


Figure 5. Proteomic profiling of proteins during late stage of bottle conditioning in beer using different yeast strains. Principal component analysis (PCA) of the normalised protein abundances in (**a**) Ale brewing yeast 2 and (**b**) Sparkling wine yeast using Spectronaut 18 software.

Generally, the PCA grouped biological replicates by time point in the first principle component (PC1). PC1 showed a clear separation (33.3%) between early time points (day 14 to day 90) and the late time point (day 120) for Ale brewing yeast 2. The second principle component (PC2) separated (22.0%) the early time points (day 14 to day 90). In contrast, the PC1 for Sparkling wine yeast showed a clear separation (26.9%) between day 14 and day 30 compared to day 60 and day 90. PC2 had a small separation (13.7%) between biological replicates. The separation of PC1 clearly correlates to the ability of these strains to maintain their viability as Ale brewing yeast 2 and Sparkling wine yeast had a rapid decline in viability at day 120 and day 60, respectively. Therefore, the proteomic changes associated with the separation of secondary fermentation time may explain the different yeast strain's ability to maintain viability during bottle conditioning.

To investigate this further, we evaluated significant changes in protein abundance (adj. p-value ≤ 0.05, Log_2 fold-change ≥ 0.58) at each time point (compared to day 14) for Ale brewing yeast 2 (Supplementary Table S4) and Sparkling wine yeast (Supplementary Table S5). There were fewer significant protein changes at the earlier stage of bottle conditioning (day 30) compared to the later stages of bottle conditioning (day 60–day 120) in both yeast strains (Table 2).

Table 2. Number of proteins with a significant abundance change during bottle conditioning *.

Condition	Upregulated		Downregulated	
	Ale Brewing Yeast 2	**Sparkling Wine Yeast**	**Ale Brewing Yeast 2**	**Sparkling Wine Yeast**
D30/D14	34	85	143	113
D60/D14	230	179	316	183
D90/D14	370	230	290	213
D120/D14	660	223	234	298

* Adj. p-value ≤ 0.05 and Log_2 fold-change ≥ 0.58.

Gene ontology analysis was performed to highlight the main biological functions that were altered in response for proteins that significantly changed in abundance. Interestingly, proteins involved in oxidoreductase activity and ribosome biogenesis were upregulated in Ale brewing yeast 2, whereas proteins associated with the cell wall were upregulated in Sparkling wine yeast (Table 3).

Table 3. Gene ontology of proteins that were significantly increased in abundance in Ale brewing yeast 2 and Sparkling wine yeast during bottle conditioning.

Ale Brewing Yeast 2				**Sparkling Wine Yeast**			
Term	***p*-Value ***	**Number of Proteins**	**Fold Enriched (%)**	**Term**	***p*-Value ***	**Number of Proteins**	**Fold Enriched (%)**
D30/D14							
No significant terms				Structural constituent of cell wall	2.98	4	4.7
D60/D14							
Catalytic activity	7.02	110	47.8	Glucosidase activity	2.93	7	3.9
Oxidoreductase activity	6.22	31	13.5	Hydrolase activity, hydrolyzing O-glycosyl compounds	2.74	9	5.0
Melatonin binding	4.81	6	2.6				
Electron transfer activity	3.33	8	3.5				
Proton transmembrane transporter activity	2.63	11	4.8				
Translation factor activity, RNA binding	2.13	8	3.5				
Day 90/Day14							
Catalytic activity	9.11	168	45.4	Catalytic activity	3.43	99	43.0
Oxidoreductase activity	7.12	43	11.6	Primary active transmembrane transporter activity	2.45	11	4.8
Structural constituent of ribosome	3.72	31	8.4				
Electron transfer activity	2.59	9	2.4				
Day 120/Day 14							
Catalytic activity	5.87	104	46.6	Catalytic activity	5.87	104	46.6

* Adj. p-value (log_{10}).

Proteins associated with eliminating oxidative stress such as Sod2p, Ctt1p, Prx1p, Tsa1p, and Tsa2p increased in abundance in Ale brewing yeast 2 compared to Sparkling wine yeast, where their abundance remained similar (Figure 6a). An increase in expression of *PRX1*, *CTT1*, *SOD2*, and *TSA1* was shown to occur in yeast strains that had superior fermentation performance during wine-making [30]. This suggested that an increase in expression of oxidative stress genes enabled Ale brewing yeast 2 to respond better to stresses such as ethanol [30] or nutrient deprivation [31]. Additionally, proteins involved in the electron transport chain such as Qcr2p and Atp2p were also increased from day 60 in Ale brewing yeast 2 (Figure 6b). Mitochondrial genes, including *QCR2* and *ATP2*, were also found to increase in yeast during refermentation of wine [9,32]. There has been growing evidence that an increase in genes involved in mitochondrial function and oxidative metabolism is required in ethanol stress tolerance [33]. Therefore, the increase of proteins associated with these pathways suggested that Ale brewing yeast 2 was more tolerant to long-term ethanol exposure.

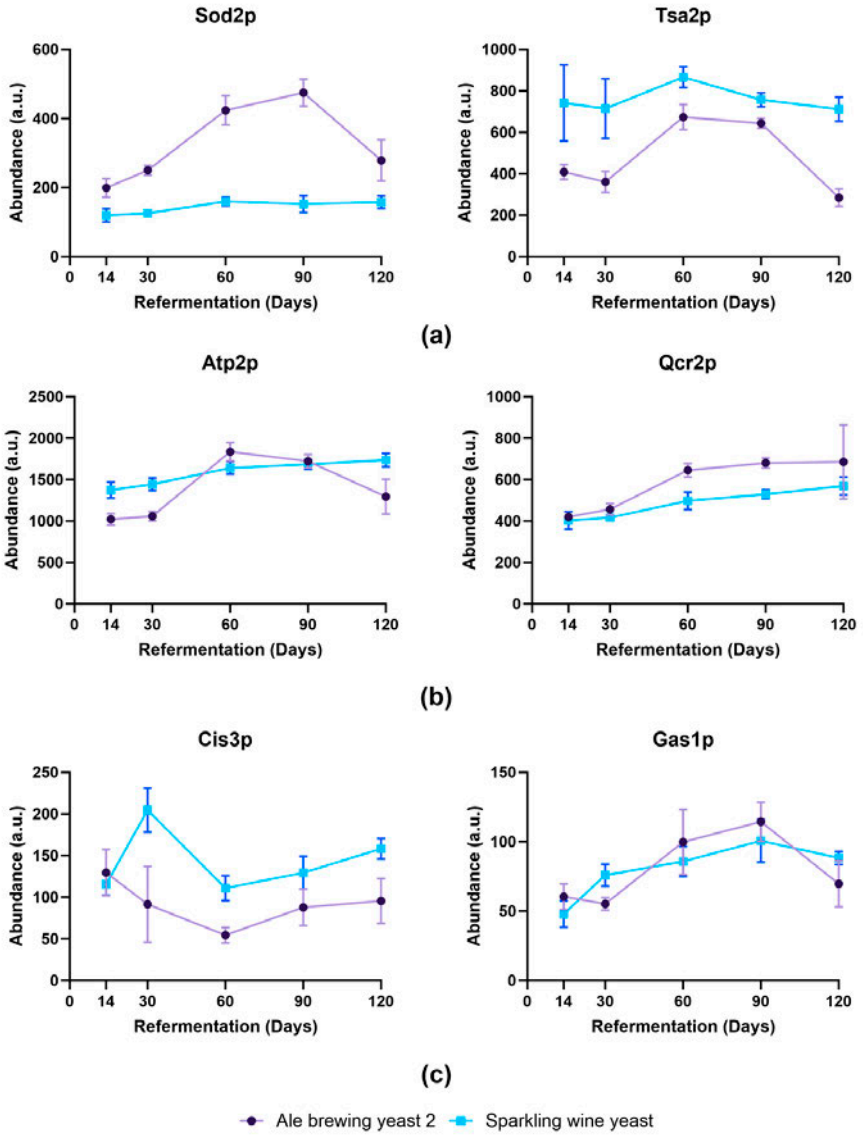


Figure 6. Specific examples of proteins that changed in abundance during bottle conditioning. Proteins shown were significantly enriched in GO term analysis (adj. *p*-value < 0.01) for different biological processes: (**a**) oxioreductase activity, (**b**) electron transport, and (**c**) cell wall organisation. Data shown as average abundance ± standard deviation of biological replicates. Protein abundance exported from Spectronaut export file. Purple: Ale brewing yeast 2; blue: Sparkling wine yeast.

Proteins associated with the cell wall were increased in Sparkling wine yeast (Figure 6c). Cis3p, Pir1p, and Hsp150p only increased at day 30 in Sparkling wine yeast. In contrast, Cis3p and Hsp150p significantly decreased at day 120 and day 30, respectively, and Pir1p was not detected after day 14 in Ale brewing yeast 2. The increase in these proteins at day 30 in Sparkling wine suggested remodeling of the cell wall in response to stress. Other proteins associated with cell wall remodeling were altered during refermentation. For example, the abundance of Crh1p increased from day 30 in Sparkling wine yeast but was not detected in Ale Brewing yeast 2, whereas Crh2p was found to significantly increase in abundance at day 90 in Ale brewing yeast 2 compared to day 60 in Sparkling wine yeast. Crh1p and Crh2p are responsible for cross-linking β-1,3-glucan to chitin in the cell wall [34]. The expression of *CRH1* has been shown to increase in response to ethanol-induced cell wall stress [35,36]. Furthermore, the expression of *CRH2* is induced as a late response to perturbations of the β-glucan network [37]. The higher abundance of Crh1p and the earlier increase in abundance of Crh2p indicated that the Sparkling wine yeast was more susceptible to cell wall stress. Furthermore, the abundance of Gas1p was significantly increased at day 60 and day 90 for Ale brewing yeast 2 but at day 30 in Sparkling wine yeast. Gas5p abundance did not change in Ale brewing yeast 2 but decreased at day 120 in Sparkling wine yeast. Gas1p encodes a β-1,3-glucanosyltransferase that is responsible for modulating the branching and elongation of β-1,3-glucan chains [38]. As *GAS1* plays an important role in increasing cell wall stiffness in response to different stresses such as acidity, this further supports that Sparkling wine yeast was more susceptible to cellular stress [39]. The increase in abundance of these proteins may promote the earlier release of cell wall components and shorten the lifespan of this strain as genes involved in cell wall are upregulated in response to autolysis in brewing yeasts [40].

Proteins associated with the structural ribosome were downregulated during refermentation in both Ale brewing yeast 2 and Sparkling wine yeast (Table 4). Many of the proteins associated with the ribosome such as Rpl34Bp were only significantly decreased at day 120 in Ale brewing yeast 2 and Sparkling wine yeast. A decrease in proteins associated with protein synthesis typically occurs at the onset of stationary phase during primary fermentation and bottle conditioning [9,41]. As many of the ribosomal proteins would have decreased by day 14, the sudden decrease at day 120 was most likely associated with autolysis and the breakdown of proteins within the cell.

Table 4. Gene ontology of proteins that were significantly decreased in abundance in Ale brewing yeast 2 and Sparkling wine yeast during bottle conditioning.

Ale Brewing Yeast 2				Sparkling Wine Yeast			
Term	***p*-Value ***	**Number of Proteins**	**Fold Enriched (%)**	**Term**	***p*-Value ***	**Number of Proteins**	**Fold Enriched (%)**
D30/D14							
No significant terms				No significant terms			
D60/D14							
RNA polymerase III activity	4.29	8	2.5	No significant terms			
Day 90/Day 14							
5′-3′ RNA polymerase activity	2.91	9	3.1	No significant terms			
Day 120/Day 14							
Structural molecule activity	5.63	32	13.7	Structural molecule activity	9.61	44	14.8
Endopeptidase inhibitor activity	2.15	3	1.3				

* Adj. *p*-value ($-\log_{10}$).

4. Conclusions

We demonstrated that six different types of yeast successfully performed secondary fermentation during the bottle conditioning of beer. Ale and Lager brewing strains were better for obtaining a standard beer aroma, while Sparkling wine and Distilling strains were more amenable to unique sensory flavour. Protein abundance changes from proteomics experiments revealed biological functions that contributed to the prolonged viability of Ale brewing yeast 2. Specifically, proteins associated with oxidoreductase activity and the electron transport chain enabled Ale brewing yeast 2 to respond to long-term ethanol stress. We anticipate that brewers could utilise a different yeast for bottle conditioning to improve beer longevity without detrimental effects on foam stability and the accompanying sensory profile. Future experiments will explore the effect of the pre-adaptation of yeast in beer or to specific environmental conditions such as high alcohol to extend their viability during bottle conditioning.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods13162593/s1, Figure S1: Resazurin production during yeast propagation in wort; Figure S2: Viability of yeast strains at time of seeding for bottle conditioning; Figure S3: Cell concentration of different yeast strains during bottle conditioning; Figure S4: Beer characteristics during early stages of bottle conditioning; Figure S5: Concentration of volatile compounds produced by different yeast strains at various stages of bottle conditioning of beer; Table S1: Apparent extract and alcohol concentration after 72 h of propagation in wort; Table S2: Panellist classification of aroma profile of pale ale bottle conditioned using different yeast strains (%); Table S3: Average protein identifications across later stages of bottle conditioning in Ale brewing yeast 1 and Sparkling wine yeast; Table S4: Significant (adj. p-value) abundance changes ($Log_2 \geq 0.58$) in Ale brewing yeast 2; Table S5: Significant (adj. p-value) abundance changes ($Log_2 \geq 0.58$) in Sparkling wine yeast.

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References

1. Dekoninck, T.M.L.; Mertens, T.; Delvaux, F.; Delvaux, F.R. Influence of Beer Characteristics on Yeast Refermentation Performance during Bottle Conditioning of Belgian Beers. *J. Am. Soc. Brew. Chem.* **2013**, *71*, 23–34. [CrossRef]
2. Marconi, O.; Rossi, S.; Galgano, F.; Sileoni, V.; Perretti, G. Influence of yeast strain, priming solution and temperature on beer bottle conditioning. *J. Sci. Food Agric.* **2016**, *96*, 4106–4115. [CrossRef] [PubMed]
3. Derdelinckx, G.; Vanderhasselt, M.; Maudoux, M.; Dufour, M. Refermentation in bottles and kegs: A rigorous approach. *Brauwelt Int.* **1992**, *2*, 156–164.
4. Saison, D.; De Schutter, D.P.; Vanbeneden, N.; Daenen, L.; Delvaux, F.; Delvaux, F.R. Decrease of Aged Beer Aroma by the Reducing Activity of Brewing Yeast. *J. Agric. Food Chem.* **2010**, *58*, 3107–3115. [CrossRef] [PubMed]
5. Saison, D.; De Schutter, D.P.; Delvaux, F.; Delvaux, F.R. Improved Flavor Stability by Aging Beer in the Presence of Yeast. *J. Am. Soc. Brew. Chem.* **2011**, *69*, 50–56. [CrossRef]
6. Vanderhaegen, B.; Neven, H.; Coghe, S.; Verstrepen, K.J.; Derdelinckx, G.; Verachtert, H. Bioflavoring and beer refermentation. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 140–150. [CrossRef]
7. Brey, S.E.; de Costa, S.; Rogers, P.J.; Bryce, J.H.; Morris, P.C.; Mitchell, W.J.; Stewart, G.G. The Effect of Proteinase A on Foam-Active Polypeptides During High and Low Gravity Fermentation. *J. Inst. Brew.* **2003**, *109*, 194–202. [CrossRef]
8. Chen, E.C.H.; Jamieson, A.M.; Van Gheluwe, G. The Release of Fatty Acids as a Consequence of Yeast Autolysis. *J. Am. Soc. Brew. Chem.* **1980**, *38*, 13–18. [CrossRef]
9. Penacho, V.; Valero, E.; Gonzalez, R. Transcription profiling of sparkling wine second fermentation. *Int. J. Food Microbiol.* **2012**, *153*, 176–182. [CrossRef]
10. Martínez-García, R.; García-Martínez, T.; Puig-Pujol, A.; Mauricio, J.C.; Moreno, J. Changes in sparkling wine aroma during the second fermentation under CO_2 pressure in sealed bottle. *Food Chem.* **2017**, *237*, 1030–1040. [CrossRef] [PubMed]
11. Martí-Raga, M.; Martín, V.; Gil, M.; Sancho, M.; Zamora, F.; Mas, A.; Beltran, G. Contribution of yeast and base wine supplementation to sparkling wine composition. *J. Sci. Food Agric.* **2016**, *96*, 4962–4972. [CrossRef] [PubMed]
12. Dilmetz, B.A.; Desire, C.T.; Meneses, J.; Klingler-Hoffmann, M.; Young, C.; Hoffmann, P. Impact of propagation time on yeast physiology during bottle conditioning of beer on an industrial scale. *Food Chem.* **2024**, *435*, 137655. [CrossRef] [PubMed]
13. Dilmetz, B.A.; Desire, C.T.; Donnellan, L.; Meneses, J.; Klingler-Hoffmann, M.; Young, C.; Hoffmann, P. Assessment of yeast physiology during industrial-scale brewing practices using the redox-sensitive dye resazurin. *Yeast* **2023**, *40*, 171–181. [CrossRef] [PubMed]
14. Wiśniewski, J.R.; Gaugaz, F.Z. Fast and Sensitive Total Protein and Peptide Assays for Proteomic Analysis. *Anal. Chem.* **2015**, *87*, 4110–4116. [CrossRef] [PubMed]
15. Bruderer, R.; Bernhardt, O.M.; Gandhi, T.; Miladinović, S.M.; Cheng, L.-Y.; Messner, S.; Ehrenberger, T.; Zanotelli, V.; Butscheid, Y.; Escher, C.; et al. Extending the Limits of Quantitative Proteome Profiling with Data-Independent Acquisition and Application to Acetaminophen-Treated Three-Dimensional Liver Microtissues. *Mol. Cell. Proteom.* **2015**, *14*, 1400–1410. [CrossRef] [PubMed]
16. Deutsch, E.W.; Bandeira, N.; Perez-Riverol, Y.; Sharma, V.; Carver, J.J.; Mendoza, L.; Kundu, D.J.; Wang, S.; Bandla, C.; Kamatchinathan, S.; et al. The ProteomeXchange consortium at 10 years: 2023 update. *Nucleic Acids Res.* **2022**, *51*, D1539–D1548. [CrossRef] [PubMed]
17. Perez-Riverol, Y.; Bai, J.; Bandla, C.; García-Seisdedos, D.; Hewapathirana, S.; Kamatchinathan, S.; Kundu, D.J.; Prakash, A.; Frericks-Zipper, A.; Eisenacher, M.; et al. The PRIDE database resources in 2022: A hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res.* **2021**, *50*, D543–D552. [CrossRef] [PubMed]
18. Wong, E.D.; Miyasato, S.R.; Aleksander, S.; Karra, K.; Nash, R.S.; Skrzypek, M.S.; Weng, S.; Engel, S.R.; Cherry, J.M. *Saccharomyces* genome database update: Server architecture, pan-genome nomenclature, and external resources. *Genetics* **2023**, *224*, iyac191. [CrossRef]
19. Stewart, G.G. Yeast Nutrition. In *Brewing and Distilling Yeasts*; Springer International Publishing: Cham, Switzerland, 2017; pp. 109–146.
20. Vanbeneden, N.; Vanderputten, D.; Vanderhaegen, B.; Derdelinckx, G.; Van Landschoot, A. Influence of the Sugar Composition of the Added Extract on the Refermentation of Beer in Bottles. *J. Am. Soc. Brew. Chem.* **2006**, *64*, 206–213. [CrossRef]
21. Werner-Washburne, M.; Braun, E.L.; Crawford, M.E.; Peck, V.M. Stationary phase in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **1996**, *19*, 1159–1166. [CrossRef]
22. Li, G.; Liu, F. Changes in Organic Acids during Beer Fermentation. *J. Am. Soc. Brew. Chem.* **2015**, *73*, 275–279. [CrossRef]
23. Van Landschoot, A.; Vanbeneden, N.; Vanderputten, D.; Derdelinckx, G. Effect of pitching yeast preparation on the refermentation of beer in bottles. *Cerevisia* **2004**, *29*, 140–145.
24. Song, L.; Chen, Y.; Guo, Q.; Huang, S.; Guo, X.; Xiao, D. Regulating the Golgi apparatus sorting of proteinase A to decrease its excretion in *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol.* **2019**, *46*, 601–612. [CrossRef] [PubMed]
25. Leisegang, R.; Stahl, U. Degradation of a Foam-Promoting Barley Protein by a Proteinase from Brewing Yeast. *J. Inst. Brew.* **2005**, *111*, 112–117. [CrossRef]
26. Michel, M.; Meier-Dörnberg, T.; Jacob, F.; Methner, F.-J.; Wagner, R.S.; Hutzler, M. Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. *J. Inst. Brew.* **2016**, *122*, 569–587. [CrossRef]
27. Pires, E.J.; Teixeira, J.A.; Brányik, T.; Vicente, A.A. Yeast: The soul of beer's aroma—A review of flavour-active esters and higher alcohols produced by the brewing yeast. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1937–1949. [CrossRef] [PubMed]

28. González-Jiménez, M.d.C.; Mauricio, J.C.; Moreno-García, J.; Puig-Pujol, A.; Moreno, J.; García-Martínez, T. Endogenous CO_2 Overpressure Effect on Higher Alcohols Metabolism during Sparkling Wine Production. *Microorganisms* **2023**, *11*, 1630. [CrossRef] [PubMed]
29. Vanderhaegen, B.; Delvaux, F.; Daenen, L.; Verachtert, H.; Delvaux, F.R. Aging characteristics of different beer types. *Food Chem.* **2007**, *103*, 404–412. [CrossRef]
30. Treu, L.; Campanaro, S.; Nadai, C.; Toniolo, C.; Nardi, T.; Giacomini, A.; Valle, G.; Blondin, B.; Corich, V. Oxidative stress response and nitrogen utilization are strongly variable in *Saccharomyces cerevisiae* wine strains with different fermentation performances. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 4119–4135. [CrossRef]
31. Gibson, B.R.; Lawrence, S.J.; Boulton, C.A.; Box, W.G.; Graham, N.S.; Linforth, R.S.T.; Smart, K.A. The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation. *FEMS Yeast Res.* **2008**, *8*, 574–585. [CrossRef]
32. Porras–Agüera, J.A.; Moreno–García, J.; García–Martínez, T.; Moreno, J.; Mauricio, J.C. Impact of CO_2 overpressure on yeast mitochondrial associated proteome during the "prise de mousse" of sparkling wine production. *Int. J. Food Microbiol.* **2021**, *348*, 109226. [CrossRef]
33. Stanley, D.; Bandara, A.; Fraser, S.; Chambers, P.J.; Stanley, G.A. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* **2010**, *109*, 13–24. [CrossRef]
34. Cabib, E.; Blanco, N.; Grau, C.; Rodríguez-Peña, J.M.; Arroyo, J. Crh1p and Crh2p are required for the cross-linking of chitin to β(1-6)glucan in the *Saccharomyces cerevisiae* cell wall. *Mol. Microbiol.* **2007**, *63*, 921–935. [CrossRef] [PubMed]
35. Udom, N.; Chansongkrow, P.; Charoensawan, V.; Auesukaree, C. Coordination of the Cell Wall Integrity and High-Osmolarity Glycerol Pathways in Response to Ethanol Stress in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **2019**, *85*, e00551-19. [CrossRef]
36. Bermejo, C.; Rodríguez, E.; García, R.; Rodríguez-Peña, J.M.; Concepción, M.L.R.d.l.; Rivas, C.; Arias, P.; Nombela, C.; Posas, F.; Arroyo, J. The Sequential Activation of the Yeast HOG and SLT2 Pathways Is Required for Cell Survival to Cell Wall Stress. *Mol. Biol. Cell* **2008**, *19*, 1113–1124. [CrossRef]
37. García, R.; Bermejo, C.; Grau, C.; Pérez, R.; Rodríguez-Peña, J.M.; Francois, J.; Nombela, C.; Arroyo, J. The Global Transcriptional Response to Transient Cell Wall Damage in *Saccharomyces cerevisiae* and Its Regulation by the Cell Integrity Signaling Pathway. *J. Biol. Chem.* **2004**, *279*, 15183–15195. [CrossRef]
38. Orlean, P. Architecture and Biosynthesis of the *Saccharomyces cerevisiae* Cell Wall. *Genetics* **2012**, *192*, 775–818. [CrossRef] [PubMed]
39. Ribeiro, R.A.; Vitorino, M.V.; Godinho, C.P.; Bourbon-Melo, N.; Robalo, T.T.; Fernandes, F.; Rodrigues, M.S.; Sá-Correia, I. Yeast adaptive response to acetic acid stress involves structural alterations and increased stiffness of the cell wall. *Sci. Rep.* **2021**, *11*, 12652. [CrossRef] [PubMed]
40. Xu, W.; Wang, J.; Li, Q. Microarray studies on lager brewer's yeasts reveal cell status in the process of autolysis. *FEMS Yeast Res.* **2014**, *14*, 714–728. [CrossRef]
41. Rossignol, T.; Dulau, L.; Julien, A.; Blondin, B. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast* **2003**, *20*, 1369–1385. [CrossRef]

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5 Conclusion and future directions

The early beginnings of beer brewing were largely a trial-and-error process as many beers were spontaneously fermented and varied in quality and taste[35,286]. The industrial revolution led to large-scale brewing and nowadays the process of brewing beer is comprised of many stages such as malting, mashing, and fermentation[287]. Although these processes are well understood from an industrial standpoint, the influence of process controls such as nutrient supply and oxygen availability on yeast growth and metabolism and therefore product quality is not fully understood[56]. This thesis focussed on utilising proteomics in combination with other biochemical assays to gain an increased understanding of yeast metabolism during propagation and bottle conditioning. This final chapter concludes the outcomes of this research, its contribution to the field of brewing biochemistry and future work that can be done to expand the field.

The physiological condition of the yeast used in brewery processes can have a significant influence on the organoleptic characteristics of beer. There are a number of assays to determine yeast physiology, and all have their limitations such as cost and time and therefore, their implementation in a brewery setting is limited[167]. The physiological quality of a yeast population is analysed by a combination of viability and vitality assays. Cell viability refers to the percentage of live cells in a population. MB is the most used dye for assessing cell viability in brewing contexts. However, environmental conditions can have a significant impact on yeast physiology during brewing that doesn't result in cellular death. The measurement of yeast vitality can be measured using a variety of methods that are based on different aspects of the cell. For example, the acidification power test is commonly employed in breweries to detect changes in vitality[288]. In this method, active yeast metabolism is determined based on the excretion of protons into the surrounding buffer. This method requires extended laboratory handling and multiple timepoints to obtain meaningful results. It is well known that different aspects of the cell are related to energy homeostasis and yeast vitality[178]. Therefore, the detection of changes

in yeast physiology is essential for not only improving yeast management but in scientific research applications.

In Chapter 2 of this thesis, we explored the application of resazurin as a vitality indicator to detect subtle changes in yeast physiology within a brewery setting. Resazurin is a blue dye that is non-toxic and routinely employed as a biochemical assay for measuring cellular metabolism[187]. It has been used in a wide range of applications such as food contamination[188] and cellular cytotoxicity[187]. Despite its widespread use, the application of using resazurin to measure cell physiology within a brewing context has not been explored. Resazurin was selected over other tetrazolium-based dyes as it can be used for real-time assessment and has fewer manual handling steps. Furthermore, this method was selected over other fluorescence-based dyes such as cFDA as it is more cost-effective and has a higher potential to be implemented in breweries without high initial investment costs. The measurement of cell vitality using resazurin involves its addition to cells in media, followed by its uptake into the cell and subsequent reduction to resorufin, a highly fluorescent pink product[187]. As the reduction of resazurin is mediated by enzymes that are located in the cytosol and mitochondria, it monitors the activity of reductive enzymes within the cell[193].

We applied the use of resazurin in an industrial scale brewery propagation to determine if it could be used to assess subtle changes in metabolism. We observed that the fluorescence produced by yeast cells decreased as they transitioned from lag to stationary phase. The resazurin assay was compared to the commercially available RealTime-Glo™ Extracellular ATP Assay where it was observed that ATP content was low in the lag phase, increased during logarithmic phase, and decreased during stationary phase. As resazurin could differentiate energy status within a certain growth stage such early and late logarithmic stage, it indicated that resazurin was more sensitive assay compared to ATP content. Resazurin was also compared to other physiological markers such as viability using MB and storage carbohydrates. A decline in fluorescence occurred prior to any detectable changes in viability and glycogen and trehalose. This showed that resazurin is a sensitive method that can be used to optimise cropping times during brewery propagation. The observed differences between resazurin and other assays such as the RealTime-Glo™ Extracellular ATP Assay or MB highlighted that the measurement of different components within the cell will lead to differences in the outcome of the assay and interpretation of results. Therefore, we recommend the use of resazurin in conjunction

with other viability and vitality assays to obtain a more holistic overview of the yeast's physiological condition.

A major advantage of using a fluorescent dye instead of a colorimetric dye is that trubs and other precipitants do not interfere with the measurement. We observed that performing the assay in beer had a significant impact on fluorescence. The high background observed when using beer was hypothesised to occur due to its low pH. To overcome this the yeast cells were resuspended in PBS but this led to the requirement of additional handling steps. Therefore, neutralising the pH of the beer (or media) within the assay could be investigated to mitigate these additional steps. This method could be adopted for use in other brewing processes such as serial repitching to predict yeast stress. Additionally, it could also be used in other beverage contexts that utilise yeast such as winemaking and distilling and in scientific research to assess yeast quality.

The primary aim of a brewery propagation is to produce yeast that has high vitality and viability, sufficient biomass yield, and is free from spoilage organisms[289]. This is important within the context of this thesis as yeast in good physiological condition is required to withstand the environment of bottle conditioning. This is also applicable to the wine industry which frequently use bottle conditioning for the production of sparkling wines. Breweries utilise a hybrid propagation to achieve this to ensure that the fermentation characteristics of the yeast are not lost[289]. Despite propagation being understood from an industrial standpoint, the underlying metabolic changes that occur in yeast and their influence on the product remains poorly understood.

Typically, yeast cells are transferred during propagation at the logarithmic phase prior to the onset of the post-diauxic shift or stationary phase. We assumed that propagation of yeast to the logarithmic phase for bottle conditioning would result in a more robust yeast during bottle conditioning. To investigate this further, we compared yeast propagated to the logarithmic and post-diauxic phase for seeding into beer for bottle conditioning in Chapter 3. Our results showed that yeast grown to the post-diauxic shift had increased longevity compared to yeast grown to the logarithmic phase. During these analyses we observed that glycogen and trehalose levels in the yeast cells could not be used as a predictive marker of yeast fermentation and longevity during bottle conditioning. This aligns with previous studies that did not find a correlation between storage carbohydrates and fermentation performance[184]. We performed proteomic analysis to gain a better

understanding of yeast physiology that enabled post-diauxic shift yeast to have improved survival characteristics. An increase in proteins associated with cellular respiration and general stress responses were observed in post-diauxic shift yeast. It has been previously shown that activation of cellular respiration pathways in yeast leads to improved stress tolerance[143]. Therefore, yeast cells propagated to the post-diauxic shift were able to more effectively initiate stress responses in the beer and survive longer. The findings of these experiments are of significant interest to breweries as it is necessary to propagate yeast until the post-diauxic shift to ensure a high-quality yeast. Further research should explore the impact of modifying other parameters such as oxygenation regime and wort concentration to optimise yeast physiological condition in bottle conditioning as well as primary fermentation. For example, it has been shown that propagation of yeast in wort at high-gravity (17.5°P) leads to poor yeast quality during high-gravity brewing[127]. Whereas propagation of yeast with wort between 7.7°P to 12.5°P is required for maintaining high viability during high-gravity brewing. Additionally, these experiments could be performed on both a small (~20 L) and an industrial scale (60 hL) to reveal physiological changes of yeast that are influenced by scale. Ideally, this would enable breweries to optimise propagation parameters at a higher throughput. This research has shown how the modification of a single environmental parameter can have significant implications on the ability of yeast to survive during bottle conditioning. Furthermore, the results obtained have broader implications within other industries that utilise yeast. For example, yeast with a strong autolytic phenotype is desired for the bottle conditioning of sparkling wines to obtain the desired organoleptic profile[290]. Therefore, propagation of yeast to the logarithmic phase could accelerate the autolytic process and reduce the ageing time for sparkling wine production.

Lastly, it has been shown that the yeast strain can impact the outcome of bottle conditioning. Furthermore, that different yeast strains can survive for different lengths of time. In Chapter 4, we evaluated six yeast strains from the brewing, wine, and distilling industries on their ability to perform bottle conditioning. We utilised the resazurin method developed in Chapter 2 to assess yeast physiology during propagation and found that all yeasts reached stationary phase prior to seeding for bottle conditioning. In this study we observed that the different strains required different lengths of time to complete bottle conditioning. Despite this, the sensory profile of the beer was similar irrespective of the brewing yeast strain (ale or lager) and had minimal effect on the aroma. In contrast,

sparkling wine and distilling yeast should be used when a beer with unique aroma is required. Furthermore, we observed that a decrease in viability of yeast only impacted foam stability when it decreased below 50%. Therefore, selection of a yeast with improved survival will help maintain foam stability. Proteomic analysis was performed on the Ale brewing yeast 2 (superior survival characteristics) compared to Sparkling wine yeast (poor survival characteristics) to gain an understanding of the processes underpinning survival. Proteins associated with oxidoreductase activity and mitochondrial ribosomes were upregulated in Ale brewing yeast 2. Interestingly, proteins associated with cell wall organisation were more rapidly upregulated in Sparkling wine yeast. We proposed that Ale brewing yeast 2 was more adept at responding to ethanol induced stress compared to Sparkling wine yeast, which enabled it to survive for longer. To explore the broader impact of these strains on the flavour profile, a metabolomic approach that is untargeted would allow for a better understanding of the complex biochemistry that is taking place during bottle conditioning and allow for further development of this process. The results obtained build on existing literature that have shown that yeasts grown under anaerobic conditions displayed an increase in the expression of oxidative stress genes and proteins as a response to cellular stress[291].

The proteomic database utilised to compare the proteome of Ale brewing yeast 2 and Sparkling wine yeast was the S288c laboratory strain. Genome sequencing has shown that yeasts from a particular fermented beverage or geographical location are more similar compared to yeasts from another environment[11]. It has been found that ale brewing yeasts diverge into two distinct clades: clade 1 contained yeast that was separated by geographical location and clade 2 that contain yeasts that were phylogenetically close to sake, wine, or bread[196]. The Ale brewing yeast 2 used in this study was similar to the WLP001 strain according to sources at the brewery. Genomic studies of this yeast have shown that it was similar to S288c, and the use of this proteome should closely align with any proteomic changes observed[292]. The Sparkling wine yeast used in Chapter 4 was the commercially available EC1118 which was shown to have 34 protein encoding genes that were not present in S288c [293]. Most of these genes were involved in the metabolism or transport of sugar or nitrogen. It was hypothesised that the increase in genes associated with sugar and nitrogen metabolism may improve the yeasts adaptation to the fermentation of high-sugar, low nitrogen environment in grape must[293]. Typically, sugar

and nitrogen are not limiting in brewing and shouldn't impact the overall proteomic outcome in this thesis.

Our findings from Chapter 3 and Chapter 4 indicate that pre-adaptation of yeast to stress conditions improves their longevity in bottle conditioning, most likely due to selective pressure. This may be a good start for future studies to improve yeast longevity during bottle conditioning. For example, evolutionary engineering or adaptive laboratory evolution could be used to obtain mutants that have an industrially relevant trait[294]. A previous study improved ethanol tolerance in yeast strains by cultivation in an aerobic turbidostat on complex medium with increasing ethanol concentrations[295]. However, this required 200 generations (~2 years) to generate yeast with improved ethanol tolerance[295]. An issue that has been identified with evolutionary engineering is that the yeast strains evolved for stress tolerance do not always show the mutant phenotype when the stress is removed[294]. Cycles of adaptive evolution with and without the selective pressure may be required to obtain a constitutive mutant phenotype[296]. Other literature has shown that short-term exposure of yeast strains to low pH during propagation enabled the yeast to perform secondary fermentation in sour beers[83]. Therefore, brewers may be able to adapt the propagation media to improve yeast longevity during bottle conditioning compared to evolutionary adaptation.

Overall, we effectively combined resazurin fluorescence vitality testing and proteomics with current brewery-based quality control measurements to provide insights into physiological differences in brewing and industrials strains of yeast. This work supplements the growing number of research papers utilising MS and proteomics within a brewing context and illustrates its application for optimising yeast physiology.

6 References

1.Balasubramanian, M. K.; Bi, E.; Glotzer, M. Comparative Analysis of Cytokinesis in Budding Yeast, Fission Yeast and Animal Cells. *Current Biology* **2004**, *14* (18), R806-R818. DOI: 10.1016/j.cub.2004.09.022.

2.Sicard, D.; Legras, J.-L. Bread, beer and wine: Yeast domestication in the *Saccharomyces* sensu stricto complex. *Comptes Rendus Biologies* **2011**, *334* (3), 229-236. DOI: 10.1016/j.crvi.2010.12.016.

3.Chapman, A. C. THE YEAST CELL: WHAT DID LEEUWENHOECK SEE? *Journal of the Institute of Brewing* **1931**, *37* (4), 433-436. DOI: 10.1002/j.2050-0416.1931.tb05346.x.

4.Pasteur, L. Mémoire sur la fermentation appelée lactique (Extrait par l'auteur). *Molecular Medicine* **1995**, *1* (6), 599–601.

5.Goffeau, A.; Barrell, B. G.; Bussey, H.*; et al.* Life with 6000 genes. *Science* **1996**, *274* (5287), 546, 563-547. DOI: 10.1126/science.274.5287.546.

6.Johnson, E. A.; Echavarri-Erasun, C. Chapter 3 - Yeast Biotechnology. In *The Yeasts (Fifth Edition)*, Kurtzman, C. P., Fell, J. W., Boekhout, T. Eds.; Elsevier, 2011; pp 21-44; DOI: 10.1016/B978-0-444-52149-1.00003-3.

7.Hou, J.; Tyo, K. E. J.; Liu, Z.*; et al.* Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*. *FEMS Yeast Research* **2012**, *12* (5), 491-510. DOI: 10.1111/j.1567-1364.2012.00810.x.

8.Libkind, D.; Peris, D.; Cubillos, F. A.*; et al.* Into the wild: new yeast genomes from natural environments and new tools for their analysis. *FEMS Yeast Research* **2020**, *20* (2). DOI: 10.1093/femsyr/foaa008.

9.Peter, J.; De Chiara, M.; Friedrich, A.*; et al.* Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. *Nature* **2018**, *556* (7701), 339-344. DOI: 10.1038/s41586-018-0030-5.

10.Sanderson, M. J.; Driskell, A. C. The challenge of constructing large phylogenetic trees. *Trends in Plant Science* **2003**, *8* (8), 374-379. DOI: 10.1016/S1360-1385(03)00165-1.

11.Gallone, B.; Steensels, J.; Prahl, T.*; et al.* Domestication and Divergence of *Saccharomyces cerevisiae* Beer Yeasts. *Cell* **2016**, *166* (6), 1397-1410.e1316. DOI: 10.1016/j.cell.2016.08.020.

12.De Guidi, I.; Legras, J.-L.; Galeote, V.*; et al.* Yeast domestication in fermented food and beverages: past research and new avenues. *Current Opinion in Food Science* **2023**, *51*, 101032. DOI: 10.1016/j.cofs.2023.101032.

13.Marsit, S.; Dequin, S. Diversity and adaptive evolution of *Saccharomyces* wine yeast: a review. *FEMS Yeast Research* **2015**, *15* (7). DOI: 10.1093/femsyr/fov067.

14.Bisson, L. F.; Fan, Q.; Walker, G. A. Sugar and Glycerol Transport in *Saccharomyces cerevisiae*. In *Yeast Membrane Transport*, Ramos, J., Sychrová, H., Kschischo, M. Eds.;

Springer International Publishing, 2016; pp 125-168; DOI: 10.1007/978-3-319-25304-6_6.
15.Alves-Jr, S. L.; Herberts, R. A.; Hollatz, C.*; et al.* Maltose and Maltotriose Active Transport and Fermentation by *Saccharomyces Cerevisiaes*[1]. *Journal of the American Society of Brewing Chemists* **2007**, *65* (2), 99-104. DOI: 10.1094/ASBCJ-2007-0411-01.
16.Stewart, G. G. Energy Metabolism by the Yeast Cell. In *Brewing and Distilling Yeasts*, Springer International Publishing, 2017; pp 77-107; DOI: 10.1007/978-3-319-69126-8_6.
17.Chen, M.; Xie, T.; Li, H.*; et al.* Yeast increases glycolytic flux to support higher growth rates accompanied by decreased metabolite regulation and lower protein phosphorylation. *Proceedings of the National Academy of Sciences* **2023**, *120* (25), e2302779120. DOI: 10.1073/pnas.2302779120.
18.DeRisi, J. L.; Iyer, V. R.; Brown, P. O. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **1997**, *278* (5338), 680-686. DOI: 10.1126/science.278.5338.680.
19.Zhang, Y.; Su, M.; Wang, Z.*; et al.* Rewiring regulation on respiro-fermentative metabolism relieved Crabtree effects in *Saccharomyces cerevisiae*. *Synthetic and Systems Biotechnology* **2022**, *7* (4), 1034-1043. DOI: 10.1016/j.synbio.2022.06.004.
20.Heyland, J.; Fu, J.; Blank, L. M. Correlation between TCA cycle flux and glucose uptake rate during respiro-fermentative growth of *Saccharomyces cerevisiae*. *Microbiology* **2009**, *155* (12), 3827-3837. DOI: 10.1099/mic.0.030213-0.
21.De Deken, R. H. The Crabtree Effect: A Regulatory System in Yeast. *Microbiology* **1966**, *44* (2), 149-156. DOI: 10.1099/00221287-44-2-149.
22.Hagman, A.; Säll, T.; Compagno, C.*; et al.* Yeast "Make-Accumulate-Consume" Life Strategy Evolved as a Multi-Step Process That Predates the Whole Genome Duplication. *PLOS ONE* **2013**, *8* (7), e68734. DOI: 10.1371/journal.pone.0068734.
23.Pfeiffer, T.; Morley, A. An evolutionary perspective on the Crabtree effect. *Frontiers in Molecular Biosciences* **2014**, *1*, Perspective. DOI: 10.3389/fmolb.2014.00017.
24.Slavov, N.; Budnik, Bogdan A.; Schwab, D.*; et al.* Constant Growth Rate Can Be Supported by Decreasing Energy Flux and Increasing Aerobic Glycolysis. *Cell Reports* **2014**, *7* (3), 705-714. DOI: 10.1016/j.celrep.2014.03.057.
25.van Rossum, H. M.; Kozak, B. U.; Niemeijer, M. S.*; et al.* Alternative reactions at the interface of glycolysis and citric acid cycle in *Saccharomyces cerevisiae*. *FEMS Yeast Research* **2016**, *16* (3). DOI: 10.1093/femsyr/fow017.
26.Remize, F.; Andrieu, E.; Dequin, S. Engineering of the Pyruvate Dehydrogenase Bypass in S*accharomyces cerevisiae*: Role of the Cytosolic Mg^{2+} and Mitochondrial K^{+} Acetaldehyde Dehydrogenases Ald6p and Ald4p in Acetate Formation during Alcoholic Fermentation. *Applied and Environmental Microbiology* **2000**, *66* (8), 3151-3159. DOI: 10.1128/AEM.66.8.3151-3159.2000.
27.Bakker, B. M.; Overkamp, K. M.; van Maris, A. J. A.*; et al.* Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **2001**, *25* (1), 15-37. DOI: 10.1111/j.1574-6976.2001.tb00570.x.
28.Zara, V.; De Blasi, G.; Ferramosca, A. Assembly of the Multi-Subunit Cytochrome bc1 Complex in the Yeast *Saccharomyces cerevisiae*. *International Journal of Molecular Sciences* **2022**, *23* (18), 10537. DOI: 10.3390/ijms231810537.
29.Zampar, G. G.; Kümmel, A.; Ewald, J.*; et al.* Temporal system-level organization of the switch from glycolytic to gluconeogenic operation in yeast. *Molecular Systems Biology* **2013**, *9* (1), 651. DOI: 10.1038/msb.2013.11.

30.Hough, J. S.; Briggs, D. E.; Stevens, R.*; et al.* Yeast Growth. In *Malting and Brewing Science: Volume II Hopped Wort and Beer*, Springer US, 1982; pp 615-643; DOI: 10.1007/978-1-4615-1799-3_7.
31.Gilliland, R. B. Yeast Reproduction During Fermentation. *Journal of the Institute of Brewing* **1962**, *68* (3), 271-275. DOI: 10.1002/j.2050-0416.1962.tb01863.x.
32.Werner-Washburne, M.; Braun, E.; Johnston, G. C.*; et al.* Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiology Reviews* **1993**, *57* (2), 383-401. DOI: 10.1128/mr.57.2.383-401.1993.
33.Stahl, G.; Salem, S. N. B.; Chen, L.*; et al.* Translational Accuracy during Exponential, Postdiauxic, and Stationary Growth Phases in *Saccharomyces cerevisiae*. *Eukaryotic Cell* **2004**, *3* (2), 331-338. DOI: 10.1128/EC.3.2.331-338.2004.
34.Lewis, J. G.; Northcott, C. J.; Learmonth, R. P.*; et al.* The need for consistent nomenclature and assessment of growth phases in diauxic cultures of *Saccharomyces cerevisiae*. *Microbiology* **1993**, *139* (4), 835-839. DOI: 10.1099/00221287-139-4-835.
35.Hornsey, I. S. *A History of Beer and Brewing*; The Royal Society of Chemistry, 2003. DOI: 10.1039/9781847550026
36.Betancur, M. I.; Motoki, K.; Spence, C.*; et al.* Factors influencing the choice of beer: A review. *Food Research International* **2020**, *137*, 109367. DOI: 10.1016/j.foodres.2020.109367.
37.Oo, S. *Beer Manufacturing in Australia*; Australia, 2021. https://my.ibisworld.com/au/en/industry/c1212.
38.Cloutman, N. *Craft Beer Production in Australia*; 2021. https://my.ibisworld.com/au/en/industry-specialized/od5071/industry-at-a-glance.
39.Steensels, J.; Verstrepen, K. J. Taming Wild Yeast: Potential of Conventional and Nonconventional Yeasts in Industrial Fermentations. *Annual Review of Microbiology* **2014**, *68* (1), 61-80. DOI: 10.1146/annurev-micro-091213-113025.
40.2 - Malts, adjuncts and supplementary enzymes. In *Brewing Science and Practice*, Briggs, D. E., Boulton, C. A., Brookes, P. A., Stevens, R. Eds.; Woodhead Publishing, 2004; pp 11-51; DOI: 10.1533/9781855739062.11.
41.Stewart, G. G. Chapter 7 - Biochemistry of Brewing. In *Biochemistry of Foods (Third Edition)*, Eskin, N. A. M., Shahidi, F. Eds.; Academic Press, 2013; pp 291-318; DOI: 10.1016/B978-0-08-091809-9.00007-8.
42.Rani, H.; Bhardwaj, R. D. Quality attributes for barley malt: "The backbone of beer". *Journal of Food Science* **2021**, *86* (8), 3322-3340. DOI: 10.1111/1750-3841.15858.
43.The Brewing Process. In *Brewing Yeast and Fermentation*, Boulton, C., Quain, D. Eds.; Blackwell Publishing, 2006; pp 19-68; DOI: 10.1002/9780470999417.ch2.
44.Stewart, G. G. Yeast Nutrition. In *Brewing and Distilling Yeasts*, Springer International Publishing, 2017; pp 109-146; DOI: 10.1007/978-3-319-69126-8_7.
45.MacWilliam, I. C. Wort Composition—A Review. *Journal of the Institute of Brewing* **1968**, *74* (1), 38-54. DOI: 10.1002/j.2050-0416.1968.tb03095.x.
46.Stewart, G. G. *Saccharomyces* species in the Production of Beer. *Beverages* **2016**, *2* (4), 34. DOI: 10.3390/beverages2040034.
47.Krogerus, K.; Gibson, B. R. 125th Anniversary Review: Diacetyl and its control during brewery fermentation. *Journal of the Institute of Brewing* **2013**, *119* (3), 86-97. DOI: 10.1002/jib.84.
48.Hucker, B.; Wakeling, L.; Vriesekoop, F. Vitamins in brewing: the impact of wort production on the thiamine and riboflavin vitamer content of boiled sweet wort. *Journal of the Institute of Brewing* **2014**, *120* (3), 164-173. DOI: 10.1002/jib.142.

49.12 - Metabolism of wort by yeast. In *Brewing Science and Practice*, Briggs, D. E., Boulton, C. A., Brookes, P. A., Stevens, R. Eds.; Woodhead Publishing, 2004; pp 401-468; DOI: 10.1533/9781855739062.401.
50.Gibson, B. R. 125th Anniversary Review: Improvement of Higher Gravity Brewery Fermentation via Wort Enrichment and Supplementation. *Journal of the Institute of Brewing* **2011**, *117* (3), 268-284. DOI: 10.1002/j.2050-0416.2011.tb00472.x.
51.Kato, T.; Takahashi, T. Studies on the Genetic Characteristics of the Brewing Yeasts *Saccharomyces*: A Review. *Journal of the American Society of Brewing Chemists* **2023**, *81* (2), 199-210. DOI: 10.1080/03610470.2022.2134972.
52.Libkind, D.; Hittinger, C. T.; Valério, E.*; et al.* Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences* **2011**, *108* (35), 14539-14544. DOI: 10.1073/pnas.1105430108.
53.Stewart, G. G. *Taxonomy of Brewing and Distilling Yeasts and Methods of Identification*; Springer International Publishing, 2017. DOI: 10.1007/978-3-319-69126-8_3
54.Basso, R. F.; Alcarde, A. R.; Portugal, C. B. Could non-*Saccharomyces* yeasts contribute on innovative brewing fermentations? *Food Research International* **2016**, *86*, 112-120. DOI: 10.1016/j.foodres.2016.06.002.
55.Quain, D. E. 8 - Yeast supply and propagation in brewing. In *Brewing New Technologies*, Bamforth, C. W. Ed.; Woodhead Publishing, 2006; pp 167-182; DOI: 10.1533/9781845691738.167.
56.Lodolo, E. J.; Kock, J. L. F.; Axcell, B. C.*; et al.* The yeast *Saccharomyces cerevisiae*–the main character in beer brewing. *FEMS Yeast Research* **2008**, *8* (7), 1018-1036. DOI: 10.1111/j.1567-1364.2008.00433.x.
57.Jakobsen, M.; Thorne, R. S. W. Oxygen Requirements of Brewing Strains of *Saccharomyces Uvarum* (*Carlsbergensis*) - Bottom Fermentation Yeast *Journal of the Institute of Brewing* **1980**, *86* (6), 284-287. DOI: 10.1002/j.2050-0416.1980.tb06882.x.
58.Kirsop, B. H. Oxygen in Brewery Fermentation. *Journal of the Institute of Brewing* **1974**, *80* (3), 252-259. DOI: 10.1002/j.2050-0416.1974.tb03614.x.
59.Piškur, J.; Rozpędowska, E.; Polakova, S.*; et al.* How did *Saccharomyces* evolve to become a good brewer? *Trends in Genetics* **2006**, *22* (4), 183-186. DOI: 10.1016/j.tig.2006.02.002.
60.Pronk, J. T.; Yde Steensma, H.; Van Dijken, J. P. Pyruvate Metabolism in *Saccharomyces cerevisiae*. *Yeast* **1996**, *12* (16), 1607-1633. DOI: 10.1002/(SICI)1097-0061(199612)12:16<1607::AID-YEA70>3.0.CO;2-4.
61.van Hoek, P.; de Hulster, E.; van Dijken, J. P.*; et al.* Fermentative capacity in high-cell-density fed-batch cultures of baker's yeast. *Biotechnology and Bioengineering* **2000**, *68* (5), 517-523. DOI: 10.1002/(SICI)1097-0290(20000605)68:5<517::AID-BIT5>3.0.CO;2-O.
62.Nielsen, O. Control of the yeast propagation process: How to optimize oxygen supply and minimize stress. 2005.
63.Moutsoglou, M. E.; Dearden, A. C. Effect of the respiro-fermentative balance during yeast propagation on fermentation and wort attenuation. *Journal of the Institute of Brewing* **2020**, *126* (3), 289-297. DOI: 10.1002/jib.621.
64.O'Connor-Cox, E. S. C.; Lodolo, E. J.; Axcell, B. C. Role of Oxygen in High-Gravity Fermentations in the Absence of Unsaturated Lipid Biosynthesis. *Journal of the American Society of Brewing Chemists* **1993**, *51* (3), 97-107. DOI: 10.1094/ASBCJ-51-0097.

65.Gibson, B. R.; Lawrence, S. J.; Leclaire, J. P. R.*; et al.* Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews* **2007**, *31* (5), 535-569. DOI: 10.1111/j.1574-6976.2007.00076.x.
66.Vanderhaegen, B.; Neven, H.; Coghe, S.*; et al.* Bioflavoring and beer refermentation. *Applied Microbiology Biotechnology* **2003**, *62* (2-3), 140-150. DOI: 10.1007/s00253-003-1340-5.
67.Saison, D.; De Schutter, D. P.; Delvaux, F.*; et al.* Improved Flavor Stability by Aging Beer in the Presence of Yeast. *Journal of the American Society of Brewing Chemists* **2011**, *69* (1), 50-56. DOI: 10.1094/ASBCJ-2011-0127-01.
68.Derdelinckx, G.; Vanderhasselt, B.; Maudoux, M.*; et al.* Refermentation in bottles and kegs. A rigorous approach. 1992.
69.Saison, D.; De Schutter, D. P.; Vanbeneden, N.*; et al.* Decrease of Aged Beer Aroma by the Reducing Activity of Brewing Yeast. *Journal of Agricultural and Food Chemistry* **2010**, *58* (5), 3107-3115. DOI: 10.1021/jf9037387.
70.van Iersel, M. F.; Eppink, M. H.; van Berkel, W. J.*; et al.* Purification and characterization of a novel NADP-dependent branched-chain alcohol dehydrogenase from *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **1997**, *63* (10), 4079-4082. DOI: 10.1128/aem.63.10.4079-4082.1997.
71.Chen, E. C. H.; Jamieson, A. M.; Van Gheluwe, G. The Release of Fatty Acids as a Consequence of Yeast Autolysis. *Journal of the American Society of Brewing Chemists* **1980**, *38* (1), 13-18. DOI: 10.1094/ASBCJ-38-0013.
72.Brey, S. E.; de Costa, S.; Rogers, P. J.*; et al.* The Effect of Proteinase A on Foam-Active Polypeptides During High and Low Gravity Fermentation. *Journal of the Institute of Brewing* **2003**, *109* (3), 194-202. DOI: 10.1002/j.2050-0416.2003.tb00159.x.
73.Marconi, O.; Rossi, S.; Galgano, F.*; et al.* Influence of yeast strain, priming solution and temperature on beer bottle conditioning. *Journal of the Science of Food and Agriculture* **2016**, *96* (12), 4106-4115. DOI: 10.1002/jsfa.7611.
74.Vanbeneden, N.; Vanderputten, D.; Vanderhaegen, B.*; et al.* Influence of the Sugar Composition of the Added Extract on the Refermentation of Beer in Bottles. *Journal of the American Society of Brewing Chemists* **2006**, *64* (4), 206-213. DOI: 10.1094/ASBCJ-64-0206.
75.Van Landschoot, A.; Vanbeneden, N.; Machtelinckx, M.*; et al.* Pecularities of seven refermented Belgian strong ales and their corresponding industrial yeasts. *Cerevisia* **2005**, *30*, 181-188.
76.Van Landschoot, A.; Vanbeneden, N.; Vanderputten, D.*; et al.* Effect of pitching yeast preparation on the refermentation of beer in bottles. *Cerevisia* **2004**, *29* (3), 140-145.
77.Tofalo, R.; Perpetuini, G.; Di Gianvito, P.*; et al.* Characterization of specialized flocculent yeasts to improve sparkling wine fermentation. *Journal of Applied Microbiology* **2016**, *120* (6), 1574-1584. DOI: 10.1111/jam.13113.
78.Kalayu, G. Serial re-pitching: its effect on yeast physiology, fermentation performance, and product quality. *Annals of Microbiology* **2019**, *69* (8), 787-796. DOI: 10.1007/s13213-019-01493-4.
79.Wang, J.; Ding, H.; Zheng, F.*; et al.* Physiological Changes of Beer Brewer's Yeast During Serial Beer Fermentation. *Journal of the American Society of Brewing Chemists* **2019**, *77* (1), 10-20. DOI: 10.1080/03610470.2018.1546030.
80.Jenkins, D. M.; Powell, C. D.; Fischborn, T.*; et al.* Rehydration of Active Dry Brewing Yeast and its Effect on Cell Viability. *Journal of the Institute of Brewing* **2011**, *117* (3), 377-382. DOI: 10.1002/j.2050-0416.2011.tb00482.x.
81.Shayevitz, A.; Abbott, E.; Van Zandycke, S.*; et al.* The Impact of Lactic and Acetic Acid on Primary Beer Fermentation Performance and Secondary Re-Fermentation during

Bottle-Conditioning with Active Dry Yeast. *Journal of the American Society of Brewing Chemists* **2022**, *80* (3), 258-269. DOI: 10.1080/03610470.2021.1952508.
82.Barazani, B.; Piercey, M.; Paulson, A.*; et al.* Rehydration of active dried yeast: impact on strength and stiffness of yeast cells measured using microelectromechanical systems. *Journal of the Institute of Brewing* **2019**, *125* (1), 53-59. DOI: 10.1002/jib.548.
83.Rogers, C. M.; Veatch, D.; Covey, A.*; et al.* Terminal acidic shock inhibits sour beer bottle conditioning by *Saccharomyces cerevisiae*. *Food Microbiology* **2016**, *57*, 151-158. DOI: 10.1016/j.fm.2016.02.012.
84.Attfield, P. V. Stress tolerance: The key to effective strains of industrial baker's yeast. *Nature Biotechnology* **1997**, *15* (13), 1351-1357. DOI: 10.1038/nbt1297-1351.
85.Causton, H. C.; Ren, B.; Koh, S. S.*; et al.* Remodeling of Yeast Genome Expression in Response to Environmental Changes. *Molecular Biology of the Cell* **2001**, *12* (2), 323-337. DOI: 10.1091/mbc.12.2.323.
86.Gasch, A. P.; Spellman, P. T.; Kao, C. M.*; et al.* Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. *Molecular Biology of the Cell* **2000**, *11* (12), 4241-4257. DOI: 10.1091/mbc.11.12.4241.
87.Marchler, G.; Schüller, C.; Adam, G.*; et al.* A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *The EMBO Journal* **1993**, *12* (5), 1997-2003. DOI: 10.1002/j.1460-2075.1993.tb05849.x.
88.Kobayashi, N.; McEntee, K. Identification of *cis* and *trans* components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **1993**, *13* (1), 248-256. DOI: 10.1128/mcb.13.1.248-256.1993.
89.Martínez-Pastor, M. T.; Marchler, G.; Schüller, C.*; et al.* The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *The EMBO Journal* **1996**, *15* (9), 2227-2235. DOI: 10.1002/j.1460-2075.1996.tb00576.x.
90.Görner, W.; Durchschlag, E.; Martinez-Pastor, M. T.*; et al.* Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes & Development* **1998**, *12* (4), 586-597. DOI: 10.1101/gad.12.4.586.
91.Beck, T.; Hall, M. N. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* **1999**, *402* (6762), 689-692. DOI: 10.1038/45287.
92.Gasch, A. P. The environmental stress response: a common yeast response to diverse environmental stresses. In *Yeast Stress Responses*, Hohmann, S., Mager, W. H. Eds.; Springer Berlin Heidelberg, 2003; pp 11-70; DOI: 10.1007/3-540-45611-2_2.
93.Marques, W. L.; Raghavendran, V.; Stambuk, B. U.*; et al.* Sucrose and *Saccharomyces cerevisiae*: a relationship most sweet. *FEMS Yeast Research* **2015**, *16* (1). DOI: 10.1093/femsyr/fov107.
94.Patel, G. B.; Ingledew, W. M. Trends in Wort Carbohydrate Utilization. *Applied Microbiology* **1973**, *26* (3), 349-353. DOI: 10.1128/am.26.3.349-353.1973.
95.Federoff, H. J.; Eccleshall, T. R.; Marmur, J. Carbon catabolite repression of maltase synthesis in *Saccharomyces carlsbergensis*. *Journal of Bacteriology* **1983**, *156* (1), 301-307. DOI: 10.1128/jb.156.1.301-307.1983.
96.D'Amore, T.; Russell, I.; Stewart, G. G. The effect of carbohydrate adjuncts on brewer's wort fermentation by *Saccharomyces uvarum (carlsbergensis)*. *Journal of the Institute of Brewing* **1989**, *95* (5), 333-336. DOI: 10.1002/j.2050-0416.1989.tb04640.x.
97.François, J.; Parrou, J. L. Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **2001**, *25* (1), 125-145. DOI: 10.1111/j.1574-6976.2001.tb00574.x.

98.Stewart, G. G.; Hill, A.; Lekkas, C. Wort FAN – Its Characteristics and Importance during Fermentation. *Journal of the American Society of Brewing Chemists* **2013**, *71* (4), 179-185. DOI: 10.1094/ASBCJ-2013-0921-01.
99.Jones, M.; Pierce, J. S. Absorption of amino acids from wort by yeasts. *Journal of the Institute of Brewing* **1964**, *70* (4), 307-315. DOI: 10.1002/j.2050-0416.1964.tb01996.x.
100.Forsberg, H.; Ljungdahl, P. O. Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. *Molecular and Cellular Biology* **2001**, *21* (3), 814-826. DOI: 10.1128/mcb.21.3.814-826.2001.
101.Conrad, M.; Schothorst, J.; Kankipati, H. N.*; et al.* Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **2014**, *38* (2), 254-299. DOI: 10.1111/1574-6976.12065.
102.Hofman-Bang, J. Nitrogen catabolite repression in *Saccharomyces cerevisiae*. *Molecular Biotechnology* **1999**, *12* (1), 35-71. DOI: 10.1385/MB:12:1:35.
103.Hinnebusch, A. G. Translational regulation of GCN4 and the general amino acid control of yeast *Annual Review of Microbiology* **2005**, *59* (1), 407-450. DOI: 10.1146/annurev.micro.59.031805.133833.
104.Stewart, G. G.; Hill, A.; Lekkas, C. Wort FAN – Its Characteristics and Importance during Fermentation1. *Journal of the American Society of Brewing Chemists* **2013**, *71* (4), 179-185. DOI: 10.1094/ASBCJ-2013-0921-01.
105.Cueto-Rojas, H. F.; Maleki Seifar, R.; Ten Pierick, A.*; et al.* In Vivo Analysis of NH_4^+ Transport and Central Nitrogen Metabolism in *Saccharomyces cerevisiae* during Aerobic Nitrogen-Limited Growth. *Applied and Environmental Microbiology* **2016**, *82* (23), 6831-6845. DOI: 10.1128/aem.01547-16.
106.Zhang, W.; Du, G.; Zhou, J.*; et al.* Regulation of Sensing, Transportation, and Catabolism of Nitrogen Sources in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews* **2018**, *82* (1), e00040-00017. DOI: 10.1128/MMBR.00040-17.
107.Ferreira, I. M.; Guido, L. F. Impact of Wort Amino Acids on Beer Flavour: A Review. *Fermentation* **2018**, *4* (2), 23. DOI: 10.3390/fermentation4020023.
108.Puligundla, P.; Smogrovicova, D.; Obulam, V. S. R.*; et al.* Very high gravity (VHG) ethanolic brewing and fermentation: a research update. *Journal of Industrial Microbiology and Biotechnology* **2011**, *38* (9), 1133-1144. DOI: 10.1007/s10295-011-0999-3.
109.Cartwright, C. P.; juroszek, J.-R.; Beavan, M. J.*; et al.* Ethanol Dissipates the Proton-motive Force across the Plasma Membrane of *Saccharomyces cerevisiae*. *Microbiology* **1986**, *132* (2), 369-377. DOI: 10.1099/00221287-132-2-369.
110.Samakkarn, W.; Ratanakhanokchai, K.; Soontorngun, N. Reprogramming of the Ethanol Stress Response in *Saccharomyces cerevisiae* by the Transcription Factor Znf1 and Its Effect on the Biosynthesis of Glycerol and Ethanol. *Applied and Industrial Microbiology* **2021**, *87* (16), e0058821. DOI: 10.1128/aem.00588-21.
111.Thomas, D. S.; Rose, A. H. Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. *Archives of Microbiology* **1979**, *122* (1), 49-55. DOI: 10.1007/BF00408045.
112.Auesukaree, C. Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. *Journal of Bioscience and Bioengineering* **2017**, *124* (2), 133-142. DOI: 10.1016/j.jbiosc.2017.03.009.
113.Ma, M.; Liu, Z. L. Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **2010**, *87* (3), 829-845. DOI: 10.1007/s00253-010-2594-3.

114.Charoenbhakdi, S.; Dokpikul, T.; Burphan, T.*; et al.* Vacuolar H^+-ATPase Protects *Saccharomyces cerevisiae* Cells against Ethanol-Induced Oxidative and Cell Wall Stresses. *Applied and Environmental Microbiology* **2016**, *82* (10), 3121-3130. DOI: 10.1128/AEM.00376-16.
115.You, K. M.; Rosenfield, C.-L.; Knipple, D. C. Ethanol Tolerance in the Yeast *Saccharomyces cerevisiae* Is Dependent on Cellular Oleic Acid Content. *Applied and Environmental Microbiology* **2003**, *69* (3), 1499-1503. DOI: 10.1128/AEM.69.3.1499-1503.2003.
116.Kim, H.-S.; Kim, N.-R.; Choi, W. Total fatty acid content of the plasma membrane of *Saccharomyces cerevisiae* is more responsible for ethanol tolerance than the degree of unsaturation. *Biotechnology Letters* **2011**, *33* (3), 509-515. DOI: 10.1007/s10529-010-0465-8.
117.Šajbidor, J.; Ciesarová, Z.; Šmogrovičová, D. Influence of ethanol on the lipid content and fatty acid composition of *Saccharomyces cerevisiae*. *Folia Microbiologica* **1995**, *40* (5), 508-510. DOI: 10.1007/BF02814733.
118.Aguilera, F.; Peinado, R. A.; Millán, C.*; et al.* Relationship between ethanol tolerance, H^+-ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. *International Journal of Food Microbiology* **2006**, *110* (1), 34-42. DOI: 10.1016/j.ijfoodmicro.2006.02.002.
119.Piper, P. W. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiology Letters* **1995**, *134* (2-3), 121-127. DOI: 10.1111/j.1574-6968.1995.tb07925.x.
120.Morano, K. A.; Grant, C. M.; Moye-Rowley, W. S. The Response to Heat Shock and Oxidative Stress in *Saccharomyces cerevisiae*. *Genetics* **2012**, *190* (4), 1157-1195. DOI: 10.1534/genetics.111.128033.
121.Piper, P. W.; Talreja, K.; Panaretou, B.*; et al.* Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma membrane Hsp30, by ethanol levels above a critical threshold. *Microbiology* **1994**, *140* (11), 3031-3038. DOI: 10.1099/13500872-140-11-3031.
122.Mansure, J. J. C.; Panek, A. D.; Crowe, L. M.*; et al.* Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1994**, *1191* (2), 309-316. DOI: 10.1016/0005-2736(94)90181-3.
123.Vriesekoop, F.; Pamment, N. B. Acetaldehyde addition and pre-adaptation to the stressor together virtually eliminate the ethanol-induced lag phase in *Saccharomyces cerevisiae*. *Letters in Applied Microbiology* **2005**, *41* (5), 424-427. DOI: 10.1111/j.1472-765X.2005.01777.x.
124.Zhuang, S.; Smart, K.; Powell, C. Impact of Extracellular Osmolality on Saccharomyces Yeast Populations during Brewing Fermentations. *Journal of the American Society of Brewing Chemists* **2017**, *75* (3), 244-254. DOI: 10.1094/ASBCJ-2017-3505-01.
125.Pratt, P. L.; Bryce, J. H.; Stewart, G. G. The Effects of Osmotic Pressure and Ethanol on Yeast Viability and Morphology. *Journal of the Institute of Brewing* **2003**, *109* (3), 218-228. DOI: 10.1002/j.2050-0416.2003.tb00162.x.
126.Morris, G. J.; Winters, L.; Coulson, G. E.*; et al.* Effect of Osmotic Stress on the Ultrastructure and Viability of the Yeast *Saccharomyces cerevisiae*. *Microbiology* **1986**, *132* (7), 2023-2034. DOI: 10.1099/00221287-132-7-2023.
127.Cahill, G.; Murray, D. M.; Walsh, P. K.*; et al.* Effect of the Concentration of Propagation Wort on Yeast Cell Volume and Fermentation Performance. *Journal of the American Society of Brewing Chemists* **2000**, *58* (1), 14-20. DOI: 10.1094/ASBCJ-58-0014.

128.Brewster, J. L.; Gustin, M. C. Hog1: 20 years of discovery and impact. *Science Signaling* **2014**, *7* (343), re7-re7. DOI: 10.1126/scisignal.2005458.
129.Hohmann, S. Osmotic Stress Signaling and Osmoadaptation in Yeasts. *Microbiology and Molecular Biology Reviews* **2002**, *66* (2), 300-372. DOI: 10.1128/MMBR.66.2.300-372.2002.
130.O'Rourke, S. M.; Herskowitz, I.; O'Shea, E. K. Yeast go the whole HOG for the hyperosmotic response. *Trends in Genetics* **2002**, *18* (8), 405-412. DOI: 10.1016/S0168-9525(02)02723-3.
131.de Nadal, E.; Posas, F. The HOG pathway and the regulation of osmoadaptive responses in yeast. *FEMS Yeast Research* **2022**, *22* (1). DOI: 10.1093/femsyr/foac013.
132.Tamás, M. J.; Hohmann, S. The osmotic stress response of *Saccharomyces cerevisiae*. In *Yeast Stress Responses*, Hohmann, S., Mager, W. H. Eds.; Springer Berlin Heidelberg, 2003; pp 121-200; DOI: 10.1007/3-540-45611-2_4.
133.Blomberg, A.; Adler, L. Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD+) in acquired osmotolerance of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **1989**, *171* (2), 1087-1092. DOI: 10.1128/jb.171.2.1087-1092.1989.
134.Vázquez-Ibarra, A.; Rodríguez-Martínez, G.; Guerrero-Serrano, G.*; et al.* Negative feedback-loop mechanisms regulating HOG- and pheromone-MAPK signaling in yeast. *Current Genetics* **2020**, *66* (5), 867-880. DOI: 10.1007/s00294-020-01089-5.
135.Aries, V.; Kirsop, B. H. Sterol synthesis in relation to growth and fermentation by brewing yeasts inoculated at different concentrations. *Journal of the Institute of Brewing* **1977**, *83* (4), 220-223. DOI: 10.1002/j.2050-0416.1977.tb03798.x.
136.Verbelen, P. J.; Depraetere, S. A.; Winderickx, J.*; et al.* The influence of yeast oxygenation prior to brewery fermentation on yeast metabolism and the oxidative stress response. *FEMS Yeast Research* **2009**, *9* (2), 226-239. DOI: 10.1111/j.1567-1364.2008.00476.x.
137.Dickinson, B. C.; Chang, C. J. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nature Chemical Biology* **2011**, *7* (8), 504-511. DOI: 10.1038/nchembio.607.
138.Delaunay, A.; Pflieger, D.; Barrault, M.-B.*; et al.* A Thiol Peroxidase Is an H_2O_2 Receptor and Redox-Transducer in Gene Activation. *Cell* **2002**, *111* (4), 471-481. DOI: 10.1016/S0092-8674(02)01048-6.
139.Toledano, M. B.; Delaunay, A.; Biteau, B.*; et al.* Oxidative stress responses in yeast. In *Yeast Stress Responses*, Hohmann, S., Mager, W. H. Eds.; Springer Berlin Heidelberg, 2003; pp 241-303; DOI: 10.1007/3-540-45611-2_6.
140.Lee, J.; Godon, C.; Lagniel, G.*; et al.* Yap1 and Skn7 Control Two Specialized Oxidative Stress Response Regulons in Yeast. *Journal of Biological Chemistry* **1999**, *274* (23), 16040-16046. DOI: 10.1074/jbc.274.23.16040.
141.Kaeberlein, M. Lessons on longevity from budding yeast. *Nature* **2010**, *464* (7288), 513-519. DOI: 10.1038/nature08981.
142.Pan, Y.; Schroeder, Elizabeth A.; Ocampo, A.*; et al.* Regulation of Yeast Chronological Life Span by TORC1 via Adaptive Mitochondrial ROS Signaling. *Cell Metabolism* **2011**, *13* (6), 668-678. DOI: 10.1016/j.cmet.2011.03.018.
143.Ocampo, A.; Liu, J.; Schroeder, Elizabeth A.*; et al.* Mitochondrial Respiratory Thresholds Regulate Yeast Chronological Life Span and its Extension by Caloric Restriction. *Cell Metabolism* **2012**, *16* (1), 55-67. DOI: 10.1016/j.cmet.2012.05.013.
144.Lillie, S. H.; Pringle, J. R. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. *Journal of Bacteriology* **1980**, *143* (3), 1384-1394. DOI: 10.1128/jb.143.3.1384-1394.1980.

145.Quain, D. E. Cambridge prize lecture: Studies on yeast physiology-impact on fermentation performance and product quality. *Journal of the Institute of Brewing* **1988**, *94* (5), 315-323. DOI: 10.1002/j.2050-0416.1988.tb04589.x.
146.Pérez-Torrado, R.; Gimeno-Alcañiz, J. V.; Matallana, E. Wine yeast strains engineered for glycogen overproduction display enhanced viability under glucose deprivation conditions. *Applied and Environmental Microbiology* **2002**, *68* (7), 3339-3344. DOI: 10.1128/aem.68.7.3339-3344.2002.
147.Somani, A.; Bealin-Kelly, F.; Axcell, B.*; et al.* Impact of Storage Temperature on Lager Brewing Yeast Viability, Glycogen, Trehalose, and Fatty Acid Content. *Journal of the American Society of Brewing Chemists* **2012**, *70* (2), 123-130. DOI: 10.1094/ASBCJ-2012-0427-01.
148.Wilson, W. A.; Roach, P. J.; Montero, M.*; et al.* Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiology Reviews* **2010**, *34* (6), 952-985. DOI: 10.1111/j.1574-6976.2010.00220.x.
149.François, J. M.; Walther, T.; Parrou, J. L. Genetics and Regulation of Glycogen and Trehalose Metabolism in *Saccharomyces cerevisiae*. In *Microbial Stress Tolerance for Biofuels: Systems Biology*, Liu, Z. L. Ed.; Springer Berlin Heidelberg, 2012; pp 29-55; DOI: 10.1007/978-3-642-21467-7_2.
150.Smith, T. L.; Rutter, J. Regulation of Glucose Partitioning by PAS Kinase and Ugp1 Phosphorylation. *Molecular Cell* **2007**, *26* (4), 491-499. DOI: 10.1016/j.molcel.2007.03.025.
151.Cheng, C.; Mu, J.; Farkas, I.*; et al.* Requirement of the Self-Glucosylating Initiator Proteins Glg1p and Glg2p for Glycogen Accumulation in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **1995**, *15* (12), 6632-6640. DOI: 10.1128/MCB.15.12.6632.
152.Farkas, I.; Hardy, T. A.; Goebl, M. G.*; et al.* Two glycogen synthase isoforms in *Saccharomyces cerevisiae* are coded by distinct genes that are differentially controlled. *Journal of Biological Chemistry* **1991**, *266* (24), 15602-15607. DOI: 10.1016/S0021-9258(18)98448-6.
153.Pederson, B. A.; Cheng, C.; Wilson, W. A.*; et al.* Regulation of Glycogen Synthase: Identification of residues involved in regulation by the allosteric ligand glucose-6-P and by phosphorylation. *Journal of Biological Chemistry* **2000**, *275* (36), 27753-27761. DOI: 10.1016/S0021-9258(19)65048-9.
154.Lin, K.; Hwang, P. K.; Fletterick, R. J. Mechanism of Regulation in Yeast Glycogen Phosphorylase. *Journal of Biological Chemistry* **1995**, *270* (45), 26833-26839. DOI: 10.1074/jbc.270.45.26833.
155.De Virgilio, C.; Hottiger, T.; Dominguez, J.*; et al.* The role of trehalose synthesis for the acquisition of thermotolerance in yeast. *European Journal of Biochemistry* **1994**, *219* (1-2), 179-186. DOI: 10.1111/j.1432-1033.1994.tb19928.x.
156.Bandara, A.; Fraser, S.; Chambers, P. J.*; et al.* Trehalose promotes the survival of *Saccharomyces cerevisiae* during lethal ethanol stress, but does not influence growth under sublethal ethanol stress. *FEMS Yeast Research* **2009**, *9* (8), 1208-1216. DOI: 10.1111/j.1567-1364.2009.00569.x.
157.Benaroudj, N.; Lee, D. H.; Goldberg, A. L. Trehalose Accumulation during Cellular Stress Protects Cells and Cellular Proteins from Damage by Oxygen Radicals *. *Journal of Biological Chemistry* **2001**, *276* (26), 24261-24267. DOI: 10.1074/jbc.M101487200.
158.Tapia, H.; Koshland, Douglas E. Trehalose Is a Versatile and Long-Lived Chaperone for Desiccation Tolerance. *Current Biology* **2014**, *24* (23), 2758-2766. DOI: 10.1016/j.cub.2014.10.005.

159.Guldfeldt, L. U.; Arnfborg, N. The effect of yeast trehalose content at pitching on fermentation performance during brewing fermentations *Journal of the Institute of Brewing* **1998**, *104* (1), 37-39. DOI: 10.1002/j.2050-0416.1998.tb00972.x.
160.Bell, W.; Sun, W.; Hohmann, S.*; et al.* Composition and Functional Analysis of the *Saccharomyces cerevisiae* Trehalose Synthase Complex *Journal of Biological Chemistry* **1998**, *273* (50), 33311-33319. DOI: 10.1074/jbc.273.50.33311.
161.De Virgilio, C.; Bürkert, N.; Bell, W.*; et al.* Disruption of TPS2, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae*, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity. *European Journal of Biochemistry* **1993**, *212* (2), 315-323. DOI: 10.1111/j.1432-1033.1993.tb17664.x.
162.Trevisol, E. T. V.; Panek, A. D.; De Mesquita, J. F.*; et al.* Regulation of the yeast trehalose–synthase complex by cyclic AMP-dependent phosphorylation. *Biochimica et Biophysica Acta (BBA) - General Subjects* **2014**, *1840* (6), 1646-1650. DOI: 10.1016/j.bbagen.2013.12.010.
163.Eleutherio, E.; Panek, A.; De Mesquita, J. F.*; et al.* Revisiting yeast trehalose metabolism. *Current Genetics* **2015**, *61* (3), 263-274. DOI: 10.1007/s00294-014-0450-1.
164.Londesborough, J.; Varimo, K. Characterization of two trehalases in baker's yeast. *Biochemical Journal* **1984**, *219* (2), 511-518. DOI: 10.1042/bj2190511.
165.Thevelein, J. Regulation of trehalose mobilization in fungi. *Microbiological reviews* **1984**, *48* (1), 42-59. DOI: 10.1128/mr.48.1.42-59.1984.
166.Dengler, L.; Örd, M.; Schwab, L. M.*; et al.* Regulation of trehalase activity by multi-site phosphorylation and 14-3-3 interaction. *Scientific Reports* **2021**, *11* (1), 962. DOI: 10.1038/s41598-020-80357-3.
167.Layfield, J.; Sheppard, J. D. What Brewers Should Know About Viability, Vitality, and Overall Brewing Fitness: A Mini-Review. *Master Brewers Association of the Americas Technical Quarterly* **2015**, *52* (3), 132-140. DOI: 10.1094/TQ-52-3-0719-01
168.Gilliland, R. B. Determination of yeast viability. *Journal of the Institute of Brewing* **1959**, *65* (5), 424-429. DOI: 10.1002/j.2050-0416.1959.tb01482.x.
169.Sahu, S. R.; Utkalaja, B. G.; Patel, S. K.*; et al.* Spot Assay and Colony Forming Unit (CFU) Analyses–based sensitivity test for *Candida albicans* and *Saccharomyces cerevisiae*. *Bio-protocol* **2023**, *13* (21), e4872. DOI: 10.21769/BioProtoc.4872.
170.Carvell, J. P.; Dowd, J. E. On-line Measurements and Control of Viable Cell Density in Cell Culture Manufacturing Processes using Radio-frequency Impedance. *Cytotechnology* **2006**, *50* (1), 35-48. DOI: 10.1007/s10616-005-3974-x.
171.McGahon, A. J.; Martin, S. J.; Bissonnette, R. P.*; et al.* Chapter 9: The End of the (Cell) Line: Methods for the Study of Apoptosis in Vitro. In *Methods in Cell Biology*, Schwartz, L. M., Osborne, B. A. Eds.; Vol. 46; Academic Press, 1995; pp 153-185; DOI: 10.1016/S0091-679X(08)61929-9.
172.Corliss, D. A.; White, W. E. Fluorescence of yeast vitally stained with ethidium bromide and propidium iodide. *Journal of Histochemistry & Cytochemistry* **1981**, *29* (1), 45-48. DOI: 10.1177/29.1.6162881.
173.Saldi, S.; Driscoll, D.; Kuksin, D.*; et al.* Image-Based Cytometric Analysis of Fluorescent Viability and Vitality Staining Methods for Ale and Lager Fermentation Yeast. *Journal of the American Society of Brewing Chemists* **2014**, *72* (4), 253-260. DOI: 10.1094/ASBCJ-2014-1015-01.
174.Williamson, C.; Kennedy, K.; Bhattacharya, S.*; et al.* A novel image-based method for simultaneous counting of *Lactobacillus* and *Saccharomyces* in mixed culture fermentation. *Journal of Industrial Microbiology and Biotechnology* **2023**, *50* (1). DOI: 10.1093/jimb/kuad007.

175.Painting, K.; Kirsop, B. A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining. *World Journal of Microbiology and Biotechnology* **1990**, *6* (3), 346-347. DOI: 10.1007/BF01201311.
176.Smart, K. A.; Chambers, K. M.; Lambert, I.*; et al.* Use of Methylene Violet Staining Procedures to Determine Yeast Viability and Vitality. *Journal of the American Society of Brewing Chemists* **1999**, *57* (1), 18-23. DOI: 10.1094/ASBCJ-57-0018.
177.Kara, B. V.; Simpson, W. J.; Hammond, J. R. M. Prediction of the fermentation performance of brewing yeast with the acidification power test *Journal of the Institute of Brewing* **1988**, *94* (3), 153-158. DOI: 10.1002/j.2050-0416.1988.tb04573.x.
178.Wang, Z.; Wang, J.; Shan, W.*; et al.* Intracellular Adenosine Triphosphate (ATP) Content Sensitively Reflects Subtle Differences in Yeast Physiology. *Journal of the American Society of Brewing Chemists* **2019**, *77* (2), 92-98. DOI: 10.1080/03610470.2019.1577706.
179.Ludovico, P.; Sansonetty, F.; Côrte-Real, M. Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. *Microbiology* **2001**, *147* (12), 3335-3343. DOI: 10.1099/00221287-147-12-3335.
180.Breeuwer, P.; Drocourt, J. L.; Bunschoten, N.*; et al.* Characterization of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*, which result in accumulation of fluorescent product. *Applied and Environmental Microbiology* **1995**, *61* (4), 1614-1619. DOI: 10.1128/aem.61.4.1614-1619.1995.
181.Opekarová, M.; Sigler, K. Acidification power: Indicator of metabolic activity and autolytic changes in *Saccharomyces cerevisiae*. *Folia Microbiologica* **1982**, *27* (6), 395-403. DOI: 10.1007/BF02876450.
182.Gabriel, P.; Dienstbier, M.; Matoulková, D.*; et al.* Optimised Acidification Power Test of Yeast Vitality and its Use in Brewing Practice. *Journal of the Institute of Brewing* **2008**, *114* (3), 270-276. DOI: 10.1002/j.2050-0416.2008.tb00338.x.
183.Crouch, S. P. M.; Kozlowski, R.; Slater, K. J.*; et al.* The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *Journal of Immunological Methods* **1993**, *160* (1), 81-88. DOI: 10.1016/0022-1759(93)90011-U.
184.Bouix, M.; Leveau, J.-Y. Rapid Assessment of Yeast Viability and Yeast Vitality During Alcoholic Fermentation. *Journal of the Institute of Brewing* **2001**, *107* (4), 217-225. DOI: 10.1002/j.2050-0416.2001.tb00093.x.
185.Grela, E.; Kozłowska, J.; Grabowiecka, A. Current methodology of MTT assay in bacteria – A review. *Acta Histochemica* **2018**, *120* (4), 303-311. DOI: 10.1016/j.acthis.2018.03.007.
186.Präbst, K.; Engelhardt, H.; Ringgeler, S.*; et al.* Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin. In *Cell Viability Assays: Methods and Protocols*, Gilbert, D. F., Friedrich, O. Eds.; Springer New York, 2017; pp 1-17; DOI: 10.1007/978-1-4939-6960-9_1.
187.O'Brien, J.; Wilson, I.; Orton, T.*; et al.* Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry* **2000**, *267* (17), 5421-5426. DOI: 10.1046/j.1432-1327.2000.01606.x.
188.Erb, R. E.; Ehlers, M. H. Resazurin Reducing Time as an Indicator of Bovine Semen Fertilizing Capacity[1]. *Journal of Dairy Science* **1950**, *33* (12), 853-864. DOI: 10.3168/jds.S0022-0302(50)91981-3.
189.Bowling, T.; Mercer, L.; Don, R.*; et al.* Application of a resazurin-based high-throughput screening assay for the identification and progression of new treatments for human African trypanosomiasis. *International Journal for Parasitology: Drugs and Drug Resistance* **2012**, *2*, 262-270. DOI: 10.1016/j.ijpddr.2012.02.002.

190.Barua, P.; You, M. P.; Bayliss, K.*; et al.* A rapid and miniaturized system using Alamar blue to assess fungal spore viability: implications for biosecurity. *European Journal of Plant Pathology* **2017**, *148* (1), 139-150. DOI: 10.1007/s10658-016-1077-5.
191.Byth, H.-A.; Mchunu, B. I.; Dubery, I. A.*; et al.* Assessment of a simple, non-toxic alamar blue cell survival assay to monitor tomato cell viability. *Phytochemical Analysis* **2001**, *12* (5), 340-346. DOI: 10.1002/pca.595.
192.Rezende, N.; Ceron Jayme, C.; Brassesco, M. S.*; et al.* Standardization of a resazurin-based assay for the evaluation of metabolic activity in oral squamous carcinoma and glioblastoma cells. *Photodiagnosis and Photodynamic Therapy* **2019**, *26*, 371-374. DOI: 10.1016/j.pdpdt.2019.05.004.
193.Chen, J. L.; Steele, T. W. J.; Stuckey, D. C. Metabolic reduction of resazurin; location within the cell for cytotoxicity assays. *Biotechnology and Bioengineering* **2018**, *115* (2), 351-358. DOI: 10.1002/bit.26475.
194.Strack, L.; Stahl, U. "Omics" Technologies and Their Input for the Comprehension of Metabolic Systems Particularly Pertaining to Yeast Organisms. In *Progress in Botany 72*, Lüttge, U. E., Beyschlag, W., Büdel, B., Francis, D. Eds.; Springer Berlin Heidelberg, 2011; pp 105-122; DOI: 10.1007/978-3-642-13145-5_4.
195.Cobb, M. 60 years ago, Francis Crick changed the logic of biology. *PLOS Biology* **2017**, *15* (9), e2003243. DOI: 10.1371/journal.pbio.2003243.
196.Gonçalves, M.; Pontes, A.; Almeida, P.*; et al.* Distinct Domestication Trajectories in Top-Fermenting Beer Yeasts and Wine Yeasts. *Current Biology* **2016**, *26* (20), 2750-2761. DOI: 10.1016/j.cub.2016.08.040.
197.Voordeckers, K.; Kominek, J.; Das, A.*; et al.* Adaptation to High Ethanol Reveals Complex Evolutionary Pathways. *PLoS Genet* **2015**, *11* (11), e1005635. DOI: 10.1371/journal.pgen.1005635 From NLM.
198.Rossignol, T.; Dulau, L.; Julien, A.*; et al.* Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast* **2003**, *20* (16), 1369-1385. DOI: 10.1002/yea.1046.
199.Gibson, B. R.; Lawrence, S. J.; Boulton, C. A.*; et al.* The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation. *FEMS Yeast Research* **2008**, *8* (4), 574-585. DOI: 10.1111/j.1567-1364.2008.00371.x.
200.Griffin, T. J.; Gygi, S. P.; Ideker, T.*; et al.* Complementary Profiling of Gene Expression at the Transcriptome and Proteome Levels in *Saccharomyces cerevisiae**S. *Molecular & Cellular Proteomics* **2002**, *1* (4), 323-333. DOI: 10.1074/mcp.M200001-MCP200.
201.Aebersold, R.; Agar, J. N.; Amster, I. J.*; et al.* How many human proteoforms are there? *Nature Chemical Biology* **2018**, *14* (3), 206-214. DOI: 10.1038/nchembio.2576.
202.Oliver, S. G.; Winson, M. K.; Kell, D. B.*; et al.* Systematic functional analysis of the yeast genome. *Trends in biotechnology* **1998**, *16* (9), 373-378. DOI: 10.1016/S0167-7799(98)01214-1.
203.Villas-Bôas, S. G.; Mas, S.; Åkesson, M.*; et al.* Mass spectrometry in metabolome analysis. *Mass Spectrometry Reviews* **2005**, *24* (5), 613-646. DOI: 10.1002/mas.20032.
204.Carisma, N. A. S.; Calingacion, M. N. Metabolomics and (craft) beers – recent advances. *Food Research International* **2025**, *205*, 116010. DOI: 10.1016/j.foodres.2025.116010.
205.Alves, V.; Gonçalves, J.; Figueira, J. A.*; et al.* Beer volatile fingerprinting at different brewing steps. *Food Chemistry* **2020**, *326*, 126856. DOI: 10.1016/j.foodchem.2020.126856.

206.Kerr, E. D.; Fox, G. P.; Schulz, B. L. Proteomics and Metabolomics Reveal that an Abundant α-Glucosidase Drives Sorghum Fermentability for Beer Brewing. *Journal of Proteome Research* **2023**, *22* (11), 3596-3606. DOI: 10.1021/acs.jproteome.3c00436.
207.Nielsen, J. Yeast Systems Biology: Model Organism and Cell Factory. *Biotechnology Journal* **2019**, *14* (9), 1800421. DOI: 10.1002/biot.201800421.
208.Wilkins, M. R.; Pasquali, C.; Appel, R. D.*; et al.* From Proteins to Proteomes: Large Scale Protein Identification by Two-Dimensional Electrophoresis and Arnino Acid Analysis. *Bio/Technology* **1996**, *14* (1), 61-65. DOI: 10.1038/nbt0196-61.
209.Wasinger, V. C.; Cordwell, S. J.; Cerpa-Poljak, A.*; et al.* Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis* **1995**, *16* (7), 1090-1094. DOI: 10.1002/elps.11501601185.
210.and, M. L. Y.; Jayaraman, A. Advances in Proteomic Technologies. *Annual Review of Biomedical Engineering* **2002**, *4* (1), 349-373. DOI: 10.1146/annurev.bioeng.4.020702.153443.
211.O'Farrell, P. H. High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* **1975**, *250* (10), 4007-4021. DOI: 10.1016/S0021-9258(19)41496-8.
212.Yates, J. R.; Ruse, C. I.; Nakorchevsky, A. Proteomics by Mass Spectrometry: Approaches, Advances, and Applications. *Annual Review of Biomedical Engineering* **2009**, *11* (1), 49-79. DOI: 10.1146/annurev-bioeng-061008-124934.
213.Tubaon, R. M.; Haddad, P. R.; Quirino, J. P. Sample Clean-up Strategies for ESI Mass Spectrometry Applications in Bottom-up Proteomics: Trends from 2012 to 2016. *Proteomics* **2017**, *17* (20), 1700011. DOI: 10.1002/pmic.201700011.
214.Toby, T. K.; Fornelli, L.; Kelleher, N. L. Progress in Top-Down Proteomics and the Analysis of Proteoforms. *Annual Review of Analytical Chemistry* **2016**, *9* (1), 499-519. DOI: 10.1146/annurev-anchem-071015-041550.
215.Tsiatsiani, L.; Heck, A. J. R. Proteomics beyond trypsin. *The FEBS Journal* **2015**, *282* (14), 2612-2626. DOI: 10.1111/febs.13287.
216.Xu, T.; Wang, Q.; Wang, Q.*; et al.* Mass spectrometry-intensive top-down proteomics: an update on technology advancements and biomedical applications. *Analytical Methods* **2024**, *16* (28), 4664-4682. DOI: 10.1039/d4ay00651h From NLM.
217.Roberts, D. S.; Loo, J. A.; Tsybin, Y. O.*; et al.* Top-down proteomics. *Nature Reviews Methods Primers* **2024**, *4* (1), 38. DOI: 10.1038/s43586-024-00318-2.
218.Melby, J. A.; Roberts, D. S.; Larson, E. J.*; et al.* Novel Strategies to Address the Challenges in Top-Down Proteomics. *Journal of the American Society for Mass Spectrometry* **2021**, *32* (6), 1278-1294. DOI: 10.1021/jasms.1c00099.
219.Takemori, A.; Kaulich, P. T.; Tholey, A.*; et al.* PEPPI-MS: gel-based sample pre-fractionation for deep top-down and middle-down proteomics. *Nature Protocols* **2025**. DOI: 10.1038/s41596-024-01100-0.
220.Takemori, A.; Kaulich, P. T.; Konno, R.*; et al.* GeLC-FAIMS-MS workflow for in-depth middle-down proteomics. *PROTEOMICS* **2024**, *24* (3-4), 2200431. DOI: 10.1002/pmic.202200431.
221.Dhenin, J.; Dupré, M.; Druart, K.*; et al.* A multiparameter optimization in middle-down analysis of monoclonal antibodies by LC–MS/MS. *Journal of Mass Spectrometry* **2023**, *58* (3), e4909. DOI: 10.1002/jms.4909.
222.Sidoli, S.; Vandamme, J.; Salcini, A. E.*; et al.* Dynamic changes of histone H3 marks during *Caenorhabditis elegans* lifecycle revealed by middle-down proteomics. *PROTEOMICS* **2016**, *16* (3), 459-464. DOI: 10.1002/pmic.201500285.

223.Pandeswari, P. B.; Sabareesh, V. Middle-down approach: a choice to sequence and characterize proteins/proteomes by mass spectrometry. *RSC Advances* **2019**, *9* (1), 313-344, 10.1039/C8RA07200K. DOI: 10.1039/C8RA07200K.
224.Wang, W.-Q.; Jensen, O. N.; Møller, I. M.*; et al.* Evaluation of sample preparation methods for mass spectrometry-based proteomic analysis of barley leaves. *Plant Methods* **2018**, *14* (1), 72. DOI: 10.1186/s13007-018-0341-4.
225.Choksawangkarn, W.; Edwards, N.; Wang, Y.*; et al.* Comparative Study of Workflows Optimized for In-gel, In-solution, and On-filter Proteolysis in the Analysis of Plasma Membrane Proteins. *Journal of Proteome Research* **2012**, *11* (5), 3030-3034. DOI: 10.1021/pr300188b.
226.León, I. R.; Schwämmle, V.; Jensen, O. N.*; et al.* Quantitative Assessment of In-solution Digestion Efficiency Identifies Optimal Protocols for Unbiased Protein Analysis*. *Molecular & Cellular Proteomics* **2013**, *12* (10), 2992-3005. DOI: 10.1074/mcp.M112.025585.
227.Ludwig, K. R.; Schroll, M. M.; Hummon, A. B. Comparison of In-Solution, FASP, and S-Trap Based Digestion Methods for Bottom-Up Proteomic Studies. *Journal of Proteome Research* **2018**, *17* (7), 2480-2490. DOI: 10.1021/acs.jproteome.8b00235.
228.Jiang, L.; He, L.; Fountoulakis, M. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *Journal of Chromatography A* **2004**, *1023* (2), 317-320. DOI: 10.1016/j.chroma.2003.10.029.
229.Koontz, L. Chapter One - TCA Precipitation. In *Methods in Enzymology*, Lorsch, J. Ed.; Vol. 541; Academic Press, 2014; pp 3-10; DOI: 10.1016/B978-0-12-420119-4.00001-X.
230.Wessel, D.; Flügge, U. I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochemistry* **1984**, *138* (1), 141-143. DOI: 10.1016/0003-2697(84)90782-6.
231.Betancourt, L. H.; Sanchez, A.; Pla, I.*; et al.* Quantitative Assessment of Urea In-Solution Lys-C/Trypsin Digestions Reveals Superior Performance at Room Temperature over Traditional Proteolysis at 37 °C. *Journal of Proteome Research* **2018**, *17* (7), 2556-2561. DOI: 10.1021/acs.jproteome.8b00228.
232.Vandermarliere, E.; Mueller, M.; Martens, L. Getting intimate with trypsin, the leading protease in proteomics. *Mass Spectrometry Reviews* **2013**, *32* (6), 453-465. DOI: 10.1002/mas.21376.
233.Doellinger, J.; Schneider, A.; Hoeller, M.*; et al.* Sample Preparation by Easy Extraction and Digestion (SPEED) - A Universal, Rapid, and Detergent-free Protocol for Proteomics Based on Acid Extraction*. *Molecular & Cellular Proteomics* **2020**, *19* (1), 209-222. DOI: 10.1074/mcp.TIR119.001616.
234.Kulak, N. A.; Pichler, G.; Paron, I.*; et al.* Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. *Nature Methods* **2014**, *11* (3), 319-324. DOI: 10.1038/nmeth.2834.
235.Wiśniewski, J. R.; Zougman, A.; Nagaraj, N.*; et al.* Universal sample preparation method for proteome analysis. *Nature Methods* **2009**, *6* (5), 359-362. DOI: 10.1038/nmeth.1322.
236.Hughes, C. S.; Foehr, S.; Garfield, D. A.*; et al.* Ultrasensitive proteome analysis using paramagnetic bead technology. *Molecular Systems Biology* **2014**, *10* (10), 757. DOI: 10.15252/msb.20145625.
237.Johnston, H. E.; Yadav, K.; Kirkpatrick, J. M.*; et al.* Solvent Precipitation SP3 (SP4) Enhances Recovery for Proteomics Sample Preparation without Magnetic Beads. *Analytical Chemistry* **2022**, *94* (29), 10320-10328. DOI: 10.1021/acs.analchem.1c04200.

238.Zougman, A.; Selby, P. J.; Banks, R. E. Suspension trapping (STrap) sample preparation method for bottom-up proteomics analysis. *Proteomics* **2014**, *14* (9), 1006-1000. DOI: 10.1002/pmic.201300553.
239.Klont, F.; Bras, L.; Wolters, J. C.*; et al.* Assessment of Sample Preparation Bias in Mass Spectrometry-Based Proteomics. *Analytical Chemistry* **2018**, *90* (8), 5405-5413. DOI: 10.1021/acs.analchem.8b00600.
240.Thompson, A.; Schäfer, J.; Kuhn, K.*; et al.* Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS. *Analytical Chemistry* **2003**, *75* (8), 1895-1904. DOI: 10.1021/ac0262560.
241.Ross, P. L.; Huang, Y. N.; Marchese, J. N.*; et al.* Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents *. *Molecular & Cellular Proteomics* **2004**, *3* (12), 1154-1169. DOI: 10.1074/mcp.M400129-MCP200.
242.Stahl, D. C.; Swiderek, K. M.; Davis, M. T.*; et al.* Data-controlled automation of liquid chromatography/tandem mass spectrometry analysis of peptide mixtures. *Journal of the American Society for Mass Spectrometry* **1996**, *7* (6), 532-540. DOI: 10.1016/1044-0305(96)00057-8.
243.Gillet, L. C.; Navarro, P.; Tate, S.*; et al.* Targeted Data Extraction of the MS/MS Spectra Generated by Data-independent Acquisition: A New Concept for Consistent and Accurate Proteome Analysis*. *Molecular & Cellular Proteomics* **2012**, *11* (6), O111.016717. DOI: 10.1074/mcp.O111.016717.
244.Lange, V.; Picotti, P.; Domon, B.*; et al.* Selected reaction monitoring for quantitative proteomics: a tutorial. *Molecular Systems Biology* **2008**, *4* (1), 222. DOI: 10.1038/msb.2008.61.
245.Hu, A.; Noble, W. S.; Wolf-Yadlin, A. Technical advances in proteomics: new developments in data-independent acquisition. *F1000Res* **2016**, *5*. DOI: 10.12688/f1000research.7042.1.
246.Li, J.; Cai, Z.; Bomgarden, R. D.*; et al.* TMTpro-18plex: The Expanded and Complete Set of TMTpro Reagents for Sample Multiplexing. *Journal of Proteome Research* **2021**, *20* (5), 2964-2972. DOI: 10.1021/acs.jproteome.1c00168.
247.Bailey, D. J.; McDevitt, M. T.; Westphall, M. S.*; et al.* Intelligent Data Acquisition Blends Targeted and Discovery Methods. *Journal of Proteome Research* **2014**, *13* (4), 2152-2161. DOI: 10.1021/pr401278j.
248.Zhang, Y.; Wen, Z.; Washburn, M. P.*; et al.* Effect of Dynamic Exclusion Duration on Spectral Count Based Quantitative Proteomics. *Analytical Chemistry* **2009**, *81* (15), 6317-6326. DOI: 10.1021/ac9004887.
249.Krasny, L.; Huang, P. H. Data-independent acquisition mass spectrometry (DIA-MS) for proteomic applications in oncology. *Molecular Omics* **2021**, *17* (1), 29-42, 10.1039/D0MO00072H. DOI: 10.1039/D0MO00072H.
250.Webb-Robertson, B.-J. M.; Wiberg, H. K.; Matzke, M. M.*; et al.* Review, Evaluation, and Discussion of the Challenges of Missing Value Imputation for Mass Spectrometry-Based Label-Free Global Proteomics. *Journal of Proteome Research* **2015**, *14* (5), 1993-2001. DOI: 10.1021/pr501138h.
251.Lou, R.; Cao, Y.; Li, S.*; et al.* Benchmarking commonly used software suites and analysis workflows for DIA proteomics and phosphoproteomics. *Nature Communications* **2023**, *14* (1), 94. DOI: 10.1038/s41467-022-35740-1.
252.Pino, L. K.; Just, S. C.; MacCoss, M. J.*; et al.* Acquiring and Analyzing Data Independent Acquisition Proteomics Experiments without Spectrum Libraries. *Molecular & Cellular Proteomics* **2020**, *19* (7), 1088-1103. DOI: 10.1074/mcp.P119.001913.

253.Zhang, F.; Ge, W.; Ruan, G.*; et al.* Data-Independent Acquisition Mass Spectrometry-Based Proteomics and Software Tools: A Glimpse in 2020. *Proteomics* **2020**, *20* (17-18), 1900276. DOI: 10.1002/pmic.201900276.
254.Tsou, C.-C.; Avtonomov, D.; Larsen, B.*; et al.* DIA-Umpire: comprehensive computational framework for data-independent acquisition proteomics. *Nature Methods* **2015**, *12* (3), 258-264. DOI: 10.1038/nmeth.3255.
255.Li, Y.; Zhong, C.-Q.; Xu, X.*; et al.* Group-DIA: analyzing multiple data-independent acquisition mass spectrometry data files. *Nature Methods* **2015**, *12* (12), 1105-1106. DOI: 10.1038/nmeth.3593.
256.Ting, Y. S.; Egertson, J. D.; Payne, S. H.*; et al.* Peptide-Centric Proteome Analysis: An Alternative Strategy for the Analysis of Tandem Mass Spectrometry Data *. *Molecular & Cellular Proteomics* **2015**, *14* (9), 2301-2307. DOI: 10.1074/mcp.O114.047035.
257.Ludwig, C.; Gillet, L.; Rosenberger, G.*; et al.* Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial. *Molecular Systems Biology* **2018**, *14* (8), e8126. DOI: 10.15252/msb.20178126.
258.Demichev, V.; Messner, C. B.; Vernardis, S. I.*; et al.* DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. *Nature Methods* **2020**, *17* (1), 41-44. DOI: 10.1038/s41592-019-0638-x.
259.Sinitcyn, P.; Hamzeiy, H.; Salinas Soto, F.*; et al.* MaxDIA enables library-based and library-free data-independent acquisition proteomics. *Nature Biotechnology* **2021**, *39* (12), 1563-1573. DOI: 10.1038/s41587-021-00968-7.
260.Yang, Y.; Liu, X.; Shen, C.*; et al.* In silico spectral libraries by deep learning facilitate data-independent acquisition proteomics. *Nature Communications* **2020**, *11* (1), 146. DOI: 10.1038/s41467-019-13866-z.
261.Gessulat, S.; Schmidt, T.; Zolg, D. P.*; et al.* Prosit: proteome-wide prediction of peptide tandem mass spectra by deep learning. *Nature Methods* **2019**, *16* (6), 509-518. DOI: 10.1038/s41592-019-0426-7.
262.Bruderer, R.; Bernhardt, O. M.; Gandhi, T.*; et al.* Extending the Limits of Quantitative Proteome Profiling with Data-Independent Acquisition and Application to Acetaminophen-Treated Three-Dimensional Liver Microtissues *[S]. *Molecular & Cellular Proteomics* **2015**, *14* (5), 1400-1410. DOI: 10.1074/mcp.M114.044305.
263.Vidova, V.; Spacil, Z. A review on mass spectrometry-based quantitative proteomics: Targeted and data independent acquisition. *Analytica Chimica Acta* **2017**, *964*, 7-23. DOI: 10.1016/j.aca.2017.01.059.
264.Peterson, A. C.; Russell, J. D.; Bailey, D. J.*; et al.* Parallel Reaction Monitoring for High Resolution and High Mass Accuracy Quantitative, Targeted Proteomics*. *Molecular & Cellular Proteomics* **2012**, *11* (11), 1475-1488. DOI: 10.1074/mcp.O112.020131.
265.Hebert, A. S.; Richards, A. L.; Bailey, D. J.*; et al.* The One Hour Yeast Proteome*. *Molecular & Cellular Proteomics* **2014**, *13* (1), 339-347. DOI: 10.1074/mcp.M113.034769.
266.Joubert, R.; Brignon, P.; Lehmann, C.*; et al.* Two-dimensional gel analysis of the proteome of lager brewing yeasts. *Yeast* **2000**, *16* (6), 511-522. DOI: 10.1002/(SICI)1097-0061(200004)16:6<511::AID-YEA544>3.0.CO;2-I.
267.Jouber, R.; Strub, J.-M.; Zugmeyer, S.*; et al.* Identification by mass spectrometry of two-dimensional gel electrophoresis-separated proteins extracted from lager brewing yeast. *ELECTROPHORESIS* **2001**, *22* (14), 2969-2982. DOI: 10.1002/1522-2683(200108)22:14<2969::AID-ELPS2969>3.0.CO;2-4.

268.Kobi, D.; Zugmeyer, S.; Potier, S.*; et al.* Two-dimensional protein map of an "ale"-brewing yeast strain: proteome dynamics during fermentation. *FEMS Yeast Research* **2004**, *5* (3), 213-230. DOI: 10.1016/j.femsyr.2004.07.004.
269.Brejning, J.; Arneborg, N.; Jespersen, L. Identification of genes and proteins induced during the lag and early exponential phase of lager brewing yeasts. *Journal of Applied Microbiology* **2005**, *98* (2), 261-271. DOI: 10.1111/j.1365-2672.2004.02472.x.
270.Rossignol, T.; Kobi, D.; Jacquet-Gutfreund, L.*; et al.* The proteome of a wine yeast strain during fermentation, correlation with the transcriptome. *Journal of Applied Microbiology* **2009**, *107* (1), 47-55. DOI: 10.1111/j.1365-2672.2009.04156.x.
271.Noti, O.; Vaudano, E.; Giuffrida, M. G.*; et al.* Enhanced arginine biosynthesis and lower proteolytic profile as indicators of *Saccharomyces cerevisiae* stress in stationary phase during fermentation of high sugar grape must: A proteomic evidence. *Food Research International* **2018**, *105*, 1011-1018. DOI: 10.1016/j.foodres.2017.12.004.
272.Trabalzini, L.; Paffetti, A.; Ferro, E.*; et al.* Proteomic characterization of a wild-type wine strain of *Saccharomyces cerevisiae*. *Biochemical Journal* **2003**, *370*, 35–46. DOI: 10.1042/BJ20020140.
273.Parapouli, M.; Fragkos-Livanios, L.; Samiotaki, M.*; et al.* Comparative proteomic analysis of alcoholic fermentation employing a new environmental strain of *Saccharomyces cerevisiae*. *Process Biochemistry* **2010**, *45* (7), 1094-1102. DOI: 10.1016/j.procbio.2010.03.028.
274.Gómez-Pastor, R.; Pérez-Torrado, R.; Cabiscol, E.*; et al.* Transcriptomic and proteomic insights of the wine yeast biomass propagation process. *FEMS Yeast Research* **2010**, *10* (7), 870-884. DOI: 10.1111/j.1567-1364.2010.00667.x.
275.Porras-Agüera, J. A.; Moreno-García, J.; Mauricio, J. C.*; et al.* First Proteomic Approach to Identify Cell Death Biomarkers in Wine Yeasts during Sparkling Wine Production. *Microorganisms* **2019**, *7* (11), 542. DOI: 10.3390/microorganisms7110542.
276.Porras–Agüera, J. A.; Moreno–García, J.; García–Martínez, T.*; et al.* Impact of CO_2 overpressure on yeast mitochondrial associated proteome during the "prise de mousse" of sparkling wine production. *International Journal of Food Microbiology* **2021**, *348*, 109226. DOI: 10.1016/j.ijfoodmicro.2021.109226.
277.González-Jiménez, M. d. C.; Mauricio, J. C.; Moreno-García, J.*; et al.* Differential Response of the Proteins Involved in Amino Acid Metabolism in Two *Saccharomyces cerevisiae* Strains during the Second Fermentation in a Sealed Bottle. *Applied Sciences* **2021**, *11* (24), 12165. DOI: 10.3390/app112412165.
278.Salvadó, Z.; Chiva, R.; Rodríguez-Vargas, S.*; et al.* Proteomic evolution of a wine yeast during the first hours of fermentation. *FEMS Yeast Research* **2008**, *8* (7), 1137-1146. DOI: 10.1111/j.1567-1364.2008.00389.x.
279.Szopinska, A.; Christ, E.; Planchon, S.*; et al.* Stuck at work? Quantitative proteomics of environmental wine yeast strains reveals the natural mechanism of overcoming stuck fermentation. *PROTEOMICS* **2016**, *16* (4), 593-608. DOI: 10.1002/pmic.201500225.
280.Di Bartolomeo, F.; Malina, C.; Campbell, K.*; et al.* Absolute yeast mitochondrial proteome quantification reveals trade-off between biosynthesis and energy generation during diauxic shift. *Proceedings of the National Academy of Sciences* **2020**, *117* (13), 7524-7535. DOI: 10.1073/pnas.1918216117.
281.de Groot, M. J. L.; Daran-Lapujade, P.; van Breukelen, B.*; et al.* Quantitative proteomics and transcriptomics of anaerobic and aerobic yeast cultures reveals post-transcriptional regulation of key cellular processes. *Microbiology* **2007**, *153* (11), 3864-3878. DOI: 10.1099/mic.0.2007/009969-0.

282.Murphy, J. P.; Stepanova, E.; Everley, R. A.*; et al.* Comprehensive Temporal Protein Dynamics during the Diauxic Shift in *Saccharomyces cerevisiae*. *Molecular & Cellular Proteomics* **2015**, *14* (9), 2454-2465. DOI: 10.1074/mcp.M114.045849.
283.Costenoble, R.; Picotti, P.; Reiter, L.*; et al.* Comprehensive quantitative analysis of central carbon and amino-acid metabolism in *Saccharomyces cerevisiae* under multiple conditions by targeted proteomics. *Molecular Systems Biology* **2011**, *7* (1), 464. DOI: 10.1038/msb.2010.122.
284.Ridder, M. d.; van den Brandeler, W.; Altiner, M.*; et al.* Proteome Dynamics During Transition From Exponential to Stationary Phase Under Aerobic and Anaerobic Conditions in Yeast. *Molecular & Cellular Proteomics* **2023**, *22* (6). DOI: 10.1016/j.mcpro.2023.100552.
285.Bisschops, M. M.; Vos, T.; Martínez-Moreno, R.*; et al.* Oxygen availability strongly affects chronological lifespan and thermotolerance in batch cultures of *Saccharomyces cerevisiae*. *Microbial Cell* **2015**, *2* (11), 429-444. DOI: 10.15698/mic2015.11.238 From NLM.
286.Alba-Lois, L.; Segal-Kischinevzky, C. Yeast Fermentation and the Making of Beer and Wine. Nature Education 2010; Vol. 3, p 17.
287.Stewart, G. G.; Russell, I.; Anstruther, A. *Handbook of Brewing*; CRC Press, 2017.
288.Burini, J. A.; Trochine, A.; Libkind Frati, D. Biomass production optimization and quality assessment of *Saccharomyces eubayanus* starter cultures for their application in the craft brewing industry. *Journal of Food Science and Technology* **2025**. DOI: 10.1007/s13197-025-06203-4.
289.Stewart, G. G. Yeast Management. In *Brewing and Distilling Yeasts*, Springer International Publishing, 2017; pp 241-258; DOI: 10.1007/978-3-319-69126-8_12.
290.Perpetuini, G.; Di Gianvito, P.; Arfelli, G.*; et al.* Biodiversity of autolytic ability in flocculent *Saccharomyces cerevisiae* strains suitable for traditional sparkling wine fermentation. *Yeast* **2016**, *33* (7), 303-312. DOI: 10.1002/yea.3151.
291.James, T. C.; Campbell, S.; Donnelly, D.*; et al.* Transcription profile of brewery yeast under fermentation conditions. *Journal of Applied Microbiology* **2003**, *94* (3), 432-448. DOI: 10.1046/j.1365-2672.2003.01849.x.
292.Borneman, A. R.; Forgan, A. H.; Kolouchova, R.*; et al.* Whole Genome Comparison Reveals High Levels of Inbreeding and Strain Redundancy Across the Spectrum of Commercial Wine Strains of *Saccharomyces cerevisiae*. *G3 (Bethesda)* **2016**, *6* (4), 957-971. DOI: 10.1534/g3.115.025692 From NLM.
293.Novo, M.; Bigey, F.; Beyne, E.*; et al.* Eukaryote-to-eukaryote gene transfer events revealed by the genome sequence of the wine yeast *Saccharomyces cerevisiae* EC1118. *Proceedings of the National Academy of Sciences* **2009**, *106* (38), 16333-16338. DOI: 10.1073/pnas.0904673106.
294.Mans, R.; Daran, J.-M. G.; Pronk, J. T. Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production. *Current Opinion in Biotechnology* **2018**, *50*, 47-56. DOI: 10.1016/j.copbio.2017.10.011.
295.Voordeckers, K.; Kominek, J.; Das, A.*; et al.* Adaptation to High Ethanol Reveals Complex Evolutionary Pathways. *PLOS Genetics* **2015**, *11* (11), e1005635. DOI: 10.1371/journal.pgen.1005635.
296.González-Ramos, D.; Gorter de Vries, A. R.; Grijseels, S. S.*; et al.* A new laboratory evolution approach to select for constitutive acetic acid tolerance in *Saccharomyces cerevisiae* and identification of causal mutations. *Biotechnology for Biofuels* **2016**, *9* (1), 173. DOI: 10.1186/s13068-016-0583-1.

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Appendix A Statement of Authorship: Aim 1

University of South Australia

Statement of Authorship

Title of Paper	Assessment of yeast physiology during industrial-scale brewing practices using the redox-sensitive dye resazurin
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PRINCIPAL AUTHOR	
Name of Principal Author (Candidate)	Brooke A. Dilmetz
Contribution to the Paper Brief description of your work in this publication	Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization.
Overall Percentage (%)	70
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature *Signatures will be redacted by the Library at the time of publication*	Brooke Dilmetz Digitally signed by Brooke Dilmetz Date: 2024.09.10 21:19:28 +09'30' Date 10-Sep-24
CO-AUTHOR CONTRIBUTIONS	
By signing the Statement of Authorship, each author certifies that: I. the candidate's stated contribution to the publication is accurate (as detailed above); II. permission is granted for the candidate in include the publication in the thesis; and III. the sum of all co-author contribution is equal to 100% less the candidate's stated contribution.	
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Appendix B Statement of Authorship: Aim 2

University of South Australia

Statement of Authorship

Title of Paper	Impact of propagation time on yeast physiology during bottle conditioning of beer on an industrial scale
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Contribution to the Paper Brief description of your work in this publication	Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization.
Overall Percentage (%)	70
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Appendix C Statement of Authorship: Aim 3

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Statement of Authorship

Title of Paper	Performance of Different Saccharomyces Strains on Secondary Fermentation during the Production of Beer
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details Relevant reference details	Dilmetz, B.A.; Brar, G.; Desire, C.T.; Meneses, J.; Klingler-Hoffmann, M.; Young, C. Hoffmann, P. Performance of Different Saccharomyces Strains on Secondary Fermentation during the Production of Beer. Foods 2024, 13, 2593. DOI: 10.3390/foods13162593
PRINCIPAL AUTHOR	
Name of Principal Author (Candidate)	Brooke A. Dilmetz
Contribution to the Paper Brief description of your work in this publication	Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization.
Overall Percentage (%)	65
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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