School of Engineering &
Physical Sciences
Research Proceedings

MSc Project

The effect of high gravity conditions and wheat malt on yeast health and foam stability.

Gareth Owen Eliot Hilton
MSc Brewing and Distillation

Abstract

Two different yeast strains revealed unique sets of data throughout the series of experiments based on yeast health, foam stability and stress response. The highest gravity beer (1.070) showed the greatest response in cell growth to an increase in wheat malt in both strains. The middle and low gravity beer showed a minimal response. Early viability of the US05 strains was restored with the addition of 20% wheat; this effect was more prominent at a lower gravity. Improvements were also seen in the viability of the saison although not as drastic. Cell growth of US05 is optimised and full attenuation was achieved more rapidly at 20% vs 0% wheat. Overall, this strain is more responsive to changes in fermentation conditions. Higher gravity correlates with greater cell growth, this translates to a higher consumption of free amino nitrogen (FAN), a possible explanation for the more obvious changes in cell growth at higher gravities. The boundary between optimal FAN concentration and excess lies between 20% and 5% wheat for both strains, although exact figure remains unknown. Saison showed no changes in foam stability regardless of gravity, whereas the US05 was significantly decreased at 1.070, this effect was only restored through the addition of 20% wheat malt. The trehalose concentration is positively correlated with gravity, but does not differ with wheat content. Overall stress is induced under high gravity conditions but does not inhibit growth as much as expected. Nitrogen consumption plays a greater role at high gravities but the experimental data limits the conclusion that can be drawn from this.

Introduction

The popularity of craft beer across the UK has seen a steady increase over the last decade, with this follows a rise in the number of independent micro-breweries (1*) seeking to establish themselves within the expanding market. High gravity brewing simply describes the process of brewing a beer using a high gravity wort, which is generally considered around 14-20°P depending on the brewer. The subsequent beer can then be packaged or further diluted to produce the desired flavour profile or alcohol by volume (ABV), effectively moving the addition of liquor from the mash to post fermentation (Anderson and Kirsop, 1975). From an economic standpoint this method of brewing offers several benefits. Firstly, an increased brewery production (Bamforth and Stewart, 2010) due to the larger volume of finished beer being produced without the necessity for additional fermentation vessels. There is also a higher ethanol yield per unit of fermentable extract (Stewart *et al*, 1993); this is related to cell growth as a higher proportion of sugars are converted to ethanol rather than being used to fuel new cell mass. Additionally, this method results in a reduced energy usage during the mash and boil (Stewart, 1999). The appeal can therefore be expected from breweries with limited funding and brewhouse capacity looking to break into the craft beer industry. The beer produced under high gravity conditions is also considered to be of higher quality, showing improvements in flavour stability and reduced haze formation (Stewart, 2004). High gravity brewing clearly carries numerous advantages, however several problems are encountered that have been repeatedly observed when brewing with this method, largely due to changes in the behaviour of the yeast which will be discussed in detail later on.

Problems arise during fermentation relating to losses in the viability of yeast, reduction in cell population growth and early flocculation (Stewart, 2018). As a result, fermentations are often slow or incomplete, in order to overcome this, pitching rates are increased. Elevations in ester production are also common in high gravity beers (Erten *et al*, 2012). Inefficient oxygen utilization contributes to an accumulation of fatty acids normally used during cell growth, instead they become precursors for ester synthesis (Thurston *et al*, 1984). Increased ester production contributes towards the difficulty faced when matching the flavour profiles of high gravity beers to their low gravity counterparts after subsequent dilution (Casey and Ingledew, 1983). The cause of this is generally accepted to be a combination of the high osmotic pressure, high ethanol content and variation in the available nutrients that negatively impact the behaviour of the yeast (Cunningham and Stewart, 1998). With regards to the mash, there is also a drop in efficiency (Andrews *et al*, 2011), observed due to the viscosity of the wort and thickness of the grain bed; this is why high gravity brewing is typically partnered with the use of a mash filter which bypasses the need for grain bed filtration. One of the major disadvantages is an overall loss in head retention (D'Amore *et al*, 1991) attributed to a greater loss of hydrophobic polypeptides in the beer. There are several factors that significantly contribute to loss of hydrophobic polypeptides under high gravity conditions. Losses are largely due to a greater formation of hot break/cold break from higher polyphenol concentrations and increased secretion of proteinase A caused by the yeast responding to the pressure of osmotic stress (Cooper *et al*, 2000).

Saccharomyces cerevisiae is a free living, single cellular organism that has evolved alongside humankind for thousands of years (Legras *et al*, 2007). Due to the fermentative capacity of this yeast strain it has found a place worldwide in the alcoholic

beverage industry. Modern strains of *S. cerevisiae* have been phenotypically selected over many years, providing brewers with a large range of highly fermentative yeast each expressing unique flavour profiles. Natural differences exist within strains of the same species, here the growth rates and several other parameters are profiled to compare the difference between two yeast strains.

Yeast have evolved an adaptation known as the general stress response (Gibson *et al*, 2007), allowing them to respond to environmental changes in a nonspecific manner that confers enhanced survivability to the cell. Typically, environmental changes such as increased osmotic pressure, heat shock, pH changes and nutrient starvation require the cell to respond appropriately or die. Physiological changes occur within the yeast in response to the high osmotic pressure present during high gravity brewing. High solute concentrations in the surrounding beer threaten a loss of intracellular water and subsequent turgor. Additionally, major water loss will cause inhibition of a number of enzymes (Kim *et al,* 1996) that function in cellular activity, such as respiration, halting critical cell processes. Yeast exhibit a general stress response to minimise the effects of osmotic pressure, nutrient starvation and heat shock. One such change in gene expression is the upregulation of enzymes involved in the synthesis of trehalose and glycerol (Hohmann 2002); this serves to increase carbohydrate reserves and synthesise stress protectants. Trehalose is a dissacharide composed of two glucose molecules, normally produced when almost all external glucose has been consumed. The role of trehalose is yet to be fully understood, however it is known to act as a stress protectant through the stabilisation of cellular chaperones involved in the denaturation of enzymes (Hohmann 2002), optimising cellular activity in response to environmental stress. Trehalose also has a dual function as a carbohydrate reserve. The accumulation of trehalose and glycogen during low nutrient levels creates a store of immediate energy. This has been demonstrated to allow the yeast to rapidly enter cell division upon a sudden increase in available sugars (Sillié *et al*, 1999) in comparison to mutant yeast unable to synthesise these molecules. Elevated concentrations of trehalose are one of several indicators of stress in yeast (Mahmud *et al*, 2010), despite this the effects of trehalose serve largely to preserve the continuation of normal cell growth under stress conditions, rather than limit it. In particular the enzymes associated with trehalose synthesis, hence trehalose accumulation, were crucial in the preservation of high growth rates under ethanol stress conditions (Mahmud *et al*, 2010). In high gravity beers there is an apparent increase in the ethanol concentration brought about by an overall increase in rates of fermentation discussed previously. This elevation was seen to increase the leakage of ions from cells, causing a drop in cell viability (Mansure *et al*, 1994). The addition of exogenous trehalose inhibited this effect and restored viability, demonstrating the importance of this carbohydrate in ethanol tolerance.

Yeast exhibit two categories of response to osmotic stress (Gibson *et al*, 2007). Firstly, an intrinsic part of the cell's resistance is known as osmotolerance, and includes traits such as membrane structure and vacuolar function (Cooper *et al*, 2013) which appear to be strain specific. This behaviour is distinct from osmoadaptation, instead it describes a broad set of biochemical and genetic changes that occur through signalling pathways in response to the environment including the reactive synthesis of trehalose. Regulation of gene transcription in response to stress is one method that gives rise to changes in the yeast's behaviour (Estruch, 2000), altering levels of enzymes involved in cell growth and nutrient consumption. Proteolysis can affect results by degrading enzymes that are needed during normal growth, but harmful under stress conditions, and has been adopted by yeast as a survival mechanism (Hilt and Wolf, 1992). Degrading intracellular enzymes also conserves energy by recycling smaller peptides and amino acids, it is here we see the vacuole act as a lysosome harbouring a number of non-specific peptidases (Teichert *et al*, 1989). During high gravity fermentation morphological changes to the vacuole, consisting mainly of dramatic swelling (Pratt *et al*, 2007), have been observed and are an additional indication of cell stress. Vacuolar enzymes are often proteolytically activated by proteinase A through the removal of propeptides (Van den Hanzel *et al*, 1996). Interestingly, the presence of proteinase A is significantly higher in beer brewed under high gravity conditions. The elevated concentrations of proteinase A are attributed to the cell's stress response and are secreted in quantities double that of low gravity beers (Cooper *et al,* 2000). The presence of proteinase A during fermentations causes the degradation of foam positive proteins and polypeptides, inadvertently having a negative overall effect on foam stability and head retention (Cooper *et al*, 2013). The effect of proteinase A continued to decrease foam stability in beer over a five-month period. This may be counteracted through pasteurisation and hence inactivation of the enzyme; however, it remains a problem if the beer is unpasteurised.

Cell growth, viability and vitality are all known to decrease in high gravity beer compared to lower gravities (Casey and Ingledew, 1983), however there remains some speculation as to the exact cause. The precursors of trehalose and glycogen are synthesised initially from products of glycolysis and have been linked to controlling rates of glycolysis (Hohmann 2002) in the cell, linking fermentation condition to changes in cell growth and respiration. In contrast to the effect of stress, 'Casey *et al*, 1984' suggests that the cause is a nutritional deficiency, namely nitrogen, ergosterol and oleic acid, instead of osmotic pressure and high ethanol concentration. This work also demonstrates that the bulk of wort attenuation is done by growing cells, utilising a majority of the available sugars. Therefore, when synthesis of new cell mass ceases, the fermentation is dramatically slowed down. For maximum growth rates, the optimised utilisation of FAN is essential.

Preliminary research into the effect of nitrogen starvation on the growth of *S. cerevisiae* has previously been conducted. The response of the yeast was typical of cells under nutrient limited conditions, reduced cell growth and early cell arrest from growth phase (Johnston *et al*, 1977). Interestingly the halting of cell growth was accompanied by non-specific degradation of intracellular proteins and RNA to compensate for the lack of available nitrogenous compounds in the beer, allowing cells to complete the current cell cycle without hinderance. FAN limitation has been observed in the brewing of sorghum beers wherein the use of higher proportions of starchy adjuncts causes an overall decrease in the amount of available amino acids (Pickerell, 1986). Here it is observed that a minimum ratio of FAN to sugars, rather than a threshold content of one nutrient alone, must be met to achieve maximum attenuation in the first 48 hours of fermentation. With this in mind it is reasonable to hypothesise that the high gravity (1.070 and 1.055) beers will exhibit more dramatic changes in overall cell growth with regards to the abundance of FAN, as the ratio of FAN:Sugars decreases growth limitations will become more exasperated.

The purpose of the research is to identify whether the stress response, nitrogen starvation or a combination of both factors are impacting the yeast health and foam stability of the beer. Original gravity and wheat malt act as independent variables to study the effects of wort composition on yeast health, stress, foam stability and nutrient consumption. Although reduction in foam stability is commonly observed in high gravity beer it must first be established whether it can be directly correlated with an increase in original gravity of the beer brewed here. The wheat content provides greater FAN through a higher soluble protein content than the base malt, Extra Pale (Despraetere *et al,* 2012). By varying the percentage of wheat malt this research hopes to demonstrate that the effects of nitrogen starvation, if present, can be reduced and the diminishment in foam stability may be restored through the addition of more wheat malt. Two strains of *Saccharomyces cerevisiae* have been selected to provide two unique data sets that represent the variation found to exist between genetically similar, yet distinct strains. These strains include the Belgian Belle Saison and US05 American ale yeast. Measuring cell growth, viability and vitality will provide baseline evidence towards the impact of each gravity and grain composition on the health of the yeast. After this, the levels of trehalose in the yeast and FAN in the beer will be measured in order to assess if they can be linked to changes in yeast performance.

Materials and methods

Wort production

The wort was produced using a 30L Grainfather All Grain Brewing system (Bevie, Auckland, New Zealand) to achieve a minimal original gravity (OG) of 1.070. In total three brews were completed to obtain wort for each grain bill using a blend of 'Crisp Extra Pale' (Crisp Malting Group, Norfolk, UK) and 'Crisp Wheat malt' (Crisp Malting Group, Norfolk, UK). The mash duration was 1 hour at 65°C, stirring every 15 minutes to increase extract efficiency, followed by a 10-minute rest at 75°C. The boil also lasted a total of 1 hour before being force chilled to 23°C. The wort was frozen and stored in bulk in 2L inert, plastic bottles until required. After defrosting, wort was diluted using previously sterilized water to meet the target gravity for each fermentation, basic filtration was carried out using a fine linen cloth before transferring 500ml of wort into 1L Duran bottles.

Laboratory scale fermentation.

Fermentations were carried out in triplicate, under laboratory conditions and at a room temperature of approximately 22°C. 1L Duran bottles were autoclaved prior to any contact with wort. Commercial yeast strains, Belle Saison® (Lallemand, Montreal, Canada) and SafaLE US-05® (Fermentis, Marcq-en-Barœul, France), were rehydrated from dry packets using a ratio of 1g/10mL of distilled water and a Magnetic Motion stir plate (2mag, Munich, Germany) for a duration of two hours. Pitching at a rate of 10^6 cells/mL/°P, the wort was inoculated immediately after taking the cell count and viability of the rehydrated yeast. Fermentation was allowed to proceed over the following 96 hours. The fermentations were disturbed for sampling every 24 hours by taking 15mL aliquots before being replaced in a cool storage cupboard out of direct sunlight.

Analytical methods

Cell count and viability

Samples of 100µL used for the cell count were pipetted from the 15mL aliquot, transferred to test tubes and diluted at a ratio of 1:10 in distilled water. Following dilution, 0.5mL of these samples were pipetted into 1.5mL Eppendorf tubes and mixed with 0.5mL methylene blue before mixing thoroughly using a IKA lab dancer vortex mixer. The solution of methylene blue and beer was left to rest for 5minutes before mixing again and pipetting 200µL onto a haemocytometer. Cell counts were taken using a bright field light microscope by counting cells in the four corner squares and central square of the 5x5 central grid, noting cells stained blue as dead. The haemocytometer was then washed and dried thoroughly in between readings before proceeding to calculate cells per mL and viability using the equations below.

$$\frac{Cells}{mL} = Live\ cell\ count \times 5 \times dilution\ factor\ (10) \times 10^3$$

$$Viability\ (\%) = \frac{Total\ unstained\ cells}{Total\ cells}$$

The remainder of the 15 mL undiluted sample was filtered through filter paper to remove suspended yeast and debris. The gravity and pH were recorded every 24 hours using an Anton Paar 4100 density meter (Anton Paar, Graz, Austria) and calibrated Hanna Instruments 83141 pH meter (Hanna Instruments, Woonsocket, USA) respectively.

Cell vitality (Acidification power test)

20.2% Glucose solution was made using D-Glucose anhydrous (Fisher Scientific, Perth, UK) and distilled water at a ratio of 1 2.02g/10mL and stirring until the glucose had dissolved. The solution was then sterilized by autoclaving (121°C for 60 minutes) held at 25°C until ready to use.

Cell vitality measurements were carried out on the rehydrated yeast before pitching and at the end of the 96-hour fermentation. Samples containing 50ml of rehydrated yeast were poured into Falcon tubes. Yeast from the fermenters was extracted by first pouring out the majority of the beer, leaving a thin layer of beer and flocculated yeast at the bottom. This solution was mixed up and also poured into 50mL centrifuge tubes, subsequent treatment of beer and rehydrated yeast samples is identical. Samples were centrifuged using a MSE Mistral 1000 centrifuge (DJB Labcare Ltd, Newport Pagnell, UK) at 4000 rpm for 10 minutes. The supernatant was discarded and the Falcon tubes refilled with distilled water, using a vortex mixture to re-suspend the yeast, repeating this process twice more. 2.7g of centrifuged yeast is then weighed out, taking 0.9g from each triplicate, into a 25mL Duran bottle.

Next a 100mL beaker of distilled water was placed onto a stir plate for 5 minutes and the pH was measured. After this 15mL of the distilled water at 25°C was added to the yeast and mixed by gently shaking the container. The pH was measured at 1 minute intervals for 10 minutes. Finally, 5mL of the 20.2% glucose was added, repeating the measurements every 1 minute for an additional 10 minutes. Acidification power is calculated using the equation below.

$$AP = pHdistilled\ water - final\ pH\ after\ 20\ minutes$$

Foam collapse

200mL beer samples were collected at the end of fermentation in 300mL Erlenmeyer flasks and placed into a FS3000 Frequency Sweep ultrasonic bath (Decon Laboratories Ltd, Brighton, UK) dissolved CO_2 was removed. The high gravity beers were diluted appropriately to an ABV of 4.5% that matched that of the lowest gravity beer.

Initially the column is filled to 100mL and gas regulator set to a CO_2 pressure of 1 bar/15 psi. Downstream of valve 1, the CO2 flow rate is adjusted to 100 units on the Rudin gas valve against a sample of beer. Gas flow is then shut off by closing valve 1 and the column of the Rudin apparatus is rinsed through with the beer sample and discarded. The column was then filled again with 100mL of sample beer and valve 1 opened. Once the foam has reached 325mL, close tap 1 and begin the timer. The time is noted when the foam has collapsed to 50mL and 75mL from the bottom up. The sample is disgarded, rinsed through with distilled water and the next beer sample before repeating.

Free amino nitrogen (FAN)

Following a standardized method (ASBC Methods of Analysis wort-12) FAN was measured from samples of each wort and beer at the start and end of fermentation. Reagents used included glycine (Fisher Scientific, Perth, UK), ninhydrin (Fisher Scientific, Perth, UK) and potassium iodate (Fisher Scientific, Perth, UK). Subsequent analysis was carried out using a Genesys 6 spec spectrophotometer (Thomas Scientific, Swedesboro, USA).

Trehalose

Yeast samples were gathered from the centrifuged yeast obtained during cell vitality (see above). 0.75g of centrifuged yeast was placed into 1.5mL Eppendorf tubes and re-suspended in 1mL of distilled water before being stored at -20°C until required.

Trehalose was analysed using the K-TREH kit (Megazyme, Bray, Ireland) following the manufacturer's microplate analysis method. Microplate readings were obtained using a SpectraMax M5 plate reader (Molecular devices, San Jose, USA). Additional calculations, detailed below, were carried out on the absorbance values to determine trehalose concentrations for each yeast sample.

Statistics and Software

(1) Trehalose concentrations were calculated using the trehalose (K-TREH) Mega-Calc (Megazyme, Bray, Ireland).
(2) Graphs and statistics were carried out using Graphpad Prism software (San Diego, USA), all statistical analysis is taken with $P \leq 0.05$.
(3) Tables were made using Microsoft Excel 2016 (Microsoft Corporation, Albuquerque, USA).

Results and Discussion

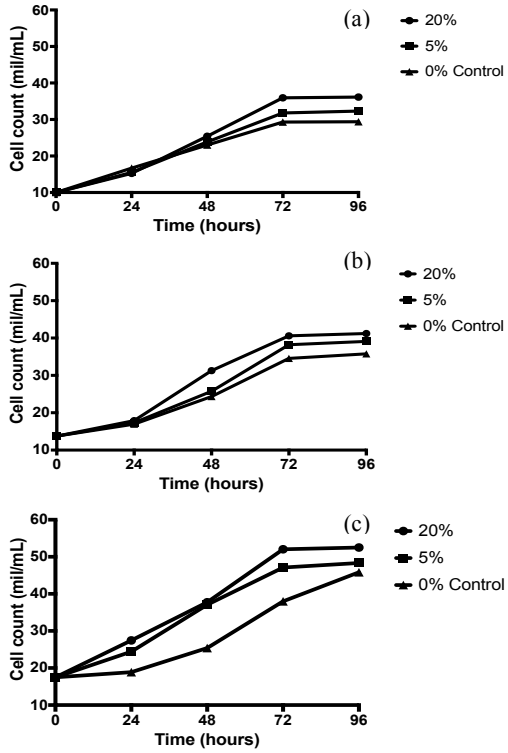

Figure 1: Cell count of US05 yeast over 96 hours at three original gravities: 1.040 (a), 1.055 (b), 1.070 (c). Wheat content for the beer is indicated in each figure legend.

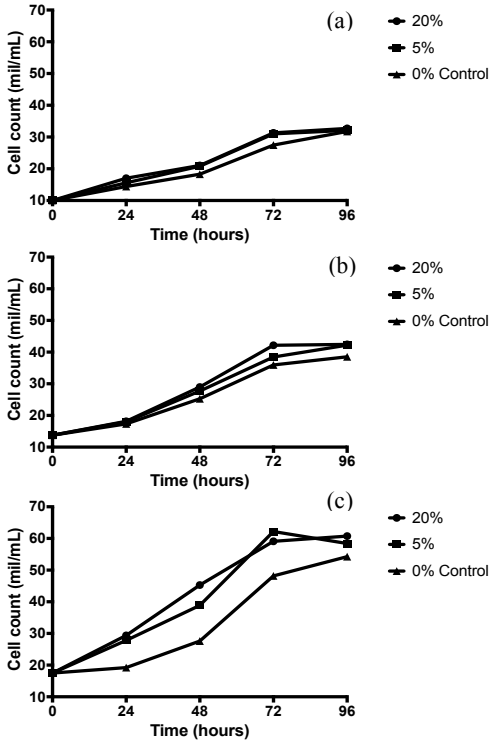

Figure 2: Cell count of Saison yeast over 96 hours at three original gravities: 1.040 (a), 1.055 (b), 1.070 (c). Wheat content for the beer is indicated in each figure legend.

The initial cell count in each beer differs depending on the original gravity, therefore the highest gravity beers (1.070, Fig 1c and 2c) end with the highest cell counts. The lowest gravity beers (Fig 1a and 2a) show similarities in terms of their rates of growth, with the Saison (Fig 2a) yeast ending fermentation on a consistent value of roughly 32 mil cells/mL, whereas the US05 (1.040, Fig 1a) shows a little more variation depending on the content of wheat malt. For the US05 there appears to be little difference between the '0% control' and the '5%' or '20%' beers when the original gravity is 1.040 and 1.055, albeit the beers with higher wheat content finish on a slightly higher cell count. However, in Fig 1b (1.055) the '20%' beer shows a deviation above the cell count of the other two at 48 hours, a feature not visible in Fig 1a (1.040). Further disparity between the cell growth can be seen in Fig 1c where the control has a substantial gap in cell growth between 48-72 hours compared to the beers with 5/20% wheat malt, this is coupled with cell growth lacking a clear plateau which is visible in the other two beers at the end of fermentation. Fig 2a-c showing the Saison yeast are largely similar to their US05 counterparts in Fig 1a-c, although the effect of high gravity limiting cell growth is slightly less apparent. The cell growth for Saison yeast appears more consistent and robust perhaps indicating that they are able to tolerate the stress of high gravity more easily than US05. The trends in Fig 1c and 2c suggest that at higher gravities the wheat malt, and therefore available nitrogen, plays a more important role in the optimization of cell growth. Though the data fail to pinpoint the exact cause of this, limited nutrients, the ratio of FAN to sugars and an alleviation on the stress caused by the high gravity conditions may have contributed to the observed effect.

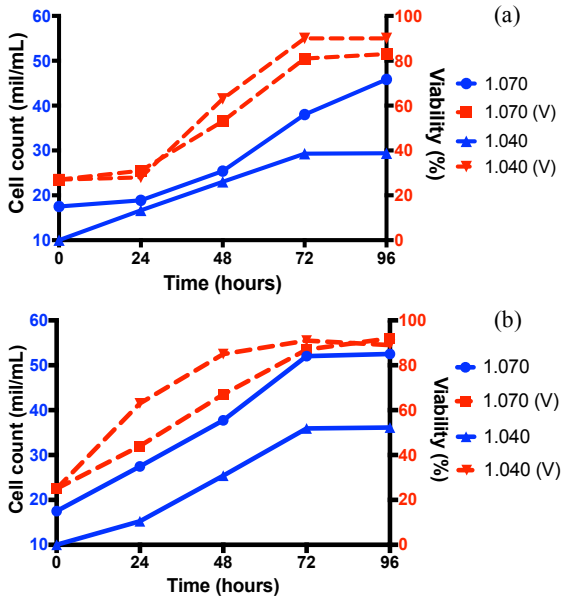

Figure 3: Comparison of US05 cell growth (blue) and viability (red) between the beers at OG 1.040 (a) and 1.070 (b).

The viability of the US05 yeast (Fig 3a, 3b) started much lower than the Saison after rehydration at the beginning of fermentation, at roughly 25%. Both cell growth and viability are slow to increase in Fig 3a. The viability picks up as the fermentation progressed, finishing consistently above 90% (Fig 3a, 3b); however, cell growth remains restricted in the beer containing 0% wheat. Early cell growth of the 1.070 is more clearly a problem at 0% wheat, where the cell count is comparable to that of the 1.040 beer at 24 and 48 hours, although continues to increase after this period. The cell count in the 20% wheat malt beers in Fig 3b rise in a linear fashion. Both beers finish attenuation 72 hours into fermentation (data not shown), this is mirrored in the discontinuation of cell growth past 72 hours. Early viability of the 1.040 beer in Fig 3b is notably higher than the high gravity beer, therefore although the 1.070 beer has an elevated viability compared to Fig 3a it is still impaired compared to that of the lower gravity beer. Overall, cell count and viability for both gravities are enhanced by the inclusion of 20% wheat malt, although the effects of high gravity stress are still present in the 1.070.

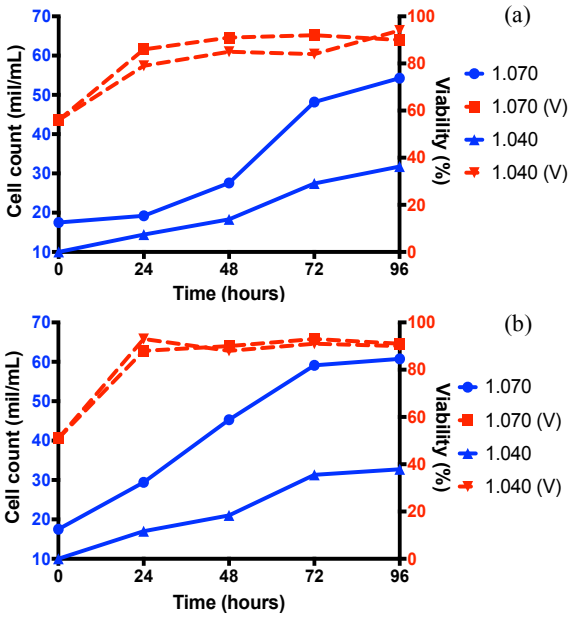

Figure 4: Comparison of Saison cell growth (blue) and viability (red) between the beers at OG 1.040 (a) and 1.070 (b).

The Saison yeast (Fig 4a, 4b) started the fermentation at roughly 40% viability post rehydration; this may provide a further indication of their resistance to stress compared to the US05. Viability quickly increases and also stabilizes at roughly 90% by 96 hours. Fig 4b shows a greater recovery of the viability, reaching the peak viability after only 24 hours. In comparison to the US05 the cell growth of the 1.070 beer does better at 0% wheat (Fig 4a), and is very similar to the US05 at 20% wheat. The cell growth of the 1.040 beer shows almost no difference whether the wheat malt is present or not, which is unexpected since the US05 increased cell growth under the same conditions.

Wheat malt has a positive relationship with cell growth when it comes to both Saison and US05, the latter finishing fermentation after 72 hours as a result of the wheat addition. Early viability also shows improvement for both yeast, although it shows no difference at the end of fermentation after the cell population has had time to establish itself.

Table 1: Results of the acidification power test. Rehydrated yeast was measured before pitching, Measurements for each additional condition were taken post fermentation at 96 hours.

Condition	US05	Saison
Rehydrated	1.08 (±0.04)	1.31 (±0.04)
0% Wheat	1.59 (±0.04)	1.95 (±0.03)
5% Wheat	2.31 (±0.08)	2.11 (±0.07)
20% Wheat	2.17 (±0.05)	2.14 (±0.05)

Table 1 displays the results of the vitality (acidification power) test. For a brewery to fully understand the fermentative capacity of the yeast it must examine both the number of viable cells and the condition of these cells. The experiment is designed to measure metabolic activity through the ability of the yeast to reduce extracellular pH in response to the addition of glucose (Kara *et al*, 1987). The data (Table 1) indicates that vitality is lowest in the rehydrated yeast, and particularly low in US05. The vitality increases with the addition of wheat but not drastically. Table 1 shows mixed results, with the Saison showing a weak increase in vitality as wheat content is increased and US05 exhibiting the greatest value at 5%. No obvious conclusions can be drawn from Table 1 since the trends between the yeast strains differ and figures from the results are mostly very similar. The rehydrated yeast will be greatly affected by the starting viability, since the yeast sample used is measured by weight and not cell count, this offers an explanation as to why the rehydrated yeast vitality is so low. In addition to the cell count, flocculation will negatively affect the perceived vitality and is not taken into consideration. The acidification power test is of great importance to the brewer, however on this occasion several factors could be improved to increase the reliability of this experiment.

Table 2: Measurements of free amino nitrogen (FAN) in both wort and finished beer in US05 and Saison beers.

Gravity	Wort	End (US05)	End (Saison)	Total utilization (US05)	Total utilization (Saison)
1.070 (0%)	113.4	35.2	32.1	78.2	81.3
1.055 (0%)	89.1	30.5	28.4	58.6	60.7
1.040 (0%)	65.8	32.3	20.7	33.5	45.1
1.070 (5%)	123.3	48.9	41.6	74.4	81.7
1.055 (5%)	97.7	45.5	42.4	52.2	55.3
1.040 (5%)	71.1	32.1	37.2	39	33.9
1.070 (20%)	169.3	58.7	57.8	110.6	111.5
1.055 (20%)	133	42.6	52.1	90.4	80.9
1.040 (20%)	96.7	35.2	40	61.5	56.7

As previously discussed, FAN is essential for providing amino acids for protein synthesis and cell growth. Increase in wheat malt content increases FAN in the wort as predicted (Table 2). When looking at the FAN utilization it is important to consider that starting concentrations differ between each original gravity and wheat content, therefore representing the data as a percentage utilization misrepresents the total consumption of FAN during fermentation. Here we see utilization increase following an increase in gravity, this is largely due to the higher overall cell growth and starting concentrations of FAN present in the 1.070 and 1.055 beers, this trend is true for both yeast strains. The end concentration of FAN for also increases dramatically at high gravities, this appears due to the increase in wheat content increases. The same cannot be said for the lower gravities, for example Saison 1.040 shows an increase of 14.5 UNIT between 0% and 5% but only 2.8 UNIT between 5% and 20%. Together with the total utilization it indicates that at high gravities (1.070) 20% wheat provides excess FAN to the yeast and it is unable to be fully utilized before fermentation is complete. The optimized condition between maximal growth and excess FAN therefore lies somewhere between 5% and 20% wheat for both Saison and US05.

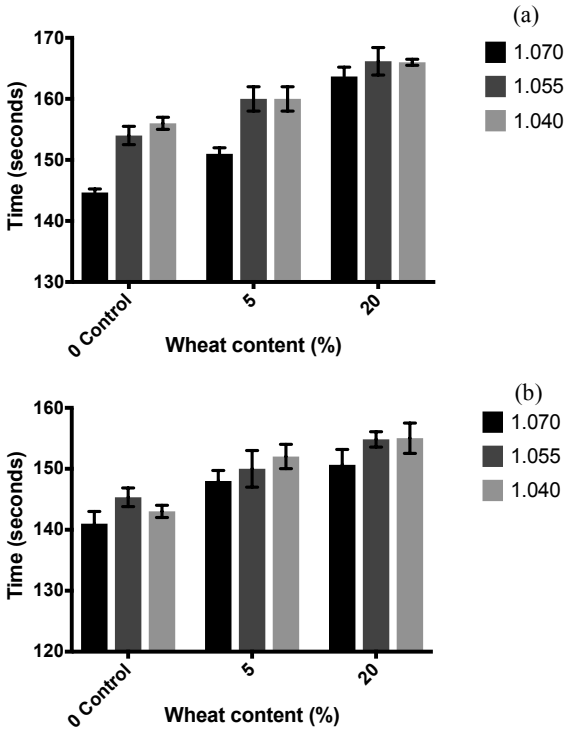

Figure 5: Data from the foam collapse experiment comparing the times taken for 75ml of foam to collapse; US05 (a), Saison (b).

The foam collapse experiment used the time taken for the foam to collapse to the 75ml point of the column. This is because the time taken for the foam collapse at 50ml was almost identical across the two variables, whereas the 75ml shows clear differences. The data seen in Figure 5 was largely consistent between triplicates which is favourable in determining a statistical significance. Statistical analysis was carried out using two-way ANOVA (P 0.05) to test whether significant differences existed between each beer under different conditions of wheat content. Firstly, the beer brewed with US05 (Fig. 5a) showed an increase in time as wheat content increased. The 1.070 beer was found to be significantly different to the 1.040 control at both 0% and 5%, whereas the 1.055 showed no significant difference in comparison to the 1.040. Once the wheat content reached 20% no significant difference was found between the 1.040 and either of the high gravity beers. Therefore, we may accept the conclusion that foam stability is negatively affected under high gravity (1.070) conditions and may be restored through the addition of 20% wheat malt. However, the Saison (Fig. 5b) did not display a similar trend. Although the foam collapse time appears to increase as the wheat content increases, no significant differences between the beers were detected at either 0% or 5%. Upon reaching 20% there was a significant difference between the 1.070 and 1.040, this was unexpected as it directly opposes the results of the US05 and was likely caused by human error when carrying out the experiment. The results of the Saison yeast beers suggest that no increase in proteolysis was occurring at higher gravities.

In the case of the US05 yeast, the results confirm the hypothesis that wheat malt may restore foam stability. The experiment could be improved further through quantifying the concentration and activity of proteinase A in the beer, since this is the factor affecting head retention and signal of the stress response. Similarly, the concentrations of hydrophobic polypeptides that are responsible for foam stability would have been of interest to measure. Through this a better understanding of how each factor differs between brewing conditions, and subsequently impacts foam stability, could be achieved.

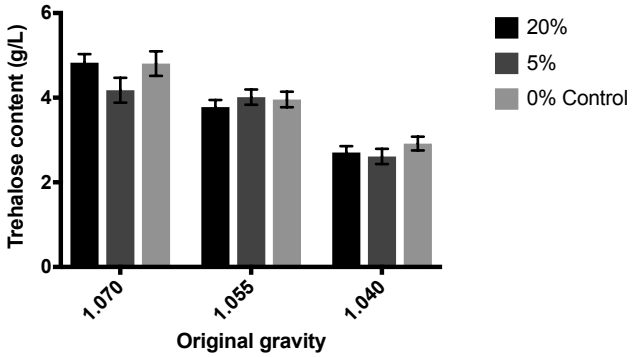

Figure 6: Comparison of the concentration of trehalose between gravities

Further ANOVA two-way analysis (P 0.05) finds a significant difference between the trehalose values at '1.040 vs 1.055' and and '1.040 vs 1.070' seen in Figure 6. One value found at 1.070, 0% wheat strayed greatly from the other two triplicates and was therefore considered an outlier. No significant differences were found between different wheat contents of the same gravities, showing that the stress response is a function of gravity and not nitrogen starvation. This finding confirms that stress is induced at high gravities, likely through the high osmotic pressure and ethanol concentration.

The trehalose concentration is linked to gravity and not wheat content, therefore stress alone cannot be attributed to the reduction in cell growth (Fig 1c, 2c) under high gravity. Clearly wheat content and FAN play a role in cell growth at 1.070, however the drop in cell growth and viability occurred largely during the early stages of fermentation. In order to fully evaluate the effect FAN consumption had on these parameters more data needs to be gathered and statistically analysed using linear regression against cell growth every 24 hours. The method of data gathering used is sufficient to identify broad trends but not to conclusively prove the link between FAN consumption and restoring cell growth in high gravity fermentation.

In conclusion the two yeast strains differ in their growth patterns and response to stressful stimulus. The Saison proved particularly robust in comparison to the US05, which seemed sensitive to changes in the conditions of fermentation. Upon repetition of this series of experiments more measurements could be taken, including a daily FAN, proteinase A and hydrophobic polypeptide reading, although the workload would require a great deal of efficient time management. I believe it may also be of interest to pitch a single quantity of yeast into three worts of different gravity, thereby creating a more comparable data set with regards to how high gravity effects cell count. Refinement of the vitality experiment, through more accurate methods of sampling live cells, could also be useful in understanding the condition of the yeast at the end of fermentation. The findings here justify further research into what is likely a complex relationship between yeast health, nutrients consumption and stress response.

Acknowledgements

I would like to thank my supervisor, Dawn Maskell, for all her help in bringing this project together and for her valuable guidance.

I am also grateful to Alasdair Brown, Maarten Cornelus, and Rachel Sutherland for their daily support in the labs throughout the data collection.

Finally, I would like to thank Helinor Johnson for assisting in the set-up of the trehalose experiment.

References

Anderson, R. and Kirsop, B. (1975) Quantitative aspects of the control of oxygenation on acetate ester concentration in beer obtained from high gravity wort, *Journal of the Institute of Brewing. 81*, 286-301.

Andrews, J. Hancock, J. Ludford-Brooks, J. Murfin, I. Houldsworth, L. Phillips, M. (2011) 125th Anniversary review: Some recent engineering advances in brewing and distilling, *Journal of the Institute of Brewing. 117,* 23-32.

Bamforth, C. and Stewart, G. (2010) Brewing – the transformation of a craft into a technology, *The Biologist*. *57,* 138-147.

Casey, G. Ingledew, W. (1983) High gravity brewing: Influence of pitching rate and wort gravity on early yeast viability, *Journal of the American Society of Brewing Chemists*.

Casey, G. Magnus, C. Ingledew, W. (1984) High-gravity brewing: effect of nutrition on yeast composition, fermentative ability and alcohol production, *Applied and Environmental Microbiology. 48,* 639-646.

Cooper, D. Stewart, G. Bryce, J. (2000) Yeast proteolytic activity during high and low gravity wort fermentations and its effect on head retention. *Journal of The Institute of Brewing. 106,* 197-202.

Cooper, D. Stewart, G. Bryce, J. (2013) Some reasons why high gravity brewing has a negative effect on head retention, *Journal of the Institute of Brewing. 104,* 83-87.

Cunningham, S. and Stewart, G. (1998) Effects of high-gravty brewing and acid washing on brewers' yeast, *Journal of the American Society of Brewing Chemists. 56*, 12-18.

D'Amore, T. Celotto, G. Stewart, G. (1991) Advances in the fermentation of high gravity wort, *Proceedings of the European Brewing Convention, Congress, Lisbon.* 337-344.

Despraetere, S. Delvaux, F. Coghe, S. Delvaux, F.R. (2012) Wheat variety and barley malt properties: influence on haze intensity and foam stability of wheat beer, *Journal of the Institute of Brewing. 110,* 200-206.

Erten, H. Tanguler, H. Cakiroz, H. (2012) The effect of pitching rate on fermentation and flavour compounds in high gravity brewing, *Journal of The Institute of Brewing. 113*, 75-79.

Estruch, F. (2000) Stress-controlled transcription factors, stressinduced genes and stress tolerance in budding yeast, *FEMS Microbiology Reviews. 24*, 469-486.

Gibson, B. Lawrence, S. Leclaire, J. Powell, C. Smart, K. (2007) Yeast responses to stresses associated with industrial brewing handling, *FEMS Microbiology Reviews. 31*, 535-569.

Hilt, W. Wolf, D. (1992) Stress-induced proteolysis in yeast, *Molecular Microbiology. 6*, 2437-2442.

Hohmann, S. (2002) Osmotic stress signalling and osmoadaptation in yeast, *American Society for Microbiology*. *66*, 300-372

Johnston, G. Singer, R. McFarlane, S. (1977) Growth and cell division during nitrogen starvation of the yeast *Saccharomyces cerevisiae*., *Journal of Bacteriology*. *132*, 723-730.

Kara, B. Simpson, W. Hammond, J. (1987) Prediction of the fermentation performance of brewing yeast with the acidification power test, *Journal of the Institute of Brewing*. *94,* 153-158.

Kim, J. Alizadeh, P. Harding, T. Hefner-Gravink, Ann. Klionsky, D. (1996) Disruption of the yeast ATH1 gene confers beter survival after dehydration, freezing and ethanol shock: potential commercial applications, *American Society of Microbiology*. *62,* 1563-1569.

Legras, J. Merdinoglu, D. Cornuet, J. Karst, F. (2007) Bread, beer and wine: *Saccharomyces cereisiae* diversity human history, *Molecular Ecology. 16,* 2091-2102.

Mansure, J. Panek, A. Crowe, L. Crowe, J. (1994) Trehalose inhibits ethanol effects on intact yeast cells and liposomes, *Biochimica et Biophysica – Biomembranes. 1191,* 309-316.

Pickerell, A. (1986) The influence of free alpha-amino nitrogen in sorghum beer fermentations, *Journal of the Institute of Brewing*. *92*, 568-71.

Pratt, P. Bryce, J. Stewart, G. (2007) The yeast vacuole: A scanning electron microscopy study during high gravity wort fermentations, *Journal of the Institute of Brewing. 113*, 55-60

Silljé, H. Paalman, J. Schure, E. Olsthoorn, S. Verkleij, A. Boonstra, J. Verrips, C. (1999) Function of trehalose and glycogen in cell cycle progression and cell viability in *Saccharomyces cerevisiae*, *Journal of Bacteriology. 181*, 396-400.

Stewart, G. and Russell, I. (1993) Fermentation – "black box" of the brewing process, *Tech. Q. Master Brew. Assoc. Am 30*, 159-168.

Stewart, G. (1999) High gravity brewing, *Brew guard*, *128,* 31-37.

Stewart, G. (2004) The chemistry of beer instability, *Journal of Chemical Education. 81,* 963-968.

Stewart, G. (2018) Yeast flocculation – sedimentation and flotation, *Fermentation*, 259-308.

Teichert, U. Mechler, B. Müller, H. Wolf, D. (1989) Lysosomal (Vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation and cell survival, *Journal of Biological Chemistry. 264*, 16037-16045.

Thurston, P. Quain, D. Tubb, R. (1984) Lipid-metabolism and the regulation of volatile ester synthesis in *Saccharomyces cerevisiae, Journal of the Institute of Brewing*. *88*, 90-96

Van den Hanzel, H. Kielland-brandt, M. Winther, J. (1996) Review: Biosynthesis and function of yeast vacuolar proteases, *Yeast. 12,* 1-16.

Online references

(1*) (Date of access 20/08/2018)
https://www.gov.uk/government/news/britain-becomes-brewing-powerhouse