COLLEGE OF HEALTH & HUMAN SCIENCES
COLORADO STATE UNIVERSITY

Detection of *Brettanomyces* vs *Saccharomyces* with Oculyze BB 2.0

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KATHINKA LABS
brewers, lab rats & consultants

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ABSTRACT

Brettanomyces use in beverages has increased drastically over recent years[1]. Due to this it is important for brewers and vintners to have control over variables like pitch rates to introduce the optimal concentration of cells into mixed or pure culture fermentations. In order to test if the Oculyze BB 2.0 and its *Brettanomyces* Beta image recognition software is capable of counting *Brettanomyces* cells, three pure *B. bruxellensis* cultures were propagated to pitchable quantities using different nutrient conditions. The propagation that had the greatest growth was then pitched into a mixed fermentation with a *Saccharomyces* yeast. The cell count and density data showed that Oculyze had the ability to count *B. bruxellensis* yeast cells but with limited accuracy due to their ability to form pseudohyphae[2] making it difficult to achieve accurate cell counts. With further development toward counting accuracy and viability of *Brettanomyces* cells, this technology could be a useful alternative to more expensive and time consuming yeast counting techniques in order for breweries to accurately control *Brettanomyces* fermentations.

INTRODUCTION

While *Brettanomyces* spp. are often considered spoilage organisms in beer and wine, the sensory attributes they provide are desired in some beer styles[3] like Lambic and Saison[4]. With the increased use of *Brettanomyces* yeast in beer, accurate cell counting throughout all stages of propagation and fermentation are vital to healthy and repeatable fermentations[5]. In order to increase the accuracy and speed of cell counting, automatic cell counting methods have been developed. Oculyze BB 2.0 is a portable microscope and image recognition software combination designed to rapidly detect and enumerate yeast cell concentration accurately[6].

OBJECTIVES

This experiment sought to determine if the Oculyze BB 2.0 system (Figure 1.) is capable of counting *B. bruxellensis* cells in pure and mixed culture fermentations. Different yeast nutrients were used in the propagations to establish which propagations' conditions were best for increasing cell counts in order to grow pitchable quantities more quickly.

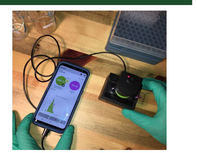


Figure 1. Oculyze BB 2.0

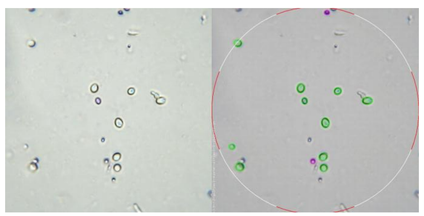


Figure 2. Oculyze *B. bruxelensis* detection in mixed fermentation A. Left side is raw image, right side depicts counted cells in green.

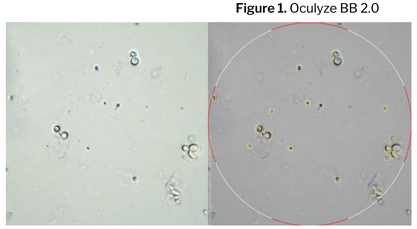
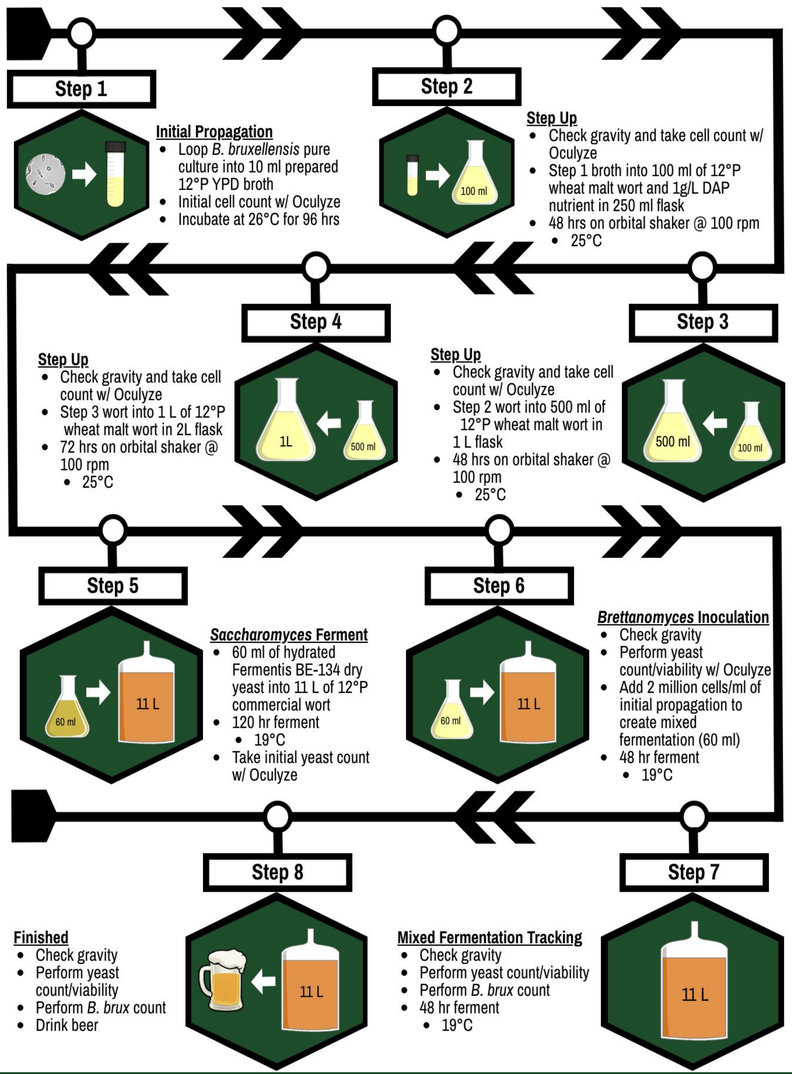


Figure 3. Oculyze *S. cerevisea* detection in mixed fermentation A. Left side is raw image, right side depicts cells counted in green, dead cells in violet.

METHODS

An initial 10 mL of YPD broth was inoculated with Kathinka KAT-W4001 *B. bruxellensis* and stepped up with 12°Plato wheat malt wort every 48 hours to a final volume of 1L over a five day period. A sample was taken every 48 hours for gravity readings using an Anton Paar DMA 35 and cell counts using the Oculyze BB 2.0 and Samsung Galaxy S8 (Figure 1). The propagation was then used in a mixed fermentation with Fermentis BE-134 yeast in 11L of diluted New Belgium Fat Tire wort to a gravity of 12°Plato.

Figure 4. *B. bruxellensis* - From Propagation to Mixed Fermentation



RESULTS

All three propagations showed long attenuation times with the majority of cell growth occurring between four and seven days (Figure 5). While previous research suggested some *Brettanomyces* species showed greater attenuation when grown in acidified media of 1000 ppm lactic acid[5], our trial did not show similar results. The acidified propagation performed considerably worse than propagations 1 and 2 which were grown with yeast nutrient. DAP as a yeast nutrient performed better than Springferm with Prop 1 achieving twice the cells/ml compared with Prop 2. The mixed fermentations performed comparably with some variation in cell counts between six and ten days with Mixed Ferm B showing an increase in Fermentis BE-134 cell counts despite previous decline and increase in *Brettanomyces* cells (Figure 6). The Oculyze system did show some difficulty distinguishing individual *Brettanomyces* cells due to their propensity to form pseudohyphae chains[2] (Figure 2), however it was able to distinguish between *Brett.* and *Sacc.* strains of yeast. (Figures 2 and 3)

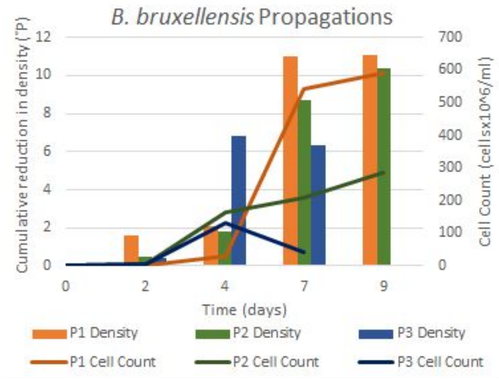


Figure 5. Cell growth and cumulative reduction in density over time in days. P1 was propagation cultured with DAP, P2 was propagated with springferm and P3 was propagated with 1000 ppm lactic acid.

APPLICATION

More brewers and vintners are utilizing *Brettanomyces* spp. yeast for the complex flavors that they impart in beer[3]. Accurate cell counts are a vital way to recreate sensory attributes that yeast impart and the Oculyze technology allows for automated cell counting with less variability. More accurate and robust cell enumeration techniques could result in greater control of desired sensory attributes in production facilities.

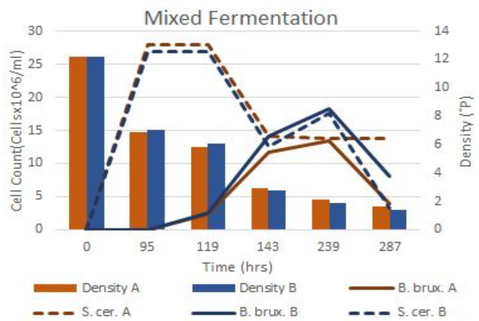


Figure 6. Cell growth of *S. cerevisiae* and *B. bruxellensis* and total density over time in hours for mixed fermentation trials A and B.

CONCLUSION

Oculyze is capable of counting *Brettanomyces* yeast cells and distinguishing between *Saccharomyces* and *Brettanomyces* strains of yeast due to morphological differences. Areas of further research that could improve the technology are: investigating sample preparation techniques and fine tuning recognition software to improve the accuracy and precision of Oculyze and developing techniques for determining viability such as fluorescent staining methods.

ACKNOWLEDGEMENTS

We would like to first and foremost thank Floris Delee and Lauren Sandell from Kathinka Labs for creating and financially supporting this research project . Their time, mentorship and guidance made this project a reality and was paramount in its completion. We would also like to thank the New Belgium Brewing Company for supplying the wort for our experiments as well as a special thanks to Oculyze (https://www.oculyze.de/en/) and Fermentis (https://fermentis.com/en/).

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Liberation of Glycosidically-Bound Aromatics in Hops by "Rapidase Revelation"

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A special thanks:
To our wonderful mentors Stacey Williams and Justin Alexander. Both of whom guided us, provided us with knowledge, and never failed to be enthusiastic and kind. To New Belgium Brewery for use of their resources and beautiful quality control lab.

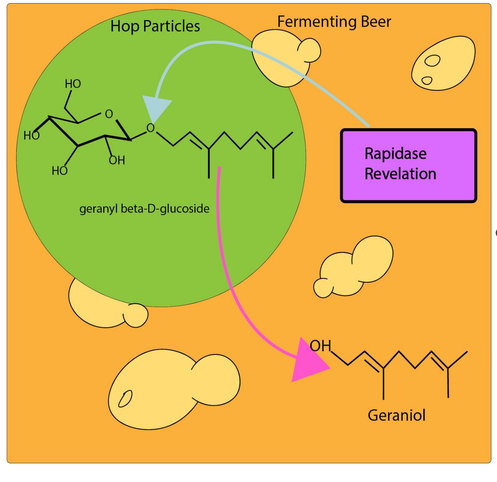
INTRODUCTION

Increasing the efficiency of hop flavor compound extraction is of great interest to the brewing industry. Hops contain both free and glycosidically bound monoterpene alcohols. These compounds are more soluble than other terpenes (i.e. Myrcene). Two commonly desired monoterpenes are geraniol and linalool, known to contribute to floral and citrus sensory characteristics in beer. **This experiment tests the use of the α & β glycosidase 'Rapidase Revelation', a common enzyme used in winemaking, added along with hops during active fermentation using ale yeast in hopes to release bound monoterpenes from hops and achieve an increase in desired aroma attributes from less hops.**

METHODOLOGY

To test the efficacy of this enzyme for the purpose of liberating glycosidically bound monoterpenes:

- Three small scale (5L) fermentations were started using freshly knocked out Fat Tire wort from the New Belgium Brewhouse. These fermentations were pitched with yeast at 1 million cells/mL/°P at 95% viability.
- Rapidase Revelation is deactivated in the presence of glucose, and inhibited in the presence of fructose and sucrose, to account for this, fermentations were not dosed until they had reached a gravity of below 6°P. At this gravity enzyme inhibiting sugars would mostly be consumed by the yeast.

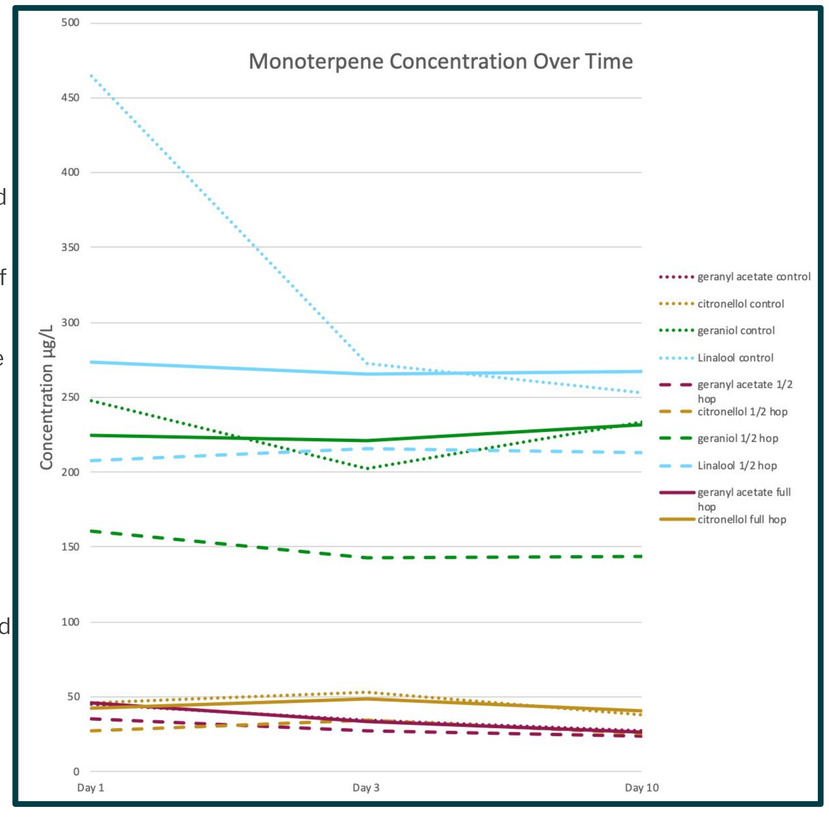


- The fermentations were dosed with amarillo hops (chosen for their high concentration of bound geraniol) in quantities of 25g to the control and full hop test, and 12.5g to a half-hop test, to compare monoterpene alcohol concentrations.
- Dosage for the enzyme was based off of instructions for industrial winemaking calling for 3g/hL at 10X dilution. Scaled down to the 5L size dosage was .15g at 10X dilution. Out of the three fermentations, two were dosed with enzyme at that rate, and all were dry hopped.
- After the addition of hops and enzymes, the fermentations were periodically sampled, centrifuged, and assayed using Gas Chromatography-Mass Spectroscopy (GCMS) to analyze the concentrations of monoterpenes.

ANALYSIS OF DATA

Physical Properties: Carbohydrates, pH, EA, and ABV	Chemical Properties: Hop Aromas and Yeast Volatiles
• Waters Acquity H-Class UPLC with Refractive Index (RI) Detector	• Agilent GC 7890A equipped with an olfactory port
• Carbohydrates: Waters UPLC BEH Amide 1.7µm 2.1 x 50mm column	• 2 Inlets: DB-5 and Wax GC columns
• Anton Paar DMA 5000M Densitometer	• Gerstel Autosampler with Headspace and SPME
• Anton Paar Alcolyzer Beer ME	• Detector: Agilent 5975C Single Quad Mass Spec
• Anton Paar Xsample 122 Autosampler	• SPME Fiber: 3 Phase Fiber (DVB/CAR/PDMS)

DATA



RESULTS

The results were deemed inconclusive. There was a release of monoterpenoids with Rapidase Revelation added, however there was no significant difference between our control and enzyme-added samples. The Null hypothesis was conclusive: The addition of Rapidase Revelation in this method will have no significant effect on the liberation of geraniol and linalool from hops, resulting in no increase of overall floral and citrus sensory characteristics of beer.

FUTURE CONSIDERATIONS

After considering the data various potential causes for error have been determined, and present opportunities to modify and improve the experimental design of this test.

- Results could be the sugar inhibitory effect of Rapidase Revelation. This enzyme was designed for winemaking and is not expected to interact with maltose or maltotriose. Potentially the various sugars present in fermenting beer at 6°P could have an impact on the efficacy of the enzyme.
- Usage of the enzyme could be experimented with. Rather than adding it to fermenting beer, it could be added post crash. Alternatively the hops could be experimentally treated with the enzyme before being dosed to the fermenter.
- Dosage amount, given that this enzyme is designed for wine, there may just be certain parameters for its usage which would not be the same in a beer. Perhaps a series of dose response experiments to determine the quantity of enzyme needed for full functionality.
- Additional experiments may be conducted in smaller, benchtop fermenters. The "Fast Ferment 7.9gal Fermenters" used in this experiment may not have been optimal. Furthermore smaller fermentations will likely allow for more sample collection. The sample size of 1 for each test was insufficient.

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Difference Testing on Hot Steep Malt Extraction

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Special thanks to Root Shoot Malting of Loveland, Colorado for malt donation and mentorship

ROOT SHOOT MALTING

*All data and results are hypothetical

INTRODUCTION

- The hot steep method is a rapid and standardized wort preparation method for the sensory evaluation of extractable malt flavor.
- Triangle test: Method of difference testing involving the simultaneous presentation of three coded samples, two of which are identical.
  1. Which one of the three samples is different?
  2. Which two samples are similar?
- The null hypothesis for the triangle test expresses that (P_t) the long-run probability of making a correct selection with no perceptible difference in the selected samples is one in three.
- Starch is converted into sugar when steeping mash in a process called saccharification. The temperature used during steeping will affect the enzymatic activity of alpha and beta amylases in the malted barley.

Figure 1.

Poirier, 2019

RESEARCH QUESTION

Can untrained sensory panelists distinguish a difference in malt held at different steeping temperatures based on perceived olfactory and gustatory characteristics?

METHODS

- The first step was to extract the flavor from barley malt by performing a hot steep. Setup can be seen in Figure 1.
  - Malt grains were ground into a flour-like powder, added to water at 65℃, and then allowed to steep for 5 minutes to induce equal levels of saccharification.
  - After the initial steep, the mash was divided so that ⅓ of the liquid was kept at 65℃. One was brought to the temperature 69℃, and the remaining ⅓ was brought to 73℃.
  - They were then kept at these temperatures for 15 minutes. The mash was then filtered out to keep the wort and was allowed to cool to room temperature.
- A sensory analysis was then performed on the three worts steeped at different temperatures.
  - The sample size was 25 participants, each performing three triangle tests. The samples were given masking numbers.
  - Samples were randomized and given masking numbers.
  - For uniformity, participants were treated equally. Colored lights were used to mask color differences in the wort, and testing setups were all identical (see Figure 2 below) .
  - Participants performed one triangle test at a time by sampling each of the three samples and noting on a response sheet which sample was different from the other two.

Figure 2.

RESULTS

- Triangle test data was compiled into an excel spreadsheet.
  - The test found that out of twenty-five panelists, nineteen could correctly identify the different sample.
- The data through a program called Sensory data analysis/Sensory discrimination test on excel.
  - A p-value lower than 0.05 was calculated.
  - The null hypothesis can be rejected
- The data interpreted suggested a significant statistical difference in which the tested panelists could identify the difference in samples.

CONCLUSIONS

- Finding a p-value lower than 0.05 concludes the null hypothesis can be rejected and there is a statistical difference between the two samples (A&B).
- Maltsters and researchers can use this data and make important decisions for the process and production of malt and beer as to how temperature can affect the final product.

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ANALYTICAL AND SENSORY COMPARISON OF HOPS AGED FOR 1-3 YEARS IN THE PRODUCTION OF MÉTHODE TRADITIONELLE STYLE BEER

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Introduction

Méthode Traditionelle is a beer style that follows the production methodology of traditional Lambic beers, but is produced outside of Belgium[1]. Méthode Traditionelle beers undergo a spontaneous barrel fermentation after overnight cooling in a traditional open-top coolship. There is a need for more in-depth scientific research into the production methods of Méthode Traditionelle beers as the popularity of sour beers continues to rise in the American craft beer market.

In order to call a beer Méthode Traditionelle, the process must follow a specific list of standards. Included in these standard is the use of 90-100% of hops aged in a warm, non-airtight environment for a minimum of one year[1]. Aged hops are used in Méthode Traditionelle beer to provide flavor, low bittering potential and to provide a means to prevent microbial spoilage from mainly lactic acid bacteria[2,3]. A large quantity of hops must be used to prevent spoilage, which would usually render the beer too bitter[2]. This is why the hops are aged for such an extended period of time. Additionally, over periods of aging and oxidation hop oils such as terpenes will convert to other unique flavor active compounds previously unavailable[4].

Currently Primitive Beer in Longmont, CO ages all of their production hops for at least three years, a traditional practice in Lambic brewing[2]. Being that the standards for Méthode Traditionelle only requires aging of one year, three year aging regimes may not be necessary. If it was found that Primitive could achieve the same desired outcome from shorter aging times, then there could be an opportunity to save time and money during production.

Objective

- Provide a time frame for alpha and beta acid concentration for aged hops
- Determine if increasing hop aging time impacts the sensory perception of chosen hop attributes
- Provide evidence that aging hops for less time could be an opportunity for saving during production for Primitive Beer

Method

To test the hop aging process, we collected 30g hop samples at Primitive from consecutive crop years (2017,2018, and 2019). All samples were of the Willamette variety and grown by the same farmer. Samples were packaged in vacuum sealed bags and stored in the freezer until analysis could be conducted.

All samples underwent both physical and sensory analysis. Physical analysis was performed to measure alpha and beta acid concentration using the ASBC spectrophotometry method for hop analysis. To perform spectrophotometric analysis we extracted 5g of hops with 100mL of toluene, and 5mL of the extract was then diluted to 100mL (dilution A). 3mL of dilution A was then diluted to 50mL with alkaline methanol (dilution B) and spectrophotometer readings at wavelengths 275, 325 and 355 were taken immediately. Absorbance readings were then put into the ASBC provided formulas to calculate alpha and beta acid concentrations.

For sensory analysis we adhered to the ASBC hop tea sensory method for sample preparation, and descriptive analysis sensory testing to get statistical data. 30 panelists were trained for three weeks prior to sensory testing to become experts on chosen attributes and standardize a ranking system. On sensory day 25g of each hop sample were ground into a fine powder. The powder was then added along with 1L of filtered water at 25℃ to a clean coffee french press with a magnetic stir bar in the bottom. The french press was then placed on a stir plate and stirred at 160RPM for 20 minutes. After stirring the the hop tea was strained out of the french press using the press plunger to remove hop particulate. Samples were placed in glass sample jars and used immediately for sensory. After sensory analysis T-test statistical tests were performed to compare the means of the various samples and determine if there were any significant difference in sensory perception.

Results

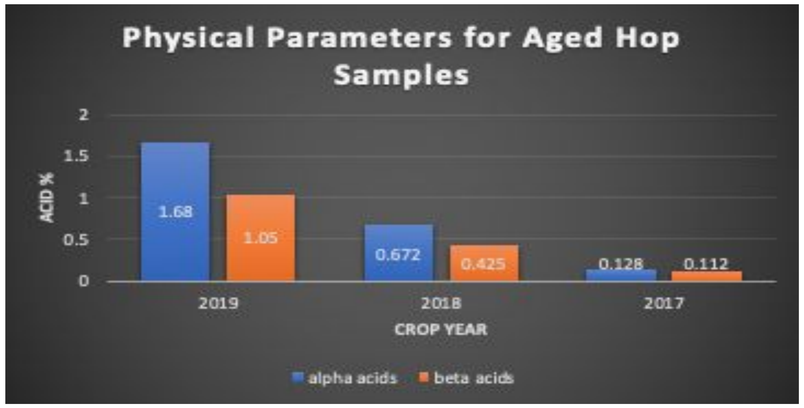

Figure 1: Physical analytics of alpha and beta acid concentration

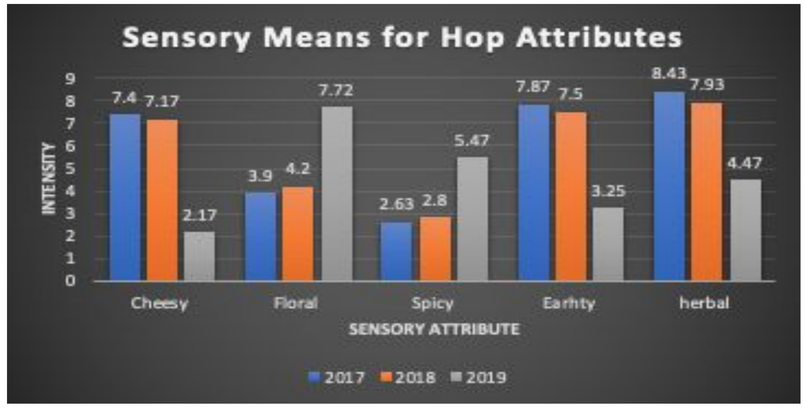

Figure 2: Sensory data as means from all 30 panelists

T-test Scores

Cheesy Attribute

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	7.16666667	7.4
Variance	0.76436782	0.66206897
Observations	30	30
Pearson Corr	-0.2908375	
Hypothesized	0	
df	29	
t Stat	-0.9421095	←
P(T<=t) one-	0.17695958	
t Critical one	1.69912703	
P(T<=t) two-	0.35391917	
t Critical two	2.04522964	

Figure 3: T-test for cheesy attribute

Floral Attribute

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	4.2	3.9
Variance	0.16551724	0.64482759
Observations	30	30
Pearson Corr	0.16888013	
Hypothesized	0	
df	29	
t Stat	1.96396101	←
P(T<=t) one-	0.02959233	
t Critical one	1.69912703	
P(T<=t) two-	0.05918466	
t Critical two	2.04522964	

Figure 4: T-test for floral attribute

Spicy Attribute

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	2.8	2.63333333
Variance	0.16551724	0.24022989
Observations	30	30
Pearson Corr	-0.0345857	
Hypothesized	0	
df	29	
t Stat	1.40936204	←
P(T<=t) one-	0.08468105	
t Critical one	1.69912703	
P(T<=t) two-	0.1693621	
t Critical two	2.04522964	

Figure 5: T-test for spicy attirbute

Earthy Attribute

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	7.5	7.86666667
Variance	0.25862069	0.18850575
Observations	30	30
Pearson Corr	0.15617376	
Hypothesized	0	
df	29	
t Stat	-3.2658312	←
P(T<=t) one-	0.00140158	
t Critical one	1.69912703	
P(T<=t) two-	0.00280315	
t Critical two	2.04522964	

Figure 6: T-test for earthy attribute

Herbal Attribute

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	7.93333333	8.43333333
Variance	0.06436782	0.46091954
Observations	30	30
Pearson Corr	0.17350306	
Hypothesized	0	
df	29	
t Stat	-4.0138649	←
P(T<=t) one-	0.00019264	
t Critical one	1.69912703	
P(T<=t) two-	0.00038528	
t Critical two	2.04522964	

Figure 7: T-test for herbal attribute

Discussion

Physical measurements showed that both alpha and beta acids degrade over long term storage time. As figure 1 shows, after only one year of aging the alpha acid concentration decreased from 5.6% to 1.68%, and after three years there were hardly left at 0.128%. This shows a degradation rate for both alpha and beta acids of roughly 70% per year when stored in a non airtight package at room temperature. If the hops were stored at a lower temperature or in an oxygen free environment, then the rate of degradation would be decreased substantially. Ultimately, it will be up to the brewer for when the hops are ready for use in production. Bitterness is not necessarily a prominent trait in Méthode Traditionelle beer, so higher alpha acid percentages will call for less hops added during the boil. The difference between alpha acid concentration from year 2 to year 3 is slight in bittering potential, showing that 2 year aged hops could be a feasible option in production.

Figure 2 shows the mean for perceived intensity from the 30 panelists for each sensory attribute on a scale from 1-10. The values show a drastic change from year 1 to year 2 for all hop samples, but little difference from year 2 to year 3. Certain attributes increase such as cheesy, herbal and earthy, while others decrease like spicy and floral. Because there was such a large change in intensity from year 1 to year 2, these results were not factored into the T-test analysis. Statistical T-test results showed that it is plausible that there is not perceivable difference for the floral, spicy, and cheesy attributes between the 2 and 3 year old hops, while it is plausible that there is a perceivable difference in the earthy and herbal attributes. Thus, the majority of the chosen attributes showed that the third year of aging made no difference in sensory perception.

Based upon our physical and sensory results, we can be confident in saying that aging hops for 3 years in Méthode Traditionelle beer production may not be necessary. As long as the brewer is comfortable with a slight amount of increased alpha acids, the 2 year hops have the potential to perform the same. With this data Méthode Traditionelle breweries can make production decisions going forward that may save them money and time when undergoing hop aging regimes.

This study is limited in the fact that it does not take into account the sensory changes that may take place in the brewhouse during production (especially the boil) or during fermentation. This research may be continued by future groups by producing actual small batches of Méthode Traditionelle beer and conducting physical and sensory analysis in the finished product to determine if the hops do in fact perform the same.

Acknowledgements

We would like to thank Brandon and Lisa at Primitive Beer for the amazing hospitality and assisting us through the completion of this study during this difficult time. We would also like to thank the FST program for providing lab space and equipment.

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Head Retention of Draft Beers and How Prior Glass Treatments Affect This

Spring 2020

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McClellan's BREWING CO. FORT COLLINS, CO

Background

Head retention is defined as the ability of a beer to produce foam and hold the foam in a stable state. The purpose of the study was to investigate how head retention of draft beers is affected by glass shape and prior glass treatments to glassware. Until recently, the general attitude about beer foam is that foam quality (determined by head retention and foam height) is dependent on the brewing process as well as the presence of intrinsic and/or extrinsic foam-positive components or foam-negative components in beer. Foam-positive components improve beer foam quality while foam-negative components deteriorate foam quality. The most commonly studied foam-positive components of beer are malt proteins, sorbents. The most commonly studied foam-negative components are lipids, polyphenols, and metal cations. Using higher mashing temperatures (>65ºC) promotes the formation of glycoproteins and other beer proteins that contribute to foam stability, and on the other hand high wort boiling temperatures are associated with decreased foam stability. When wort is boiled at higher altitude and thus a lower temperature, more foam-promoting proteins are preserved than wort boiled at sea level. Brewing yeast have shown to have an impact on head retention as well. Flocculant yeast strains produce a more stable beer foam than non-flocculent yeast strains. (Bamforth and Evans 2009, Evans and Sheehan 2002, Branyik et. al. 2018, Bamforth et. al. 2008).

Beer components, brewing processes, and even storage methods have been intensely studied for their effect on beer foam, but it wasn't until 2013 that glass treatment was taken into serious consideration as a factor influencing beer foam. Barth concluded that glass cleanliness was pertinent to a high quality beer foam (Barth 2013). Glass treatment was further studied by Bojas and Sulc in 2018 with their studies on glass temperature, detergent residues, and pH of glass. There still exists gaps in the research about how glass treatment affects foam height and retention. This project aims at filling some of those gaps by providing the industry with data about how glass shape, sanitizing treatments, and rinsing effects head retention in a Irish red ale, a pilsner, and a Belgian ale.

(Photo by: Camden Walters)

Methods

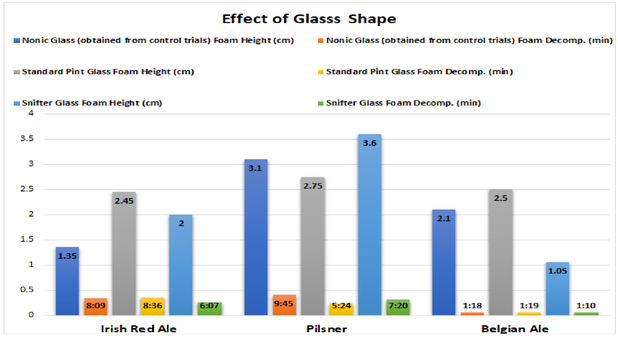
1. Question to be answered: how do glassware characteristics affect head retention of draft beers?
2. Standardize a pouring method for the draft beers.
3. Establish how to measure head retention: collect foam height & foam decomposition time immediately after pouring.
4. Establish control values using McClellan's current glassware protocol and shape (Nonic pint).
5. Test the effect of glass shape: pour beers into traditional American pint glass and Snifter glass.
6. Test the effect of rinsing the glass: pour beers into glasses that have been rinsed with tap water inside.
7. Test the effect of glass sanitation: pour beers into glasses that have been sanitized with a bleach solution.
8. Repeat steps 4-7 once, average foam heights and decomposition times from each trial. Compare data to control values.

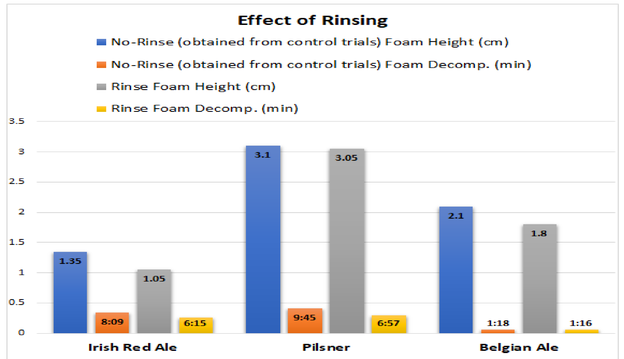
https://www.etsy.com/il-en/listing/235588505/custom-reddit-gift-exchange-single-glass

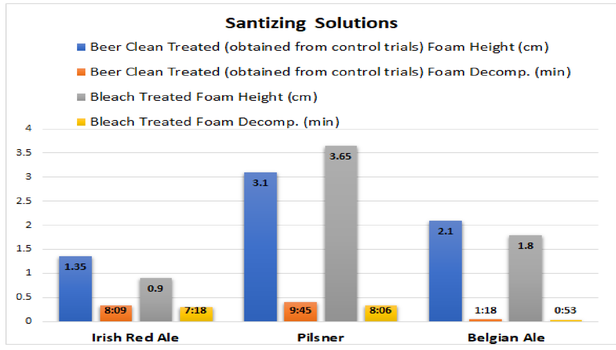
(Photo by: Marie Comstock)

(Photos by: Camden Walters)

Results







Objectives

- To provide the beer industry with information on how to maximize the head retention of draft beers.
- To provide the beer industry with information on how to enhance consumers' beer drinking experience in bars, pubs, and microbreweries.
- To determine which glassware characteristic has the most impact on draft beer head retention, whether it be a positive or negative impact.

Conclusions

- Rinsing the inside of the glass with cold water decreased foam heights and increased rate of foam decomposition for all three draughts tested.
- Bleach treatment increased the rate of foam decomposition for all three draughts.
- Bleach treatment decreased the foam heights of the Irish red ale and the Belgian ale, and improved the foam height of the pilsner.
- The snifter glass significantly increased the foam height of the pilsner, and the standard pint increased that of the Irish red ale.

Acknowledgements

We would like to thank our mentor, Graeme Hirstwood, for his commitment to our research project. Graeme consistently showed a deep interest in our project, offering advice, suggestions, and his expertise throughout the entire process. We would also like to thank all of the staff at McClellan's Brewery of Fort Collins for allowing us to run our trials at the brewery. The staff at McClellan's provided us with all the necessary equipment and even helped us run our experimental trials, all while customers were present at the brewery!

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How Microbial Spoilers in Kombucha & Alcoholic Kombucha Impact Final Product Organoleptic Properties

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Background

This study facilitated the genetic sequencing of a complete microflora of a kombucha batch to identify any irregularities or presence of spoilage organisms in an otherwise symbiotic community of lactic and acetic acid bacteria, and yeast. Multiple fermentation cycles may exhibit microbial drift of the beverage, causing inconsistencies.

Purpose

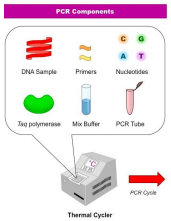
The purpose was to determine the longevity of a kombucha starter culture until it no longer resembled the original as a result of microbial drift.

Hypothesis

A kombucha sample with off-flavors or off-odors will have an unwanted microbial spoiler present in the kombucha symbiotic culture of bacteria and yeast or SCOBY, which competes with traditional kombucha SCOBY microbes via different metabolic pathways and protective measures.

Methods & Materials

Genetic Sequencing and Polymerase Chain Reaction (PCR):
PCR is a sequencing technique that makes copies of a specific region of DNA in a test tube so it can be analyzed.



Basic mechanics of PCR:

1. Denaturing double strands of DNA
2. Annealing of primers and Polymerase assembly
3. Extension of target DNA

https://cdn.apartmenttherapy.info/image/upload/f_auto,q_auto:eco,c_fit,w_1060,h_1325/k%2Farchive%2F549aa993461e462712b033881d6f4355185a13f2

- PCR utilizes DNA primers designed to target the region of DNA - the primers used for this specific research are GTGYCAGCMGCCGCGGTAA.
- These primers were chosen to target Lactobacillus, Gluconacetobacter, Acetobacter.
- These primers were used to sequence a portion of the SCOBY while a Gas Chromatography (GC assay was used to detect volatile Short Chain Fatty Acids (SCFA) that might be causing off-odors and/or off flavors.

Results

Most common Bacteria found via DNA sequencing:

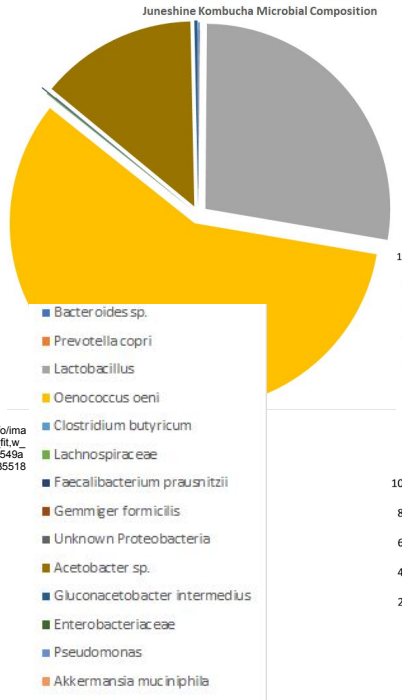
- *Oenococcus oeni*
- *Lactobacillus sp.*
- *Acetobacter sp.*
- *Gluconacetobacter sp.*
- *Clostridium butyricum*

Volatile SCFA found via GC:

- Acetate was the highest compound detected
- Butyrate was below detection

Supplemental Data

Juneshine Data



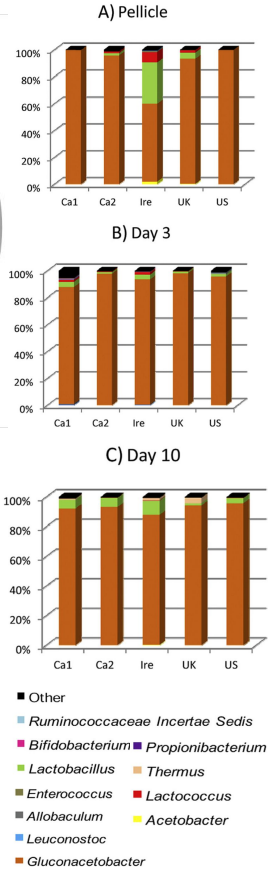


Fig.2 16S phylogenetic composition of the bacterial comopnent of the kombucha pellicle (A) and the tea at day 3 (B) and day 10 (C) of fermentation, at genus leve. (Alan Marsh, 2014)

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COLLEGE OF HEALTH & HUMAN SCIENCES
COLORADO STATE UNIVERSITY

CHARACTERIZING BIOACCUMULATION OF ZINC IN *SACCHAROMYCES SP.* IN A PRODUCTION BREWERY OVER MULTIPLE GENERATIONS BY ICP-MS

Spring Semester 2020

Presented by:
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Supervised by:
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Rob Christiansen, New Belgium Brewing Co

Sponsored by:
New Belgium Brewing Company

ABSTRACT

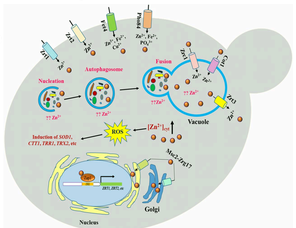
Zinc plays an integral part in yeast performance during beer fermentation. It is a cofactor for alcohol dehydrogenase, stimulates maltose and maltotriose uptake, increases ester production, and governs protein and phospholipid synthesis for yeast membranes. As a result of this knowledge, it has become common practice for brewers to dose zinc at concentrations of 0.050 – 0.300ppm to chilled wort to aid in faster and more consistent fermentation times. Research shows there is a bioaccumulation effect when dosed over multiple generations however, the quantity and characterization of bioaccumulation of zinc in *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* in production brewing is not well understood. Thus, the focus of this collaborative study between New Belgium Brewing (NBB) and Colorado State University (CSU was the development of a sample preparation methodology for future zinc analysis.
Yeast samples were supplied from harvesting tanks by NBB. Samples were washed to remove the beer matrix and then prepared in triplicate for storage at -80˚C until chemical analysis.

BACKGROUND

These amounts are where yeast have shown the highest fermentation performance (Nicola). Brewers typically incorporate zinc into their wort through zinc salt powders, on the blades of a mash mixer, or by placing a zinc plate in the boil kettle (Nicola). Adding more zinc than is needed for the best yeast performance can result in undesired organoleptic effects and added brewhouse costs.

Zinc uptake by yeast cells occurs in two different stages:

- Passive capture:
  - involves zinc cations being retained on the outer surface of the cell wall where they are absorbed through anionic binding sites into myofibrillar structures.
- Active capture: utilizes specific membrane transporters.
  - This process is a metabolism-dependent type of intracellular uptake (Wietstock).
  - Relies on specific membrane transporters to bring metal ions into the yeast vacuole for storage (Schothorst).
  - These ions are stored for use in replication and other metabolic processes (Shariatmadari).
- Research has found that zinc added to a yeast culture enhances the cell growth because this element helps regulate metabolic activity such as flocculation and cell division.
- This information has been used to analyze what happens during the different stages of the yeast's life cycle, more specifically during the lag phase of the life cycle.
- Yeast accumulate zinc at different rates depending on aerobic or anaerobic environments.
- Zinc uptake is much faster in an anaerobic pathway
  - Zinc is taken up completely by yeast within the first 48-96 hours (Shariatmadari).
  - This is due to yeast being in a favorable environment where they can begin synthesizing components for cell composition that are best suited for rapid growth.
- Yeast are relatively specific when it comes to zinc requirements and are strain dependent
  - Lager yeast: 0.05-0.150 parts per million of zinc
  - Ale yeast: 0.150-0.300 parts per million of zinc



Zinc uptake by Saccharomyces pastorianus

METHODS

In order to accurately assess and measure the concentration of zinc present in the yeast samples collected, samples are to be separated from the beer matrix prior to running on ICP-MS.. The ICP-MS device is designed to pick up trace concentrations of minerals and metals in small sample sizes, making it an ideal instrument to test our yeast cell samples on (Wei).Each yeast sample was washed of any residual beer or wort compounds in solution. Samples of ale and lager yeast were provided by NBB Each sample was coded with its original propagation date, the date it was sampled, and the generation sample number. All samples from NBB were also provided with their corresponding viability, cell concentration, and pH.

Additionally all yeast samples were harvested from similar batches and brands of beer, preferably from successive generations.

- This is in order to trace zinc accumulation as it relates directly to cell maturity.
- In total, 12 individual samples of yeast were provided by NBB, resulting in 6 total generations over the span of one month.
- Each sample was stored in both Milli-Q water as well as 40% Glycerol in triplicate.
- These samples will be run through the ICP-MS equipment in the CSU Animal Sciences building.

In the future continuation of this project, data trends between zinc concentrations, health, and viability will be compared against each other to determine if the bioaccumulation of zinc in the sample correlates to overall yeast health and performance.

OUTLINE OF THE SAMPLE PREPARATION PROCESS

Collection — Yeast samples pulled from harvesting tanks at NBB and provided in 250mL jars with corresponding viability, cell concentration, and pH, then stored in refrigeration for no more than 96 hours before washing.

Washing — 15mL of homogenized sample was placed in a 50mL conical centrifuge tube. Separation was achieved through centrifugation, and then the yeast was washed 3 times in Milli-Q water, decanting off liquid in between each washing.

Dilution — After final washing and decanting, the conical centrifuge tube was filled back to 50mL and homogenized. 1mL of this homogenized solution was placed in a separate tube and diluted further with 9mL of Milli-Q. A cell count of this dilution was taken on a Nexcelom cellometer.

Storage — 1mL of 1:10 diluted sample was moved into a 1.5mL microcentrifuge tube and 0.5mL of 1:10 diluted sample was moved into a separate 1.5mL microcentrifuge tube and topped with 0.5mL of 40% Glycerol. This was performed in triplicate. Samples were placed in a cryo storage box and stored at -80˚C.

ICP-MS — All samples are to be run on the ICP-MS device located in the CSU Agriculture Chemistry Laboratory. To be completed in the second part of this study.

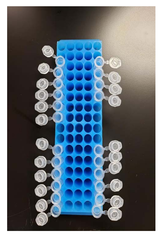
PICTURES OF THE SAMPLE PREPARATION PROCESS

Left: 15mL of sample in 50mL conical centrifuge tube

Right: 250mL jars containing sample from NBB

Separation after first centrifugation

Separation after third washing



Sets of 1.5mL microcentrifuge tubes for 3 samples, along with blanks

CONCLUSION

This research will potentially lead us to a more accurate and deeper understanding of how yeast are functioning at generational levels. While there has been a good amount of research done between the relationship of zinc and yeast, this study will add another element of our understanding to the mix. If we can examine and quantify exactly how much zinc is being passed on generationally from mother cell to daughter cell, as well as how much is actually being utilized, we will be able to make our zinc additions more precise for fermentations. Since we have examined that yeast fermentations typically slow down and decrease in efficiency during the fourth through sixth generations, we will hopefully be able to identify if it is because of an excess or lack of zinc. In turn, this could increase brewhouse efficiency, decrease mineral addition costs, lead to the production of desirable esters in greater quantities, create higher rates of fermentability, and enhance yeast health for professional brewers around the world.

This study is not without its limitations. While we were able to acquire yeast samples from NBB from multiple generations, they were not-coming from the same yeast lineage further studies should follow one yeast propagation through its entire propagation tree.-Repeating a study in this way with multiple independent props of the same strain would allow us to tighten down the data set of means, coming to a more accurate data set on which to base zinc addition predictions and decisions on going forward.

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The Effect of pH and Titratable Acidity on Perception of Acidity in Sour Beers

By: Samuel Jones, Samuel Krason, Tayla Osborne, Michael Tran
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Hypothesis

Hypothesis: Titratable acidity will better correlate to the perceived acidity in sour beer over pH.

Null Hypothesis: pH will have the greatest correlation to the perceived acidity in sour beer compared to titratable acidity.

Abstract

This experiment tested whether pH or titratable acidity (TA) was a better representative measure of the sensory perception of the intensity of sourness within sour beer. To conduct this experiment, TA, pH, and sour perception sensory scores were collected from four traditional aged barrel beers and five beers spiked with two different concentrations of individual organic acids, as well as a blend of four organic acids; Variable A consisted of a 1,000ppm addition of the chosen organic acid and Variable B contained a 3,000ppm addition of each chosen organic acid. The four traditional barrel aged beers were included in the experiment in order to increase statistical relevance of data and as a comparison in flavor complexity. The TA, pH, and total acidity via HPLC for the barrel aged sour beers (Brands: Citrus Hour, Professor Funk, Flanders Red and Fliek) were also recorded. The organic acids evaluated in this experiment were Citric, Lactic, Malic, Tartaric, and a blend consisting of 25% of each aforementioned organic acid. After testing, the barrel-aged sour, Fliek, had the highest TA measuring at 63 g/L and was rated with the highest intensity score averaged at 2.73 on a scale of 1-3 (1 =non-sour, 3=intensely sour). Fliek had a pH of 3.1 which was equivalent to another barrel aged sour, Professor Funk, which had a lower TA at 49 g/L. The Professor Funk intensity score was averaged at 2.27, correlating with the lower TA value. Products with a higher titratable acidity appeared to have more direct relationships in correlation with the perception of sour tastes, whereas pH offered fluctuating, inconclusive results. The data collected shows that the TA of a specific beer has a direct correlation to the perceived sourness of a sour beer regardless of the measurable concentration of any organic acids present.

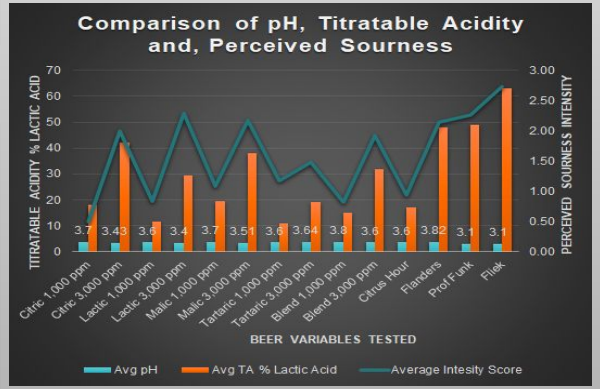
Background

In past sensory perception data, titratable acidity has the greatest impact on the intensity of perceived sourness over pH[2][3][6]. Hydrogen ions bind with taste receptors on the tongue which in turn causes the sensation that is associated with sour taste[6]. Titratable acidity is the measurement of the total concentration of acids in a solution that can be neutralized with the addition of alkali solution[11]. The amount of alkali solution required to neutralize the substrate is then used to calculate the equivalent percentage of lactic acid in the solution, whereas pH measures only free hydrogen ions[9]. Since taste receptors detect both free and bound hydrogen ions, using titratable acidity may be a better measurement of perceived sourness over pH[1]. In common studies, low pH is associated with higher perceived sourness and the body's interactions with organic acids[7], but in counter studies, pH has inaccurate relationships with perceived sour taste[4]. Acids prevalent in food and beverages are only partially ionized, meaning that both bound and free hydrogens must be considered[11]. In order to solve this dispute of which measurement tool is more indicative to overall sour perception, both pH and titratable acidity must be evaluated concurrently.

Methods

To conduct this experiment a basic "control lager" was used. The beers tested were decarbonated via a coffee filter before the experiment was performed. This was because CO2 in aqueous solution forms carboxylic acid which would affect the results. Five groups of beer were collected with each group of consisting of an A & B variable. The A variable consisted of a 1,000ppm addition of the chosen organic acid and subsequently, the B variable was 3,000ppm of the chosen organic acid. The organic acids variables tested in this experiment were; Citric, Lactic, Malic, Tartaric, and a blend consisting of 25% of each mentioned acid. To ensure the accuracy of this experiment, the control lager was used in conjunction with 4 additional barrel aged beers, being; Citrus Hour, Flanders, Professor Funk and, Fliek. Barrel aged beers were included in this experiment to bolster both the statistical relevance of the data, but also to determine the impact that the addition of certain organic acids in specified amounts have on the sensory characteristics of beer. Each individual beer was tested for pH, TA, and intensity of sourness via an attribute-specific sensory analysis. pH was tested for each undiluted beer sample with a calibrated pH meter three times and then averaged for accurate measurements. Group members worked simultaneously to calculate TA measurements for each sample with a RedCheck Acidity Kit. Using a 1 mL pipette, 1 mL of an individual sour beer sample was added to 100 mL of distilled water. The diluted sample was then checked for TA by adding one drop of NaOH in succession until dilution turned red. While TA was checked duplicately and then averaged in the beginning of the experiment, results were consistent, therefore the averaging process was null and TA was calculated once for the remaining samples. The recorded number of drops was used to determine acid percentage per sample. For sensory testing, 6 panelists evaluated each sample on a scale of 0 (non-sour) to 3 (intensely sour). Answers from each panelist were then averaged and recorded in Google Sheets along with the pH and TA measurements for evaluation of the data.

Data



Conclusions

The primary goal of this experiment was to determine whether pH or TA has the greatest correlation to the perceived sourness of a beer. Additionally, the successful addition of organic acids to beers would negate the traditional aging process and, subsequent fermentations typically required with production of sour beers. In testing the hypothesis, each variable was tested for not only pH and TA, but also subjected to a sensory analysis panel. After analyzing the results of all samples using the recorded measurements of pH, TA, and perceived sourness intensity scores, it was found that the measurements of titratable acidity and perceived sourness intensity scores correspond with one another more than pH and perceived sourness intensity scores. With the range recorded pH of all samples only differentiating by 0.72, titratable acidity offers a larger scale that allows clearer correlations between total acidity and perceived sourness intensity scores. From the collected data, by using TA to compare the perceived intensity of sour beer rather than just pH measurements, it allows more accurate predictions of the intensity of sourness in a sample. The hypothesis was accepted and the null hypothesis was rejected. This data can be used to expand the research and identify the gaps of comparing TA to perceived sourness by including more variables to indicate what other factors may influence perceived sourness intensity and if those variables correspond with TA.

Acknowledgements

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