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Investigation of Fermentation Efficiency in Saccharomyces cerevisiae Through Telomere Integrity and Maltase Expression

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By

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Abstract

Many microbreweries practice serial fermentation, or serial repitching. Serial repitching is the process by which one yeast culture is reused for multiple batches of beer. Each batch of subsequent beer is called a "pitch." This technique helps breweries limit production costs. However, fermentation is difficult to predict throughout serial fermentation. This leads to beer quality issues such as fluctuation in alcohol production, buildup of unwanted flavor compounds, and decreased carbon dioxide production, which results in reduced profit. To combat this issue, many breweries will attempt to predict fermentation efficiency through viable cell counting in order to ensure a consistent number of viable cells are inoculated into each pitch.

However, it has been previously demonstrated by researchers and brew masters that viability and other morphological characteristics are not a reflection of metabolic competence in brewer's yeast (*Saccharomyces cerevisiae*). For this reason, a better understanding of serial fermentation at a molecular level is necessary. Once the molecular impacts of serial repitching are understood, specialized strains of brewer's yeast can be designed to provide more stable, predictable fermentation.

This study investigated whether *MAL* genes, which encode the enzyme maltase, exhibits a decrease in mRNA expression throughout serial fermentation via RT-qPCR. Additionally, the present study investigated telomere integrity throughout serial fermentation to determine whether telomeres degrade throughout serial repitching via telomere restriction fragment analysis (TRF). No significant decrease in *MAL* mRNA expression was observed as pitch number increased. This indicates that reduced maltase expression is not the cause of decreased fermentation efficiency. Telomeres shortened and became more heterogeneous in length throughout serial fermentation. These observations indicate that increased pitch number results in cell aging and an overall decline in cell health.

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Introduction

As the number of microbreweries increase across the United States, there is a demand for improved understanding of yeast biology. Yeast is the most important component of brewing because it dictates the quality of beer through various fermentation products, such as ethanol, carbon dioxide, and flavor compounds. Fermentation performance directly impacts brewery resources, such as time and profit. One common practice to retain profitability is serial repitching. Serial repitching is when the yeast from one batch of beer is collected, stored, and reused for the next brewing cycle (1). This practice helps keep waste and production costs low (2,3). However, the more a culture is reused, the less efficient fermentation becomes (2, 4, 5). In addition to decreased fermentation efficiency, there may also be an increase in undesirable compounds resulting from yeast metabolism (6). Most breweries limit the number of times a culture can be reused to ten and fifteen pitches in an attempt to prevent batch loss (7). Most breweries try to address culture aging by focusing on culture viability. This is usually achieved through viable cell counting. However, it has been found that viability is not a predictor of fermentation efficiency (8). Hence, analytical techniques beyond viability testing are necessary to evaluate potential fermentation efficacy prior to pitching.

In order to develop new methods of testing fermentation efficiency in viable yeast, the cause of the decline in efficiency must first be identified. Previous studies have indicated gene families that likely play critical roles in beer production, namely *FLO*, *HXK*, and *MAL* (4). The *FLO* gene family encodes glycoproteins that are responsible for

cell-cell adhesion, i.e. flocculation (9). Flocculation contributes to yeast removal, but it does not impact fermentation efficiency. Rather, flocculation contributes to optical qualities of beer, so *FLO* was not evaluated in this study. There are two *HXK* loci, which encode hexokinase. The most important role of hexokinase in brewer's yeast is the conversion of glucose to glucose-6-phosphate, which is the first step in glycolysis and the addition of a phosphate prevents glucose from diffusing out of the cell (10). Maltase is responsible for breaking down maltose into two glucose molecules, making it a fermentable sugar (11). There are five *MAL* loci that encode maltase enzymes, four of which are known to be telomere-adjacent (12, 13). Maltase is a critical enzyme for brewer's yeast because 60-70% of the available sugar in wort is maltose (14). Considering the critical role of this enzyme and its position near the telomere, *MAL* expression and telomere integrity were evaluated in this study.

The objective of the present study was to determine whether the transcription of maltase, which is one of the most critical enzymes for metabolic competence in brewer's yeast, was negatively impacted by serial fermentation. Due to the telomere-adjacent location of *MAL* loci, the present study aimed to determine whether telomere integrity was compromised by serial fermentation. We hypothesized that decreased fermentation efficiency throughout serial repitching is caused by a decrease in *MAL* mRNA expression, i.e. *MAL* expression and pitch number will exhibit an inverse relationship. Further, we hypothesized that telomere integrity will decrease throughout serial fermentation.

Literature Review

Saccharomyces cerevisiae

Saccharomyces cerevisiae is one of the most highly utilized models of eukaryotic biology. It is a unicellular eukaryote that is easy to work with because of its minimal nutritional requirements, rapid growth rate, and versatility (15). *Saccharomyces cerevisiae* is the most common species used to brew ales. *S. cerevisiae* has also given rise to other common hybrid species commonly used in brewing, such as lager-producing *Saccharomyces pastorianus* (16). *S. cerevisiae* possesses sixteen chromosomes and reproduces via budding.

Budding is the asexual reproduction process by which a haploid cell will duplicate its genetic material and pinch off a smaller daughter cell, which can then grow and repeat the budding process (17). There are two types of budding yeast cells: a and α. The a and α cell types denote the type of mating pheromone that is released by the cell. When an a or α cell detects the mating pheromone of the opposite cell type, the cells begin to form projections that result in the fusion of the cells. This usually occurs under high stress, such as nutrient limitation. The diploid cell that results from the fusion of the two cell types will then undergo meiosis to yield four haploid spores, which can mate or continue the budding process upon germination (17). Other notable morphological characteristics of yeast include cell walls made of chitin and accumulation of fat globules in the cytoplasm (18).

Brewer's yeast can be divided into two groups: top-fermenters and bottom-fermenters. These terms describe where the yeast flocculate and ferment in the brewing

vessel. *S. cerevisiae* is a top-fermenting, facultative anaerobe that can ferment many types of sugars, such as maltose, fructose, and glucose (18). Most brewer's wort contains high concentrations of maltose, which was the primary focus of this study.

Maltase

Under brewing conditions, at least 60% of the available fermentable sugar is maltose (14, 18). Maltase is an enzyme encoded by the *MAL* genes that converts maltose into two glucose molecules. There are five known *MAL* loci. These loci are labeled with two numbers. The first number refers to the locus; the second number refers to genes within the locus. Each *MAL* locus encodes three genes: the permease (gene 1), maltase (gene 2), and an activator (gene 3) (12). For an example, *MAL11* denotes the maltose permease of the first *MAL* complex. The orientation of these genes (5'-3') within each locus is: activator, permease, and maltase. Thus, the gene encoding maltase gene is closest to the telomere. *MAL1* is located on chromosome VII, *MAL2* is located on chromosome III, *MAL3* is located on chromosome II, *MAL4* is located on chromosome XI, and *MAL6* is located on chromosome VIII (12). The structural maltase genes have genetically identical nucleotide sequences at all loci, whereas the permeases and activators differ with respect to each locus. Most strains do not actively carry all five loci (12).

Brewing and Fermentation

The brewing process can be summarized in four key steps: malting, wort production, fermentation/maturation, and packaging. During the malting step, barley is mechanically processed to produce malt, which is barley seeds that have been germinated and dried. This process converts starch to maltose and other fermentable sugars through the amylases in the grain. Next, wort is produced by boiling the malt with hops and water. Hops are essential in providing flavor and natural antimicrobial properties. The wort production step transforms the malt into a nutritional medium that the yeast will utilize for growth and fermentation. The wort is then inserted into a brewing cellar where the yeast— which ferment sugars into ethanol, carbon dioxide, and various flavor compounds— are added. When the fermentation process is finished, the product moves into quality testing and bottling (19).

There are several pathways and enzymes necessary for yeast to successfully convert brewer's wort into ethanol and carbon dioxide. Fermentation is the most critical step in brewing. If fermentation does not occur correctly, the product must be discarded, which is a source of economic loss for the brewery. Maltose must first be cleaved into two glucose molecules via maltase. The glucose can then enter the Embden-Meyerhof-Parnas Pathway, i.e. glycolysis. Glycolysis yields ATP, NADH, and pyruvate. In the absence of oxygen, fermentation begins after glycolysis. The pyruvate is decarboxylated, which converts pyruvate to acetaldehyde and CO_2. Then, the acetaldehyde is converted into ethanol via alcohol dehydrogenase (20). The yield of these metabolic processes

depends on yeast viability and vitality, which includes morphological changes, enzyme activity, ATP content, and mitochondrial membrane potential (21).

Serial Repitching and Fermentation Efficiency

Serial repitching, or serial fermentation, is the process by which the yeast from one batch of beer are removed and inoculated into the next batch (1). Each sequential batch of beer that a single yeast culture ferments is called a "pitch." This technique is employed by microbreweries in order to limit production costs.

Although this practice limits production costs, it comes with its setbacks. The most notable downfall of serial fermentation is the unpredictability of fermentation efficiency. Fermentation efficiency refers to the ability of yeast to convert sugar to ethanol and carbon dioxide. A decline in fermentation efficiency leads to a lower alcohol by volume (ABV), less carbon dioxide, and an accumulation of unwanted compounds that alter beer quality (3, 22). Furthermore, serial repitching can lead to increased autolysis of yeast, which results in leakage of intracellular compounds that cause unpleasant flavor and colloid formation (22). Serial fermentation affects yeast health due to the stressful conditions it provides. Stresses involved in serial repitching include shifting between anaerobic and aerobic environments, osmotic potential changes, low pH, ethanol toxicity, carbon limitation, and temperature shifts (23). When yeast fail to ferment efficiently, the brewery must dispose of the product, which results in reduced profit and delayed production time. Prior to pitching, it is difficult for a brewer to predict

whether yeast will efficiently ferment. The most commonly utilized technique to predict fermentation prior to pitching is cell viability.

Current Viability Testing

Prior to repitching, brewers should determine the number of live cells present to ensure that fermentation will be successful. Methods of determining viability include methylene blue staining, pH testing, protease activity, bud scar quantification, plating, and capacitance (5,13). Methylene blue staining is the most frequently used viability testing technique because it is rapid, inexpensive, and requires only a microscope (24). This method is able to distinguish live cells from dead cells because the methylene blue stain is reduced to a colorless compound inside the cytoplasm of living cells. Dead cells are unable to reduce the stain, thus dead cells remain blue. Methylene blue can cause an overestimation of viable cell count, especially if a culture is below 95% viability. This is due to counting inaccuracies and clumping of dead cells (25, 26). An inaccurate estimation of viable cells is a common downfall of all subjective cell counting techniques because the cells seen underneath a microscope are not guaranteed to be representative of the entire sample (24). This discrepancy has been observed when colony forming units (plate counts) were compared to their respective methylene blue estimations (26).

Regardless of the viability technique used and its accuracy, it has been found that live cells do not always ferment efficiently with increased pitch number. This is an important distinction between cell viability vs. cell vitality (21). A cell's viability does not indicate metabolic competence and adequate stress response. Although more uniform

fermentation can be attained by altering nutritional conditions, this would also alter beer quality and consistency (8). Further understanding of the molecular mechanisms of fermentation efficiency, or cell vitality, will help engineer yeast strains for extended serial repitching without compromising fermentation efficiency.

Morphological Impacts of Serial Repitching

Previous serial fermentation studies have mostly focused on morphological data because morphological characteristics are easily observed. As pitch number increases, various morphological qualities can be affected.

With increased pitch number, cell age increases. Cell age directly correlates with bud scars, which result from cell division. An older cell that has been involved in more pitches will have a higher number of bud scars. However, bud scars have not been shown to compromise cellular integrity (27). Although previous studies have shown that cell aging has a negative impact on flocculation, data remains inconclusive (4). Cell aging has been shown to cause wrinkling of the cell wall and distortion of the overall cell shape (22). In addition to the impact on cellular structure, serial fermentation also impacts levels of intracellular chemicals, such as ATP, reactive oxygen species (ROS), glycogen, and trehalose (5, 22). A buildup of reactive oxygen species is of particular concern because ROS can inactivate proteins, damage nucleic acids, harm mitochondria, and cause lipid peroxidation (22). While some studies have found that these morphological and chemical changes are associated with serial repitching, other studies have found no

impact throughout serial fermentation (23). For this reason, a greater understanding of the molecular impact of serial fermentation is needed (23).

Molecular Impacts of Serial Repitching

Molecular studies of serial fermentation remain limited and inconclusive. Additionally, molecular studies of serial fermentation have involved genome mutation investigations rather than investigations of transcription, translation, or epigenetic factors. Although minor fluctuations in expression have been found via microarray analysis for genes involved in flocculation, protein kinase production, and membrane integrity, no clear molecular indicators of fermentation efficiency have been determined (2). One previous study investigated repetitive genomic sequences, called delta regions, via Random Amplified Polymorphic DNA PCR (RAPD-PCR) (4). These regions are thought to be indicators of genetic drift because of their high mutation rates. RAPD-PCR of these regions showed that serial fermentation did not significantly impact the stability of genomic DNA as no change in the gel electrophoresis patterns were seen (4). Additionally, restriction fragment length polymorphism (RFLP) fingerprints of Ty elements (transposable elements in yeast that can cause chromosomal rearrangements or deletions) did not show significant changes (4). Due to their highly mutable nature, Ty elements are also good indicators of genomic instability. Although these techniques showed stability in the genomes of two brewer's yeast strains, these results do not apply to all strains. Additionally, it is important to note that gene expression could vary from strain to strain (4). Expression of *FLO* genes, responsible for flocculation, have been

found to both increase and decrease throughout serial fermentation (28). This variability has been strain-specific as well.

The physiological stresses caused by brewing may encourage genetic drift of *Saccharomyces* species. In other words, successive pitches may select for viable, more metabolically competent mutants, while weeding out wild types that are unable to ferment under high stress (4). As a result of this, some of the loci containing fermentation genes can be inactivated (28). This observation likely explains the inconsistencies found when one attempts to study fermentation-related gene expression.

Telomeres

Telomeres are sequences of repeated, noncoding nucleotides that serve as a protective barrier to combat chromosomal degradation. Although this is important for overall chromosomal integrity, it is critical for telomere-associated gene function. Telomerase is an enzyme that is responsible for telomere extension. As a cell ages, the telomeres may become shorter due to reduced telomerase activity, leading to apoptosis. For this reason, rapidly dividing cells experience the most telomere-related issues (29).

Although telomerase plays a critical role in telomere maintenance, it is also important to discuss the telomere position effect (TPE). The telomere position effect was first described in *S. cerevisiae.* TPE is defined as the regulatory effect of heterochromatin spreading into genes. Thus, the closer a gene is to the telomere, the more likely it is to be silenced through this effect (30). Multigene families, such as *MAL,* are often found near telomeres (31).

Telomere integrity as a function of serial fermentation has not previously been studied. In this investigation, the goal was to determine whether a change in fermentation efficiency specifically related to telomere integrity. We hypothesized that telomere length would have an inverse relationship with pitch number, which would decrease telomere-associated *MAL* expression by degradation of chromosomal ends.

This thesis investigates whether maltase expression and telomere integrity are affected by serial fermentation. We hypothesized that maltase expression and telomere integrity would decrease with increased pitch number. If observed, we proposed that decreased maltase expression contributes to decreased fermentation efficiency in viable cells by hindering maltase catabolism. Furthermore, a decline in telomere integrity would suggest that overall cell health and telomere-adjacent genes, such as *MAL*, are compromised.

Materials and Methods

Brewing

For each pitch, three replicate fermentations were performed. *Saccharomyces cerevisiae* (WLP002) was used to ferment. Brewer's wort was created by the addition of 100.8 grams of sorghum malt extract syrup (Northern Brewer) to 700 mL of autoclaved, distilled water. The syrup and water mixture was heated to 80^{o}C for fifteen minutes. After fifteen minutes, the equivalent of 5 hops pellets (Citra hops pellets, Home Brew Supply) were added to the mixture. The wort mixture was held at 80^{o}C for an additional fifteen minutes. Next, the solution was cooled to 2 ^{o}C. Each of three 250 mL Erlenmeyer flasks were inoculated with 200 mL wort broth and $5x10^{6}$ viable yeast/mL. The base of a sterilized airlock was fitted into a cork. An airlock and cork were placed tightly into each Erlenmeyer flask to prevent oxygen entry throughout the fermentations. The remainder of the wort was tested with Benedict's reagent to estimate reducing sugar concentration prior to fermentation. Pitches were allowed to ferment for ten days at room temperature. Minimum and maximum temperature of the laboratory throughout the fermentation was recorded with a minimum/maximum thermometer (Appendix Figure 1). This process was repeated for ten pitches. Equipment was sterilized with StarSan (Five Star) in between pitches and flasks were autoclaved. All three replicates remained independent; yeast from one replicate was always inoculated into one corresponding sequential replicate. After each pitch, RNA and DNA were extracted, alcohol by volume was measured with a hydrometer, reducing sugar concentration was estimated with Benedict's reagent, and cell viability was assessed via flow cytometry.

RNA extraction

RNA was extracted from each replicate pitch with the Norgen Total RNA Purification Kit (Cat. 17200) according to the manufacturer's protocol. Quantification of RNA was obtained with a Thermo Scientific NanoDrop. RNA samples were stored at -80 degrees Celsius until qPCR was performed.

DNA extraction

DNA was extracted from each replicate using lithium acetate according to the protocol by Looke et al. (32). For cleanup purposes, isolated DNA was washed twice with 70% ethanol and resuspended in TE buffer. Quantification of DNA was obtained with a Thermo Scientific NanoDrop.

Estimation of Reducing Sugar Concentration

Before and after each fermentation, the concentration of reducing sugars in solution was estimated with Benedict's reagent. One milliliter of wort (pre-fermentation) or beer (post-fermentation) were boiled with 2 mL of Benedict's reagent for five minutes. The color was then rated on a scale from 1-4.

Table 1. Estimated reducing sugar concentration through approximate color rating**.**

Color	Numeric Rating	Sugar Concentration
Blue	1	0%
Green	2	0.5-1%
Orange	3	1-1.5%
Red	4	2% or more

Flow Cytometry

After each pitch, 0.5 mL of yeast was pelleted and washed twice with phosphate-buffered saline (PBS). Then, cells were stained with ViaCount™ (MilliporeSigma). Viability and cell count were analyzed with a flow cytometer (MilliporeSigma). The number of live, dead, and apoptotic cells was obtained. The equation $C_1V_{1=}C_2V_2$ was utilized to ensure that $5x10^6$ viable cells/mL were inoculated into each pitch.

cDNA synthesis

Total RNA was converted to cDNA with GoScript™ reverse transcriptase (Promega) according to the manufacturer's protocol. Each reverse transcriptase reaction received a different input concentration of total RNA. To ensure consistency, each cDNA sample was then standardized to a final concentration of 25 ng/µL. RT-qPCR was performed with 5 ng of cDNA.

Quantitative Real Time PCR (RT-qPCR)

A Step One Plus real-time PCR machine (Applied Biosystems) was used to measure *MAL* mRNA expression throughout serial fermentation. A custom TaqMan probe (ThermoFisher; Table 2) was designed for *MAL* with the Custom TaqMan Assay Design Tool. Relative amounts of RNA expression were calculated through the utilization of *ELF1* expression as an internal control. *ELF1* encodes elongation factor 1, a transcription elongation factor involved in the maintenance of chromatin structure in actively transcribed DNA (33). Expression of *ELF1* mRNA did not fluctuate throughout

serial repitching, which indicated that *ELF1* provided a valid standard to normalize *MAL1* mRNA expression. RNA was quantified using a standard curve with a 4-fold dilution scheme (18 ng/uL, 4.5 ng/uL, 1.125 ng/uL, 0.3 ng/uL). Two technical replicates were performed for each sample. Nuclease-free water was used as a negative control. The thermocycler (Applied Biosystems™ QuantStudio 5) followed the manufacturer's protocol for TaqMan probes. After an initial denaturation step at 95°C for 10 minutes, each cycle included a 15 second hold at 95°C and annealing/extension for one minute at 60°C. cDNAs were amplified through 45 cycles.

Table 2. Quantitative-PCR probes (Applied Biosystems)

Gene	Primer-Probe Reference Number
MAL	APDJ3JA
ELF1	Sc04141607_s1

Telomere Restriction Fragment Analysis (TRF)

A digoxigenin-labeled (DIG) probe was created via PCR for detection of the *MAL* loci. In the PCR reaction, dCTP, dATP, and dGTP were added at a concentration of 200 uM. In order to incorporate DIG-labelled dUTP, 133 uM of dTTP and 67 uM of DIG-labelled dUTP were added to the PCR reaction. Custom primers (Integrated DNA Technologies; Table 3) were designed for the *MAL* loci and the PCR product was sequenced (Genewiz) to ensure specificity.

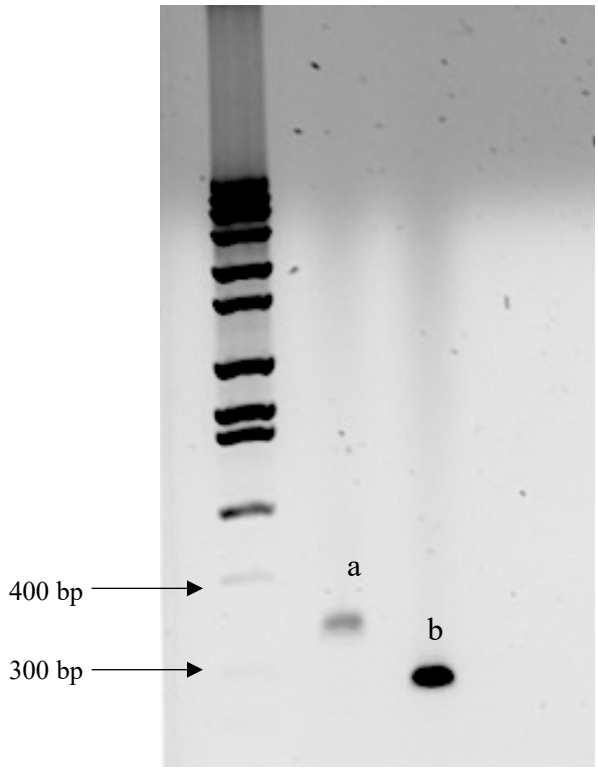


Figure 1. Successful incorporation of DIG-labelled dUTP into the PCR product. Band "a" contains the DIG-labelled dUTP PCR product. Band "b" contains the control PCR product in which dUTP was not used in the reaction. The further migration of band "b" in the 1% agarose gel to about 300 bp indicates that dUTP was successfully integrated into PCR product "a" due to its larger size, caused by incorporation of digoxigenin-dUTP.

Table 3. Maltase probe sequences.

Primer	Sequence
MAL-Forward	5'-GGT GGT TCA GCT TGG ACT TT-3'
MAL-Reverse	5'-GTA CCA ACT TCA ACG TGC GT-3'

Genomic DNA samples were digested with three restriction enzymes (New England BioLabs): BsaB1 (catalog R0537S), BamH1-HF (R3136S), and Nhe1-HF (R3131S). These enzymes digested upstream genomic DNA, but did not digest DNA

from the *MAL* locus through the end of the telomere. After the restriction digest, DNA was run on a 0.9% agarose gel. The gel was then denatured, neutralized, washed, and transferred onto a nylon membrane. Next, DNA was crosslinked to the nylon membrane with a UV-Crosslinker (Daigger Scientific) at 120 millijoules/cm^2. Next, the nylon membrane was treated with hybridization solution for one hour at 43^oC in order to prehybridize the membrane. After prehybridization, the solution was discarded and replaced with fresh hybridization solution. The DIG-labelled DNA probe was added to the hybridization solution (40 uL probe, 20 mL hybridization solution). The membrane was incubated for 18 hours in the hybridization mixture at 43^oC. Following hybridization, the membrane was rinsed with 6X and 1X stringency buffers for 15 minutes each. After stringency washes, the membrane was washed in washing buffer for 5 minutes, blocking solution for 30 minutes, antibody solution for 30 minutes, washing buffer for another 30 minutes, then detection buffer for 5 minutes. Recipes for all solutions used are found in Appendix Table 1. Anti-digoxigenin-AP Fab fragments (Enzo) were used as the antibody to detect the digoxigenin-labelled probe. The membrane was incubated with 1.5 mL of CSPD™ (Invitrogen™) for 10 minutes at 37^oC to detect the antibody. Lastly, detection of the probe was performed with the Odyssey® Fc Imaging System.

Data Analysis

A one-way ANOVA was test performed on qPCR data in order to determine if *MAL* expression was impacted by serial fermentation. A one-way ANOVA was performed on cell viability, apoptosis, and death to determine if cell viability decreased throughout serial repitching. The Shapiro-Wilk and Kolmogorov-Smirnov tests were performed to test normality of all statistically analyzed data. Levene's Test was performed to evaluate homogeneity of variances between pitches for apoptosis and *MAL* expression data. The Brown and Forsythe ANOVA was performed to evaluate variations between replicates for apoptosis and *MAL* expression.

Results

Evaluation of beer quality throughout serial fermentation

Quality of the beer product was estimated by reducing sugar concentration and alcohol by volume (ABV). Alcohol concentration gradually increased throughout serial repitching until pitch 4 and 5. After this halfway point, alcohol production gradually decreased throughout the serial fermentation process. In the first several pitches, it was evident that yeast rapidly metabolized the reducing sugars present in the brewer's wort. After the first half of serial fermentations, the presence of residual reducing sugars increased.

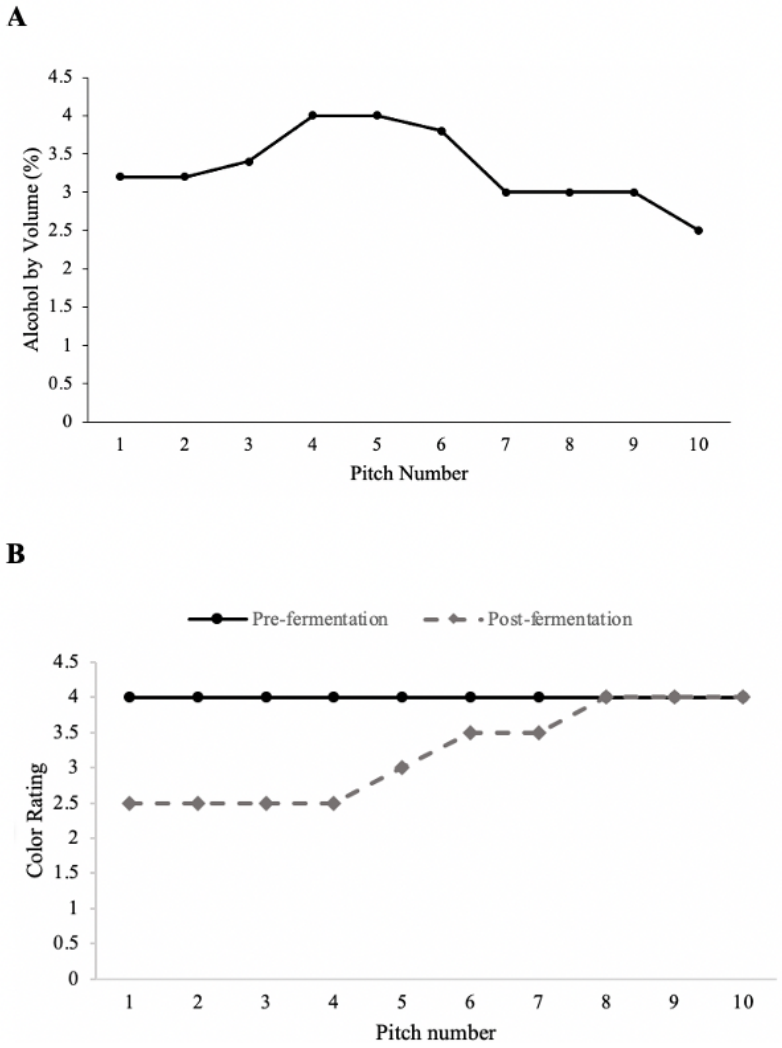


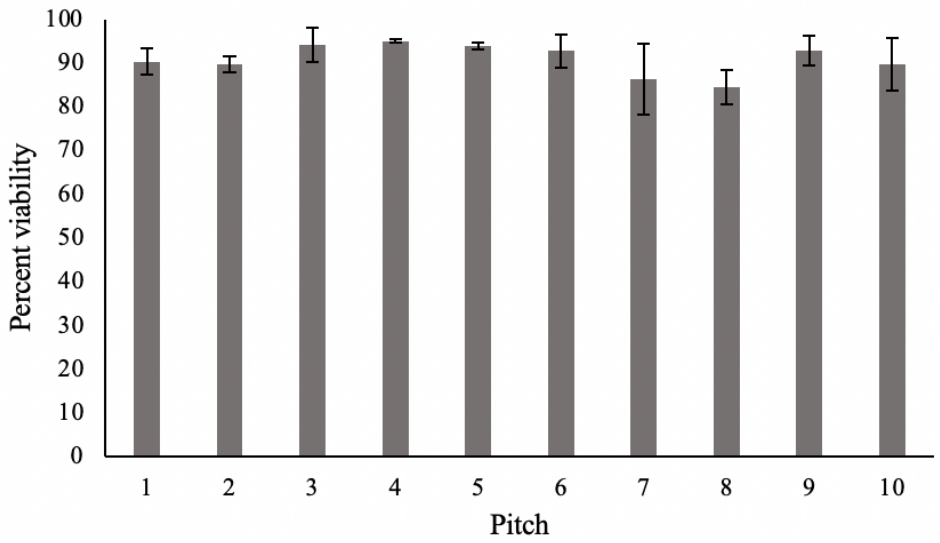
Figure 2. Alcohol by volume and reducing sugar measurements throughout serial repitching. (A) Alcohol by volume gradually increased from 3.2% to 4% between pitches 1 and 4. Alcohol concentration remained steady around 4% until pitch 7. After pitch 7, alcohol production decreased to 3% until pitch 10, which yielded 2.5% the lowest alcohol concentration of 2.5%. **(B)** Each batch of wort contained the highest detectable concentration of reducing sugars, which was evident by a bright red color of Benedict's reagent. Yeast steadily utilized a large amount of reducing sugars through pitch 4. This was exhibited by a dull orange color of the Benedict's reagent. After pitch 5, a slightly higher amount of reducing sugar remained in the beer product, as indicated by a red-orange color. From pitch 6 to pitch 10, reducing sugar concentration remained high after fermentation.

Effect of serial fermentation on cell viability

Cell counts were obtained via flow cytometry after each pitch. No significant decrease in viability throughout serial repitching was found. Additionally, no significant increase in apoptotic or dead cells throughout serial repitching was found. Viable cell counts obtained via flow cytometry enabled us to ensure that a consistent concentration of viable cells ($5x10^6$ cells/mL) were inoculated into each pitch. These observations indicate that cell viability, apoptosis, and cell death are not responsible for the decline in fermentation efficiency. Additionally, no significant decrease in overall cell count was found. Although no overall statistical difference in cell apoptosis was found, it is important to note the visual increase in apoptosis between pitches 7 and 8 (shown in Figure 2B).

The Shapiro-Wilk and Kolmogorov-Smirnov tests confirmed that the apoptosis data followed a Gaussian distribution. Levene's test for equal variances revealed that the apoptosis data did not exhibit equal variances between pitch replicates. For this reason, the Brown and Forsythe ANOVA was chosen to investigate whether there were significant differences in apoptosis between each pitch replicate. The Brown and Forsythe ANOVA did not show any significant differences between replicates.

A



B

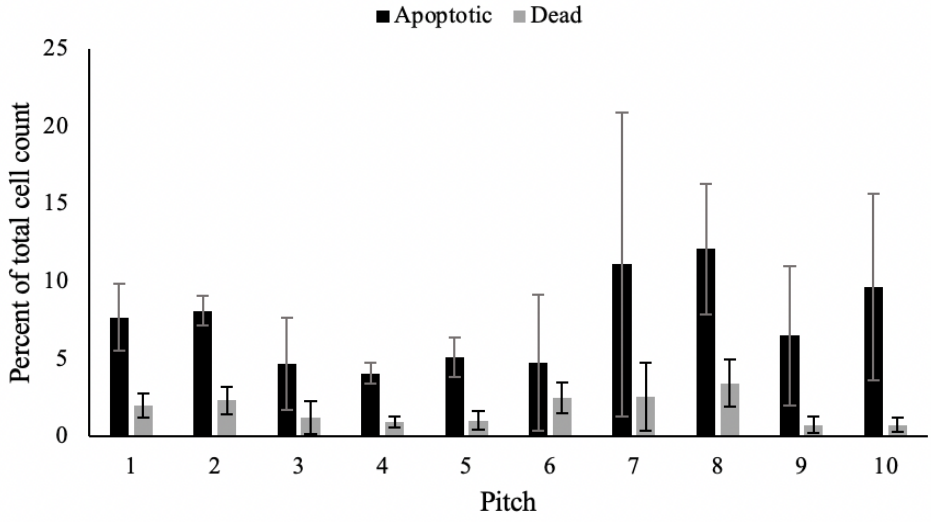


Figure 3. Analysis of cell viablility, apoptosis, and death via flow cytometry. Although cell viability fluctuated throughout serial fermentation, there was no significant decline in cell viability ($F_{(9.20)}$=1.976, p=0.098). Additionally, no significant increase in apoptotic ($F_{(9.20)}$=1.096 p=0.319) or dead cells ($F_{(9,20)}$=2.289, p=0.059) was found.

Quantification of MAL mRNA throughout serial fermentation

A one-way ANOVA test was performed for relative *MAL* mRNA expression as a function of pitch number. Expression of *MAL* mRNA measured via qPCR did not significantly change throughout serial fermentation ($F_{(9,18)}$=2.266, p=0.067). This observation indicates that *MAL* expression is not directly responsible for the evident decline in fermentation efficiency throughout serial repitching.

The Shapiro-Wilk and Kolmogorov-Smirnov tests confirmed that *MAL* mRNA expression data followed a Gaussian distribution. Levene's test for equal variances revealed that the data did not exhibit equal variances between pitch replicates. For this reason, the Brown and Forsythe ANOVA was chosen to investigate whether there were significant differences in *MAL* mRNA expression between each pitch replicate. The Brown and Forsythe ANOVA did not show any significant differences between replicates.

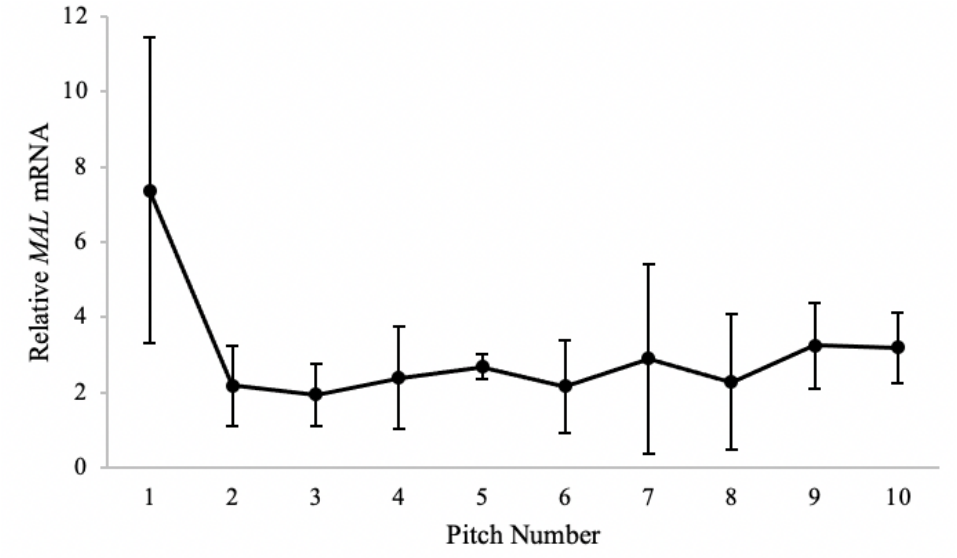


Figure 4. *MAL* mRNA expression throughout serial fermentation. There was no significant change in maltase expression ($F_{(9,18)}$=2.266, p=0.067) throughout ten serial repitches. *MAL* expression was maintained at low, steady level after Pitch 1. The steep decline observed after Pitch 1 is likely due to acclimation to the stressful, anaerobic conditions that serial fermentation provides. Expression of *MAL* fluctuated between pitch replicates, which resulted in large standard deviations. Replicates exhibited the most uniform *MAL* expression during Pitch 5.

Effect of serial fermentation on telomere integrity

The telomere restriction fragment analysis shows five distinct smear distributions (Figure 5). Pitch 1 (lane 1) and pitch 3 (lane 2) exhibit their smears distributed towards the top of the gel. Pitch 5 (lane 3) shows a migration of the smear towards the bottom of the gel, which suggests shorter fragments when compared to pitch 1 and 3. In pitch 8 (lane 4), the smear is distributed towards the middle of the gel. Lastly, in pitch 10 (lane 5), the smear is shown to be the most heterogenous in length and is spread vertically across the lane with no uniform distribution. Based on the smear pattern of these terminal

DNA fragments, which span from *MAL* to the end of the telomere, it is concluded that telomeres are the most uniformly distributed prior to pitch 5. Additionally, the telomeres are longer in pitch 1 and 3. In pitch 5, a shortening of telomeres can be seen, but the size distribution appears to remain distinct among the sample. A mild recovery in telomere length and homogeneity can be seen in pitch 8. Once pitch 10 was reached, telomeres are the most heterogenous in length. The most homogenous telomere length distributions were seen in pitch 1, 3, and 8. Overall, when compared to pitches 1 and 3, telomeres shortened as serial fermentation progressed.

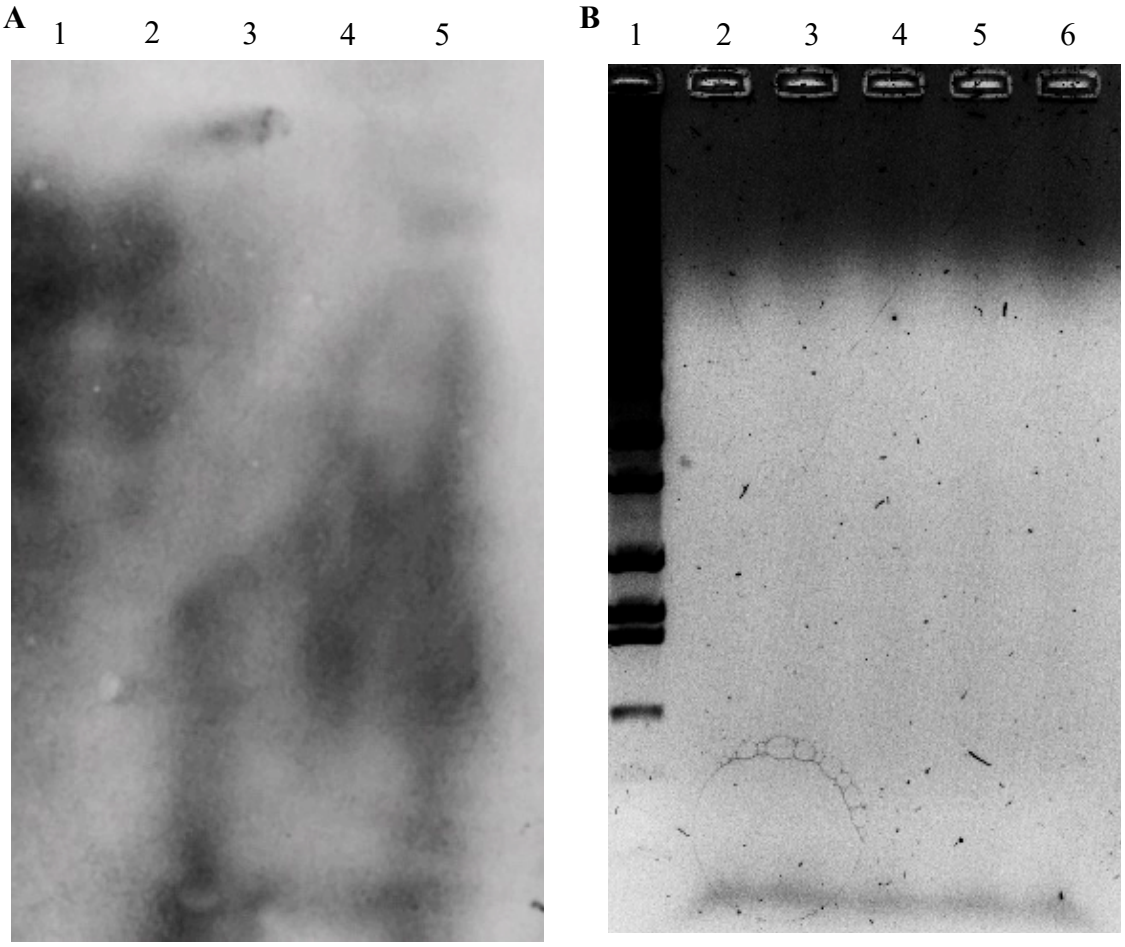


Figure 5. Impact of Serial Fermentation on Telomere Length. Panel A depicts lanes 1, 2, 3, 4, and 5 that represent telomeric DNA samples from pitch 1, 3, 5, 8, and 10, respectively. The black smears in the lanes in Panel A represent the telomeric DNA fragments. The distribution of the DNA smears indicate an overall shortening of telomeres throughout serial fermentation. Furthermore, the size distribution becomes more heterogeneous throughout serial fermentation. Telomere length is the most heterogenous in pitch 10. Panel B depicts the agarose gel image after the genomic DNA restriction enzyme digestion. The lack of distinct bands and the smears present in lanes 2-6 indicates that the genomic DNA was successfully digested.

Discussion

This study was conducted to investigate the decline in fermentation efficiency that is exhibited throughout serial fermentation. Previous studies have investigated morphological indicators of cell health; such as viability, bud scars, colony morphology, cell shape, and membrane potential (4–6, 34). Although morphological changes throughout serial repitching have been observed, these changes are not indicative of fermentation efficiency. Previous data regarding genetic impacts of serial fermentation are inconclusive. For this reason, the molecular basis of fermentation efficiency must be understood. It is important to note that the present study utilized sorghum malt syrup extract, which comes from white sorghum grain rather than barley or wheat. Grain type can influence fermentation products, such as alcohol production and sugar utilization (35). This is because each grain type has a unique chemical composition, such as amylase enzymes that hydrolyze starch to fermentable sugars. For this reason, the conclusions of this study may not be applied to other grains.

Beer Quality

To mimic microbrewing practices, ten serial repitches were performed in this study. It was important to achieve a clear decline in fermentation efficiency prior to drawing conclusions about *MAL* expression and telomere integrity. There have been inconsistencies regarding cell counts and viability in previous studies. Studies have found an increase, decrease, and no change in cell counts (21, 22, 24, 36). Our flow cytometry data (Figure 2) further solidified previous observations that cell viability is not an

adequate predictor of fermentation performance (8, 21). No significant change in cell viability was found in the present study. Further, no significant change in overall cell count was found. This supports a previous observation that there were no consistent cell viability trends between breweries (37). Although a consistent number ($5x10^6$ cells/mL) of viable *S. cerevisiae* were inoculated into each pitch and viability did not significantly decrease throughout 10 pitches, alcohol production declined after Pitch 6 (Figure 1). Furthermore, residual reducing sugar levels continued to rise throughout serial repitching (Figure 1). These data indicate that viable yeast were not efficiently fermenting the available sugars, which reiterates the previous observations that cell viability is not an accurate predictor of fermentation efficiency. Although cell viability, reducing sugar concentration, and ABV were measured for beer quality purposes, these measurements confirmed the previously discussed downfalls of unpredictable fermentation efficiency, which include product loss and reduced profit.

Maltase Expression

We aimed to determine whether the expression of maltase, one of the most critical enzymes in beer production, is negatively impacted by serial fermentation. This observation may explain the decrease in fermentation efficiency, as seen by brewers through a decrease in alcohol production and an increase in undesirable compounds. Our results indicated that *Saccharomyces cerevisiae* maintained a low and steady expression of *MAL*. This result suggests that maltase expression is not the reason for the decrease in fermentation efficiency that is observed throughout serial fermentation. However, the

presence of reducing sugars in the beer product increased as pitch number increased (Figure 1B). This observation suggests that enzyme structure, other metabolic enzymes, or epigenetic factors may be responsible for metabolic decline in *S. cerevisiae* throughout serial fermentation. There may be a discrepancy between transcription of fermentation-related genes and translation or enzymatic function.

Telomere Integrity

Telomere integrity is essential to chromosomal health. Previous studies have indicated that telomere-associated gene complexes, such as *MAL,* may be impacted by telomere integrity due to telomere shortening and telomere position effect (TPE) (31). This study aimed to determine if telomere length decreased throughout serial fermentation. Our hypothesis that telomeres would shorten throughout serial fermentation was supported by the data obtained via TRF. There was an overall shortening throughout serial repitching when compared to the pitch 1. Although there was a slight recovery in telomere length after pitch 8, these telomeres were shorter than those in pitch 1 and 3. It was observed that telomeres were longer and more homogenously distributed throughout earlier pitches. Once the cells fermented five pitches, a distinct shift in telomere length distribution was seen. Telomeres shortened after pitch 5. After pitch 8, a slight recovery in telomere length and homogeneity was seen. Prior to pitch 8, cells that experienced the most severe telomere shortening were likely unable to replicate, which would cause the minor recovery in length and homogeneity that was seen. This observation may indicate that the serial fermentation process was selecting for younger, healthier cells.

Although there was no statistically significant increase in apoptosis found via flow cytometry, an upwards trend of apoptosis was seen between pitches 7 and 10. The highest level of apoptosis was seen after pitch 8. These apoptotic cells likely contained the shortest telomeres, which lead to the temporary upward shift of the telomere smear. After the final pitch, there was no uniform distribution of telomere length. The smear spanned the entire lane. As serial fermentation progresses, the cell population becomes more heterogenous in age and health (21). Thus, pitch 10 contained the widest range of cell ages. This led to the most heterogeneous telomere length distribution seen after pitch 10.

Ten pitches may have caused the cells oxidative stress and selective pressure. When telomeres exhibited shortening after pitch 5, the presence of residual reducing sugars increased in the beer product. This indicated that the yeast were unable to utilize the wort sugars as rapidly as was seen in previous pitches. Telomeric shortening may be the direct cause of increased residual reducing sugar concentration by impacting other telomere-associated metabolic genes, such as *HXK*. Telomeric shortening may be also be a general indicator of other molecular impacts, such as telomerase expression.

Future Studies

This study reinforced the demand for research of the molecular mechanisms underlying fermentation efficiency. Previous studies have indicated that *FLO*, *MAL*, and *HXK* are the most likely to have an impact on fermentation efficiency and beer quality (13). Most molecular studies have investigated *FLO*, which is responsible in yeast flocculation. Although *FLO* genes have been shown to display genetic drift and expression variability, flocculation impacts beer quality rather than fermentation efficiency (2, 28). The present study was the first study to investigate expression of *MAL* mRNA throughout serial repitching. Although no significant change in maltase mRNA expression was observed, future studies should focus on *MAL* permease genes. If maltase is expressed steadily while maltose permease is not, then maltose is not able to enter the cell. This would result in the failure to ferment maltose. Furthermore, this might provide an explanation for the increased residual reducing sugar concentration observed in the present study as serial fermentation proceeded. All future studies should include a control culture that does not impose the fermentation environment. This control environment would allow for aerobic respiration in nutrient-rich media, such as wort broth. Viability and the variables of interest should be tested in these control cultures to determine if the results seen from serial fermentation are also seen as a result subsequent culturing under non-stressful conditions.

Hexokinase (*HXK*), responsible for the first step in glycolysis in which glucose is converted to glucose-6-phosphate, should also be investigated. As indicated by previous studies, *HXK* is another a critical fermentation-related gene that may be affected by its

position near the telomere (13). Hexokinase is also required for catabolite repression, which is important for efficient catabolism of available sugars (10). For these reasons, hexokinase mRNA expression and enzymatic function throughout serial fermentation should be evaluated.

Additionally, more than three replicate pitches should be inoculated in future studies due to the fluctuation in *MAL* expression and cell apoptosis that was seen between the replicates. An increase in replicates would help provide more uniform data as microorganisms' metabolic activity and cell health frequently vary depending on each culture.

Because telomere length appeared to shorten throughout serial fermentation, further research is needed to evaluate the expression of *TLC1*, which encodes telomerase, via qPCR. Furthermore, the integrity of this enzyme should be evaluated through enzymatic assays. Additionally, Rap1 protein concentration should be investigated throughout serial repitching. Rap1 is a DNA binding protein that is responsible for binding telomeric DNA (38). Thus, the longer a telomere is, the more Rap1 is bound. Rif1 and Rif2 proteins then bind to Rap1. The Rif proteins prevent extension of the telomeres, although the mechanism of this downregulation is still unclear (38). It is hypothesized that a telomere that needs extension will have a low concentration of Rap1 proteins bound to the telomere, which results in a low concentration of the Rif proteins. This results in the extension of the telomere (38). Given this information, Rap1, Rif1, and Rif2 expression should be assessed throughout serial fermentation. This could be performed via DNA electrophoretic mobility shift assay (EMSA).

Once the molecular basis of fermentation efficiency is understood, *Saccharomyces* strains specifically designed for serial fermentation can be genetically engineered. It is important that any significant findings are also evaluated with various grain sources, such as barley and wheat. These specially designed brewer's yeast strains are the most practical application of serial fermentation research. This is because laboratory equipment, such as flow cytometers and thermocyclers, are found in research laboratories and are not commonly at a brewer's disposal. Therefore, molecular indicators of fermentation efficiency are most useful for researchers, not brewers. Genetically engineered strains as a result of molecular research in serial fermentation will allow for more predictable brewing, which will prevent product and resource loss for microbreweries.

Although genetically engineered brewer's yeast strains would directly combat the presented issues exhibited throughout serial fermentation, other assays to assess yeast vitality may be designed. A dipstick test may also be designed to indirectly assess yeast apoptosis. This type of test may estimate leakage of intracellular components into the beer, such amino acids and fatty acids. Morphological and molecular impacts of serial fermentation must first be elucidated prior to the development of such tests.

Appendix

Table A1. TRF reagent recipes. The following reagents required for southern blotting were prepared according to the listed formulas.

Reagent	Components
Denaturing Solution	1.5 M NaCl, 0.5 M NaOH, pH 13
Neutralizing Solution	1.5 M NaCl, 1 M TRIS-HCl, pH 7.5
Prehybridization/Hybridization solution	5X SSC, 1% blocking reagent, 0.1% sarcosyl, 0.02% SDS
Washing Buffer	0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.5
Detection Buffer	0.1 M Tris-base, 0.1 M NaCl, pH 9.5
Maleic Acid Buffer	0.1 M maleic acid, 0.15 M NaCl, pH 7.5
Blocking solution	1% Blocking reagent (Enzo), maleic acid buffer
Antibody solution	1:10,000 dilution in blocking solution

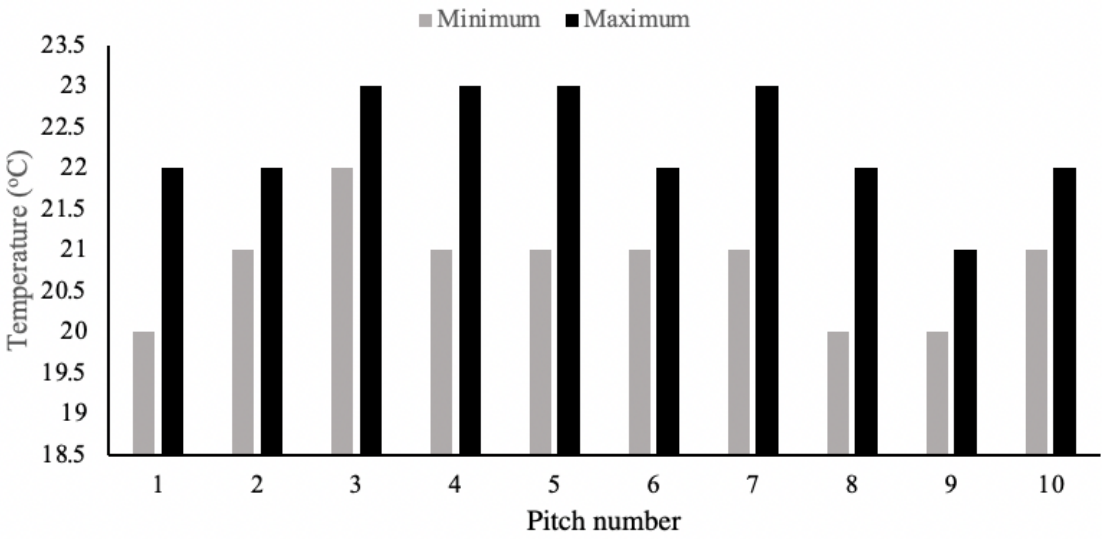


Figure A1. Temperature recordings throughout serial fermentation. No significant fluctuations in room temperature were observed.

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