# Investigating Malt Inducing Premature Yeast Flocculation: Threshold and Stability

By

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## Dedication Page

I dedicate the summation of all of my work to the very people that made it possible. To Marty, Judi and Tammy, my loving family always supported my each and every step along this journey.

I also dedicate this work to my best friend, partner and love of my life Jessica. Always supportive and encouraging, if it were not for you this would have never been completed.

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## Abstract

Premature yeast flocculation (PYF) has proven to be troublesome for the malting and brewing industries. It causes production difficulties and quality issues characterized by poor attenuation and low yeast cell counts post-fermentation. These issues result in variability in fermentation and flavor profiles. For this reason it is critical for brewers to assess their malts for PYF potential. To test for PYF potential in malt, the industries rely on a variety of fermentation assays to indicate if a sample has PYF potential. This study used a modified miniature fermentation assay to determine if malt displays PYF potential and its associated threshold. In addition to traditional wort preparation methods, a wash technique was used to extract the PYF factor for use with synthetic wort. This modification to the assay eliminated the need for a controlled mashing device and reduced the amount of fine adjustments of the wort pre-fermentation to reach target start values. Control malt known to display no PYF attributes and PYF inducing malts were used in the study. To further our understanding of the PYF factor the "PYF Solution" extracted using the wash technique was subjected to various conditions pre-fermentation. The PYF Solution was mixed with the synthetic wort with varying ratios from 0-100% of the PYF solution (mixed with distilled water) to find the threshold at which PYF characteristics was displayed. The wort was treated with a combination of pre-fermentation boiling (60, 90, 120 minutes), chilling at 5°C and Freezing at -30°C to further our understanding of the how these conditions affect the PYF factor. The trials were conducted using a 15 mL fermentation with a consistent temperature and pitch rate (21°C, 1.5 × 107 cells/mL). The change in absorbance and Plato was monitored throughout the fermentations. The control malt had no significant ($P>0.05$) difference in absorbance and Plato measurements from PYF inducing malt when the synthetic wort had <70% PYF solution. Synthetic wort containing >70% PYF solution had significant ($P<0.05$) differences from the control malt for both absorbance and Plato measurements. Boiling treatments alone did not produce significant ($P>0.05$) differences in the trials. Boiling followed by chilling yielded the same results and did not have significant changes ($P>0.05$) in fermentation characteristics. While most of the pre-fermentation treatments did not create differences in fermentation characteristics, boiling wort for >60 minutes and freezing before fermentation caused the >70% PYF solution to display the same fermentation characteristics as the control ($P>0.05$). These findings show that the PYF inducing factor may be susceptible to further processing. This thesis established two further findings. First, that the factor causing PYF must meet a threshold before it affects fermentation characteristics. Second, mashing the PYF-inducing grain may not be absolutely crucial for testing PYF potential of malt.

**List of Abbreviations and Symbols Used**

| Abbreviation / Symbol | Description |
|---|---|
| **ABV** | Alcohol by Volume |
| **ABW** | Alcohol by Weight |
| **Abs** | Absorbance |
| **AE** | Apparent Extract |
| **ADF** | Apparent Degree of Fermentation |
| **AP** | Antimicrobial Peptides |
| **DO** | Dissolved Oxygen |
| **FIAF** | Factor Induced Altered Flocculation |
| **FTU** | Formazin Turbidity Units |
| **HL** | Hectoliter |
| **HMWP** | High Molecular Weight Polysaccharides |
| **MSS** | Malt Soak Solution |
| **MFA** | Miniature Fermentation Assay |
| **Nm** | Nanometer |
| **NTU** | Nephlometric Turbidity Units |
| **PYF** | Premature Yeast Flocculation |
| **°P** | Plato |
| **R** | Rotation of the Gaussian curve |
| **SRM** | Standard Reference Method |
| **YIS** | Yeast in Suspension |
| **A** | The Absolute amplitude (AU) |

| | |
|---|---|
| $\boldsymbol{\mu}$ | Mid-point in hours |
| $\boldsymbol{\sigma}$ | Standard deviation of the curve |
| $\mathbf{P_t}$ | Particular density at a specific time |
| $\mathbf{P_e}$ | The Equilibrium asymptotic density value |
| $\mathbf{P_i}$ | The initial asymptotic density value for the density attenuation regression |
| **B** | Function of the slope at the inflection point |
| **M** | Time for a specific B value |

## Acknowledgements

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Dr. Speers, thank you for your guidance, support, mentorship and patience through this pursuit. If it were not for you I would have never had the opportunity to find my brewing science passion.

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Lastly, I would like to thank John Allen at Propeller Brewery Company for encouraging my academic endeavors and the brewery as whole for helping conduct this research.

## Chapter 1. Introduction

### 1.1 Synopsis of Malting and Brewing

Beer was one of the first prepared beverages and it was essential in the creation of modern society. The origins surrounding the production of man kind's first beer are riddled with unknowns. Dates ranging from 3500BC to 9000BC are speculated to have the earliest forms of evidence of beer production. While the exact date of inception is unknown, the ability to utilize large scale agriculture to produce grain for beer and bread production played a key role in switching from a nomadic to a sedentary human civilization (Unger, 2001).

While beer production technology has advanced drastically since its first preparation, the core processes have essentially remained constant. A grain source (usually barley however other grains can be used) is utilized to provide sugars which are fermented via yeast (generally *Saccharomyces* spp.). The fermentation converts the sugars into alcohol and carbon dioxide which is expressed in Figure 1.1. When grain is the predominant source of sugar utilized for fermentation, the resulting beverage is defined as beer (Boulton & Quain, 2008).

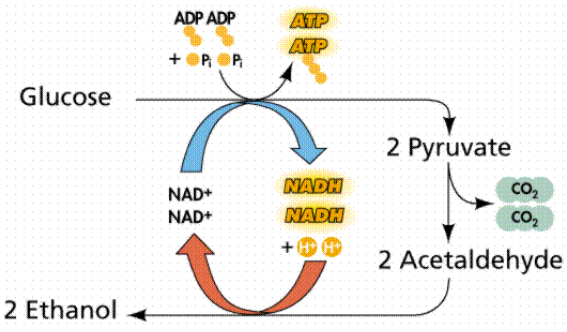



Figure 1.1 Chemical equations for the breakdown of sugar into ethanol and $CO_2$ (Stanbury et al, 2013)

The simple conversion of grain sugar to alcohol and carbon dioxide does form the basis of beer production; however, additional processes are needed to produce the finished product as we know it today. The general production guidelines of beer are depicted in a flow chart in Figure 1.1. Malting of barley is undertaken at a malting facility or malthouse. Because the barley grain itself contains starches which are not able to be utilized by yeasts, the grain must be malted to develop enzymes which will break down the starches into sugars. The malting process consists of three stages and begins with a step called “steeping”. This steeping step is where the grain is immersed in water and drained and re-immersed in a systematic fashion to develop a final moisture content of 40-46% (Priest & Stewart, 2006). This steeping step takes 2 to 3 days and causes the grain to come out of seed dormancy which will allow for further processing. The next step in malting is germination where the steeped grain is moved to an area where it is allowed to germinate under controlled conditions (Mixed and Air dried). This germination step alters the structure of the grain, produces some sugars and most importantly develops natural enzymes within the kernel which will be utilized in the brewhouse. The final stage in malting is the kilning step where warm air or smoke (traditionally 52-56 °C) is passed through the germinated grain. This step creates a notable colour change in the malt, stops the germination process, preserves the natural enzymes in the kernel and prepares the malt for long-term storage (Priest et al. 2006).

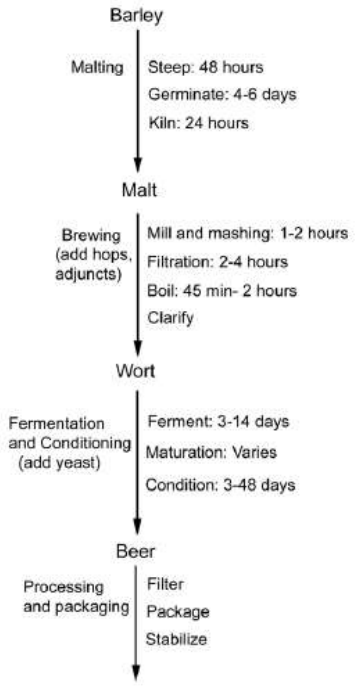



Figure 1.2 Overview of the malting and brewing processes (Lake, 2008)

Once the barley has gone through the three main malting stages, it is ready to be stored and/or shipped to a brewery. Once the malt arrives at the brewery, it is milled and mixed with water at 45-72°C inside a vessel called a mash tun. The purpose of this step is to utilize the natural enzymes developed in malting to breakdown starch into sugars, which include; the monosaccharide glucose, disaccharide maltose, the trisaccharide maltotriose, and higher sugars called maltodextrins. Depending on the brewery and the beer style being produced, the mash will "rest" at different temperatures and will optimize the enzymes to yield better starch breakdown (Stanbury et al., 2013). In Table 1, the optimal temperature for the major mashing enzymes is shown. While traditionally the natural enzymes found in malt have been used, the industry has

started to utilize artificial enzymes for flavour development and cost savings (Stanbury, 2013). The resulting mash-liquid rich in fermentable sugar is now coined "wort". The wort not only provides fermentable sugar but adds colour, viscosity and flavour to each breweries product. This wort is then separated from the "spent" grain in a Lauter Tun and moved to a kettle (Priest & Stewart, 2006).

Table 1.1 Main enzymes associated with mashing (White & , 2010)

| Temp °C | Enzyme | Breaks down |
|---|---|---|
| 40-45 °C | β-Glucanase | β-Glucan |
| 50-54 °C | Protease | Protein |
| 62-67 °C | β-Amylase | Starch |
| 71-72 °C | α-Amylase | Starch |

The wort is transferred to the kettle where it will be boiled, and hops are added. This boiling of the wort and mixing in the hops not only imparts bitterness and aromas to the product, but it works as a sterilization phase (Rückle, 2005). The boiling of the wort also reduces the levels of volatile compounds which cause off flavours in the finished product. The now boiled wort is chilled to 8-26°C (depending on the style of beer) where it is oxygenated and transferred to a fermentation vessel (Schwarz, 2011). Yeast is added to the fermentation vessel and after a lag phase the yeast begins fermentation. After yeast has converted the majority of sugars, the yeast begins to form aggregates. These aggregates of yeast are called floccs, and this natural phase in fermentation is called flocculation (Speers, 2006). Depending on the strain of yeast being used, the floccs will either settle to the bottom of the vessel (Lager) or rise to the top (Ale) (Jin, 2000). Flocculation is very important as it aids in clarifying the product for filtration. In addition, brewers rely on the flocculation characteristics of their yeast to collect and re-use the yeast from batch-to-batch (Priest & Stewart, 2006). Unlike other areas in the production of beer, the fermentation utilizes living

organisms and this step is extremely hard to control with repeatability. The wort which has undergone fermentation is now beer and ready to undergo further processing to be placed in the finished package (Bottle, Can, Cask, or Keg). Additional processes and proprietary recipes and techniques are what gives each brewery their distinct character. While brewers of varying sizes may have vastly different systems and technologies, these core beer production principles remain universal.

### 1.2 Large Scale Beer Production: Efficiency, Consistency and Quality

The fundamentals of beer production remain constant within breweries of all sizes; however, as a brewery becomes larger and reaches more clientele, it becomes imperative that the product remains the same with an accurate shelf-life to create brand trust and loyalty (Bamforth, 2011). Many further processing stages and monitoring systems are in place in large scale breweries (>250,000HL / year) to assure consistency, efficiency and quality. While microbreweries (<250,000HL / year) may pride themselves on variations from batch-to-batch, these same variations on the small scale can have detrimental effects on large scale production and sales which effect brand loyalty for the typical consumer (Stack, 2000).

The main reason why large scale brewers pay such close attention to the quality of the product is because the loss of a routine consumer equates to a yearly loss of $800-1200 per year in revenues (Bamforth, 2011). Investigating this issue further, we see that losing 10,000 customers due to a low quality product containing a defect or hazard equates to ~$150 million in lost revenues over a 15 year period. The loss of 10,000 consumers may seem drastic, however, when you look at the largest producer of beer in Canada (AB InBev) it only equates to 0.2% of their routine consumers

(Hope-Ross, 2006). Therefore, it is imperative for large breweries to establish strong brand loyalty and they do this by routinely producing a high quality and consistent product.

Consistency, efficiency and quality become intertwined in large scale brewing. Not only must the product be produced within the projected time and cost parameters, but it must meet all of the product specifications (Bamforth, 2011). The specifications that are typically analyzed at large breweries are listed in Table 1.2 (White, 2010). Finished product specification numbers 3,5,7, and 8 in Table 1.2 are all directly impacted when an occurrence of PYF (Premature Yeast Flocculation) takes place. This can lead to the product needing to be disposed of, reworked, and/or blended. KPMG estimates these losses at 14.4 million per occurrence of PYF in Canada. If the product does make its way into distribution, it may need to be recalled and disposed of which generally costs 5 to 10 times the amount of its initial production. The largest factor of financial loss due to occurrences of PYF is the stress it places on manufacturing which must hold production until issues are remedied, this causes diminished yearly capacity and severely disrupts the production stream (Soares, 2011).

Table 1.2 Typical Wort/Beer specifications analyzed at large breweries (White, 2010)

| Quality Parameter | Description |
|---|---|
| **1. Alcohol (ABV)** | Using various methods brewer's attempt to keep ABV within 0.3% of the advertised value |
| **2. Bitterness (IBU)** | By measuring the concentration of Alpha acids, the brewer can assure proper hop levels have been added |
| **3. Dissolved Oxygen (DO)** | To assure a reduction in oxidation, DO is measured in the ppb range |
| **4. Colour (SRM)** | Using absorbance, a SRM value is used to monitor the colour of beer |
| **5. Head Retention** | Many methods are used to determine if the foam in a beer glass will remain or deteriorate |
| **6. Carbonation** | Natural and injected carbonation plays a key role in the sensory experience of beer |
| **7. Aroma – Flavour Analysis** | Sensory departments in conjunction with advanced chemical analytical instrumentation are used |
| **8. Turbidity (Haze)** | Using absorbance at various wavelengths, the brewer can determine the perceived level of haze (FTU, NTU) |

### 1.3 Yeast Flocculation and Premature Yeast Flocculation

To assure the efficient production of a high quality beer with exceptional consistency, the fermentation must be controlled closely. Similar to any piece of machinery in the brewery which may need routine maintenance and safety checks, the yeast plays a fundamental role in beer production and needs to be in top working order to achieve the desired goal. While making sure yeast is healthy with high viability and vitality is a key aspect in production, the initial selection of the proper yeast for the desired beer style is essential (Palmer, 2006). Yeast not only converts sugars into alcohol and $CO_2$, but it imparts numerous flavours and aromas which must be considered before selection. In addition, one of the most desirable characteristics of yeast is its ability to form aggregates and either rise to the top or sink to the bottom of a fermentor. This ability to form aggregates once the majority of sugars have been utilized is yeasts flocculation ability (Jin et al, 2000).

Yeast flocculation is a key parameter in the production of high quality beer, and is one of the key contributors to producing beer efficiently and consistently. Brewers have harnessed this natural biochemical characteristic to aid in clarifying beer and supporting when repitching from batch to batch for centuries. Brewers themselves have played a key role of the evolution of brewing strains of yeast by unknowingly placing selective pressure on flocculation characters that they desire (Piškur, 2006). A yeast's flocculation characteristics can be placed into three classifications; high, medium and low. A brief summary of how these flocculation characteristics effect beer production are listed in Table 1.3. As seen in Table 1.3, flocculation ability affects more than just the settling and rising of yeast, it is a key parameter in the overall character of an individual beer.

Table 1.3 Flocculation Classification and the corresponding effect on beer fermentation (White, 2010). *Adapted From Yeast: The Practical Guide to Beer Fermentation

| **Flocculation Degree** | **Notes** |
|---|---|
| Low | • Fail to begin to flocculate by day 15<br>• Most wild yeast display low flocculation<br>• Suitable yeast for styles that do not require filtration<br>• Makes filtration difficult |
| Medium | • Start to flocculate between day 6 and 15 of fermentation<br>• Great for ales<br>• Generally have very clean fermentation profiles |
| High | • Start to flocculate in less than 5 days<br>• May require routine rousing<br>• Higher diacetyl production and lower attenuation rates |

While the direct need for a desirable flocculation characteristic is no longer needed for clarification of beer due to the incorporation of advanced filtration and centrifugation techniques, further flocculation effects on beer are still very much imperative. Even though flocculation has

been examined by scientists for decades, the exact mechanisms behind it are filled with controversy. What is known for sure is that flocculation of brewery yeast is a complex process which is one of the most variable in the production of beer (Speers et al, 1992). The main reason for this flocculation variability is most likely because an abundance of subtleties are known to influence flocculation. These include environmental conditions, genetic factors and physical forces which are all present in propagation, fermentation and storage of yeast (Speers et al, 1992). Another large factor affecting flocculation is the brewers cropping techniques which can put selective pressure on the yeast leading to genetic changes through selection (Powell et al., 2003).

A common issue in flocculation is when it does not occur to the extent it should (poor flocculation), leaving high levels of yeast in suspension. This results in the product requiring further processing to clarify. Another issue with flocculation is when the yeast flocculates too early, which leads to poor attenuation and flavour defects. This issue is called premature Yeast Flocculation (PYF) and it leads to substantial financial stress on brewers and malsters. Even with substantial amounts of research into PYF, the driving mechanism behind it is not fully understood (Lake, 2008). One of the main reasons it is so hard to fully understand this complex issue is because there are a multitude of yeast strains, malt varieties and brewing techniques which must be accounted for in the research. There are currently three theories governing the mechanism behind PYF, and while they all have unique interpretations of findings, they all agree that PYF is linked to a protein and/or carbohydrate portion within malt. Unlike other issues arising in industrial fermentations, PYF does not occur because of improper processing (Panteloglou, 2013). All of the prerequisites (protein and/or carbohydrate) for PYF to occur are always present during fermentation; however, they must reach a threshold level or “extreme” before PYF is actually

witnessed in the fermentation and the only current method to avoid it is to reject the use of PYF inducing malt via pre-screening (Lake, et al., 2009).

## 1.4 Objectives of the Literature Review

The purpose of this literature review is to provide an in depth understanding of yeast, flocculation and premature yeast flocculation in relation to brewing while focusing on aspects that are pertinent to the research conducted.

# Chapter 2. Literature Review

## 2.1 Brewing Yeast

Yeast is the one of the most exploited organism in modern society and it is used in numerous industries worldwide. It is used in the baking, beverage, pharmaceutical, probiotic, enzyme and supplement industries (Cereghino & Cregg, 1999). The term yeast refers to organisms within the two phylogenetic groups; Ascomycota and Basidiomycota. Altogether there are currently 1,500 yeast strains that are described, but researchers estimate that a large portion of other species exist that are not yet cultured (Legras et al., 2007). This paper will focus on yeasts which reproduce asexually via budding (yeasts within the order *Saccharomycetales*) as they are the yeasts used in brewing and generally regarded as "true yeasts".

### *2.1.1 Yeast Genetics, Structure and Physiology*

In 1996, an international community of scientists successfully sequenced the unicellular *S. cerevisiae* and found that it contained 6,000 genes. These 6,000 genes form the makeup of a yeasts 16 different chromosomes (Bacteria = 1 Chromosome, Humans = 23 Chromosomes). Generally, yeasts and even human cells are diploid, which means that they contain two copies of each chromosome (Lander, 1996). Wild yeasts are diploid and therefore have 32 chromosomes (a copy of each chromosome). These wild yeasts reproduce sexually by differentiation (formation of spores) and this leads to rapid changes in genetics which is good for health and survival of yeast (Jespersen & Jakobsen, 1996). This rapid change in genetics, while good for future survival, is detrimental to brewers whom attempt to produce a consistent fermentation. Luckily for today's brewers, older generations of brewers were selecting for desirable traits for centuries by only

reusing yeasts from desirable fermentations. This evolutionary pressure eventually caused brewer's yeast to lose the ability for sexual reproduction and caused the yeast to develop a polyploidy state and reproduce asexually. Being polyploidy means the yeast contains multiple copies (>3) of its chromosomes (Priest, et al., 1996). This is of substantial benefit to brewers because a mutation in the yeasts genes will likely not incapacitate the cell as the cell will have multiple copies encoding for the one mutated gene (Priest, et al., 1996).

Morphologically the size of a yeast cell varies substantially depending on the species (Speers et al, 2006). The range of size for yeast is between 3-40µm and brewer's yeast (*S. Cerevisiae)* are generally 7-15µm when mature. The cells are generally globose, and ellipsoid to elongate in shape (Priest et al., 1996). A generic diagram of a yeast cell structure is shown in Figure 2.1. As brewer's yeast reproduce asexual via budding, the resulting cell clones are referred to as daughter cells. The parent cell will display a permanent scar ("bud scar") on the yeasts cell wall for every daughter cell it produces (Powell, et al., 2003). The display of a yeasts bud scar is shown in Figure 2.2.

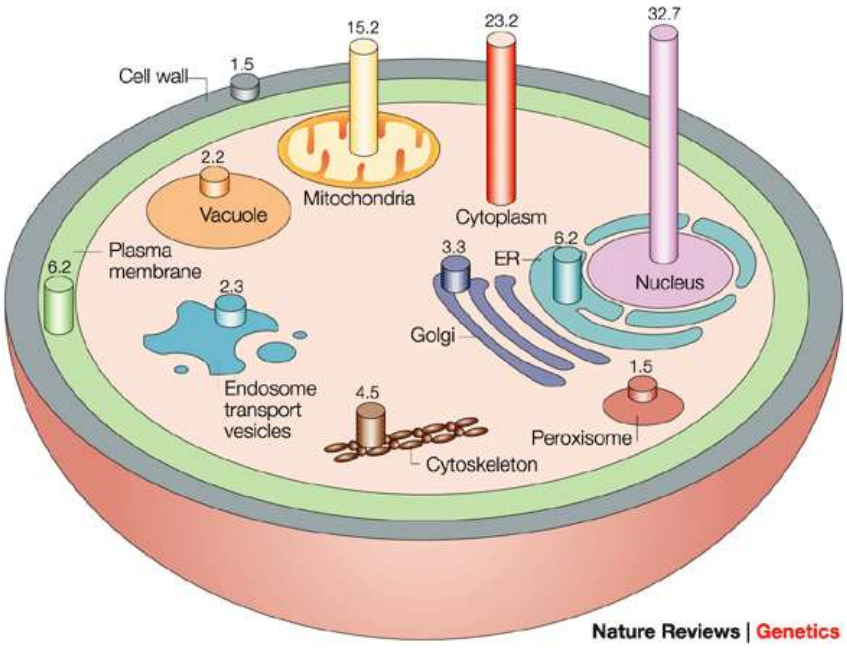



Figure 2.1 General Structure of a Yeast Cell with corresponding percentages of each component (Ouzounis et al., 2003)

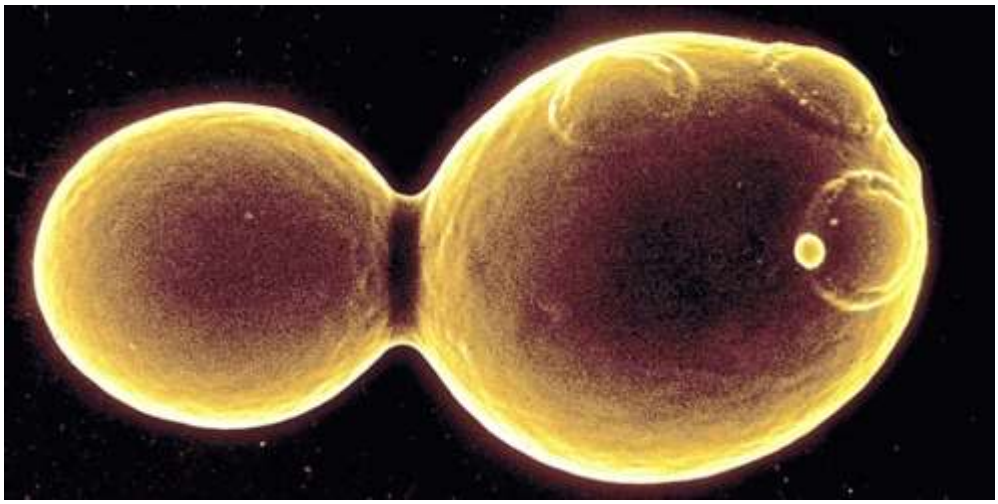


Figure 2.2: Electron microscope image of a budding yeast with previous bud scars present on the right site of the image (Slaughter, 2009)

The cell wall of yeast is a complex of carbohydrates, proteins and lipids. The wall acts as a three cross-linked layers (Lake, 2008). The outer layers is composed of predominantly mannoproteins. The intermediate layer is composed of a mixture of mannoproteins and chitins, and the inner layer is made of predominantly chitin. When a cell clones itself and produces a daughter cell, the resulting visible bud scar (Figure 2.2) is composed mainly of chitin (Speers, 1993). The cell wall's main function is to act as a barrier to protect the cells content, however, the cells plasma membrane also aids with protection.

The cells plasma membrane is located between the cell wall and the inside of the cell. This layer is permeable by some compounds and restrictive to other compounds. This semi permeable layer is composed of lipids, sterols and proteins. The composition of these three constituents provides the layer with high fluidity (Priest et al., 1996). This high fluidity makes the layer malleable which aids in the cell's ability to bud daughter cells (Priest et al, 2006).

Inside of the cells plasma membrane one finds the cytoplasm. The cytoplasm is a highly complex mixture of various organelles, nutrients, minerals and enzymes. The organelles present in the cytoplasm are; mitochondria, vacuole, endoplasmic reticulum and the golgi complex. All of these organelles play a particular role in a yeasts metabolism. The enzyme rich cytoplasm actually plays a key role in the fermentation by aiding in the conversion of glucose to energy once it enters through the plasma membrane. The cytoplasm also serves as a storage site for the cells energy reserve in the form of glycogen (an energy storage carbohydrate). The cell's membrane not only provides some environmental protection and a gateway for what transfers in and out of the cell, but it's this layers fluidity that aids in the completion of fermentation (Priest et al., 2006).

### *2.1.2 Yeasts Metabolism and Fermentation*

When yeast is inoculated into wort the cells will first utilize their glycogen reserves and also use any oxygen in the environment to replenish their cell membranes. Replenishing their cell membranes optimizes the cells permeability and aids in the transfer of nutrients and sugars. Once the cells have absorbed oxygen, they begin to absorb the nutrients and sugars from the wort. Yeast cells utilize some sugars more easily than others, and will absorb the available sugars in a specific order; glucose, fructose, sucrose, maltose and maltotriose. Some compounds will diffuse through the cell membrane, while others require transport mechanisms (Quain et al., 1981).

The start of fermentation begins with the reaction shown in Figure 2.3. Once through this pathway, in an aerobic environment the pyruvate will enter the mitochondria, go through the Kreb cycle and yield ATP, $CO_2$ and water. This pathway produces the maximum amount of energy from each molecule of glucose. While aerobic respiration produces maximum amounts of energy, it is not possible throughout an entire fermentation because traditional "pitch rates" with high viability yeast deplete oxygen levels of wort rapidly (within one hour).

Once oxygen levels have been depleted the yeast must rely on anaerobic fermentation to survive in the anaerobic environment. While twelve and a half times less efficient as aerobic respiration, anaerobic respiration gives the yeast cell the ability to survive in oxygen deprived environments. The result of this anaerobic respiration also produces alcohol, which is arguably the main reason why beer and other alcoholic beverages are so popular (Ouzounis et al., 2003).

Yeast rely on nicotinamide adenine dinucleotide (NAD+) for reduction-oxidation reactions and use it in the initial breakdown of glucose. Without oxygen the NAD+ in the initial breakdown of the glucose molecule is not present, and no pyruvate or energy is created. The yeast now focus

on the reduction of the pyruvate molecule (Figure 2.4) which goes through two reactions in the cytosol of the cell. These two reactions are shown in Figure 2.4 and display pyruvate first being converted into acetaldehyde via pyruvate decarboxylase (Reaction 1) and then to ethanol plus NAD+ via alcohol dehydrogenase (Reaction 2). While this pathway does not produce as much energy, it gives the yeast the ability to survive (Boulton et al, 2008).

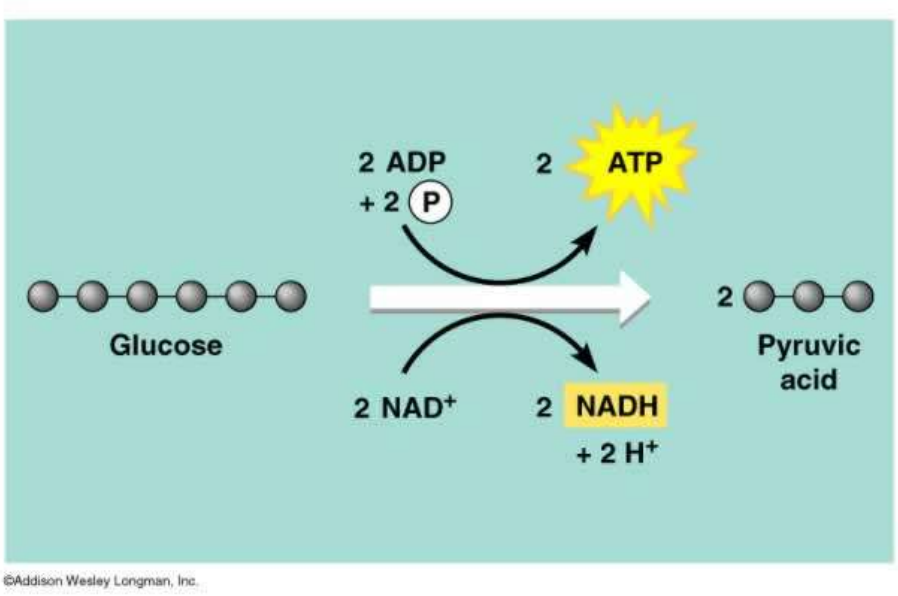



Figure 2.3 Breakdown of glucose into 2 pyruvates reaction (Boulton, 2008)

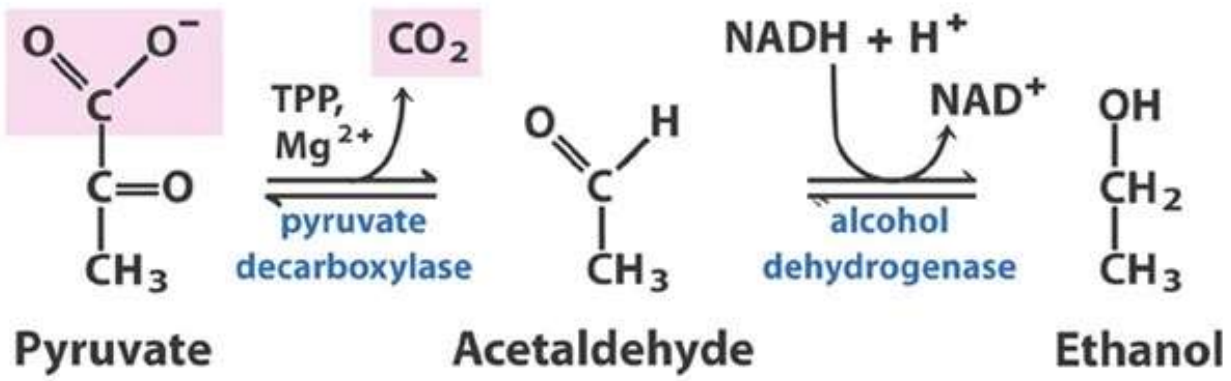



Figure 2.4. Pyruvate conversion to Ethanol via two sequential reactions (Boulton, 2008)

**2.2 Yeast Flocculation**

Flocculation is a yeasts ability to form aggregates. As mentioned in the introduction, it is a key parameter when selecting the yeast strain for use in beer production. Table 1.3 displays the differences amongst yeasts flocculation degree and shows the trends associated with them. While the exact mechanisms of flocculation are somewhat controversial and still an active area of research, a great deal of information regarding its mechanisms are available. While genetics and other factors are still debated, there is a consensus amongst scientists of the main driving mechanism behind flocculation (Speers, 2012).

### *2.2.1 Defining Flocculation*

The earliest description of flocculation was by Pasteur in 1876 and research has been conducted on it ever since (Salehizadeh et al., 2001). Various definitions have attempted to describe flocculation, however, a combination of these four definitions below best define it for the purposes of this research.

> *"Yeast flocculation is the non-sexual aggregation of yeast cells into clumps, dispersible by ethylenediaminetetraacetic acid (EDTA) or specific sugars"* (Stratford et al., 1992).
>
> *"flocculation is a reversible, asexual and calcium dependent process in which cells adhere to form flocs consisting of thousands of cells which then separate from the medium"* (Verstrepen, 2003).
>
> *"Yeast flocculation is governed by the competition between electrostatic repulsion (nonspecific interaction) and polysaccharide-protein bonds (specific interaction)* (Kihn et al., 1988).
>
> *"the phenomenon wherein yeast cells adhere in clumps and either sediment rapidly from the medium in which they are suspended or rise to the medium's surface"* (Speers et al., 1992).

A conjunction of these four definitions will serve as the definition of flocculation throughout this work.

### *2.2.2 Mechanism of Flocculation: Research and Theory*

The current theory of flocculation which is agreed upon by various yeast physiologists and brewing chemists is the zymolectin interaction theory. The theory states that yeast cell walls

contain proteins (zymolectins) and carbohydrates (mannans) that bind with one another in a lectin-like fashion.

A zymolectin is an anchored yeast cell wall protein that contains one or more mannose or gluco-mannose binding sites (Hsu et al., 2001). They are similar to glycoproteins, but differ because they have specific roles and may not be divalent. The theory is supported by research preformed using pronase (Speers et al., 1993) which degrades zymolectins. The addition of this pronase caused irreversible flocculation which was explained by the degredation of the zymolectins. Furthermore the addition of papain (protein cleaving enzyme) caused permanent deflocculation. Generally brewing yeasts have between $3.18 \times 10^6$ to $7.33 \times 10^6$ zymolectins on the cells surface.

This theory depicted in figure 2.5 has maintained validity where other theories have come up short. It not only describes how the aggregates are formed, but it states the role of calcium and describes how coflocculation (non-flocculating aggregating with flocculating) and mutual flocculation (two non-flocculants flocculating together) is exhibited. While this theory does have a lot of evidence to support it, other factors such as cell surface hydrophobicity play a role and this topic is still an area of debate.

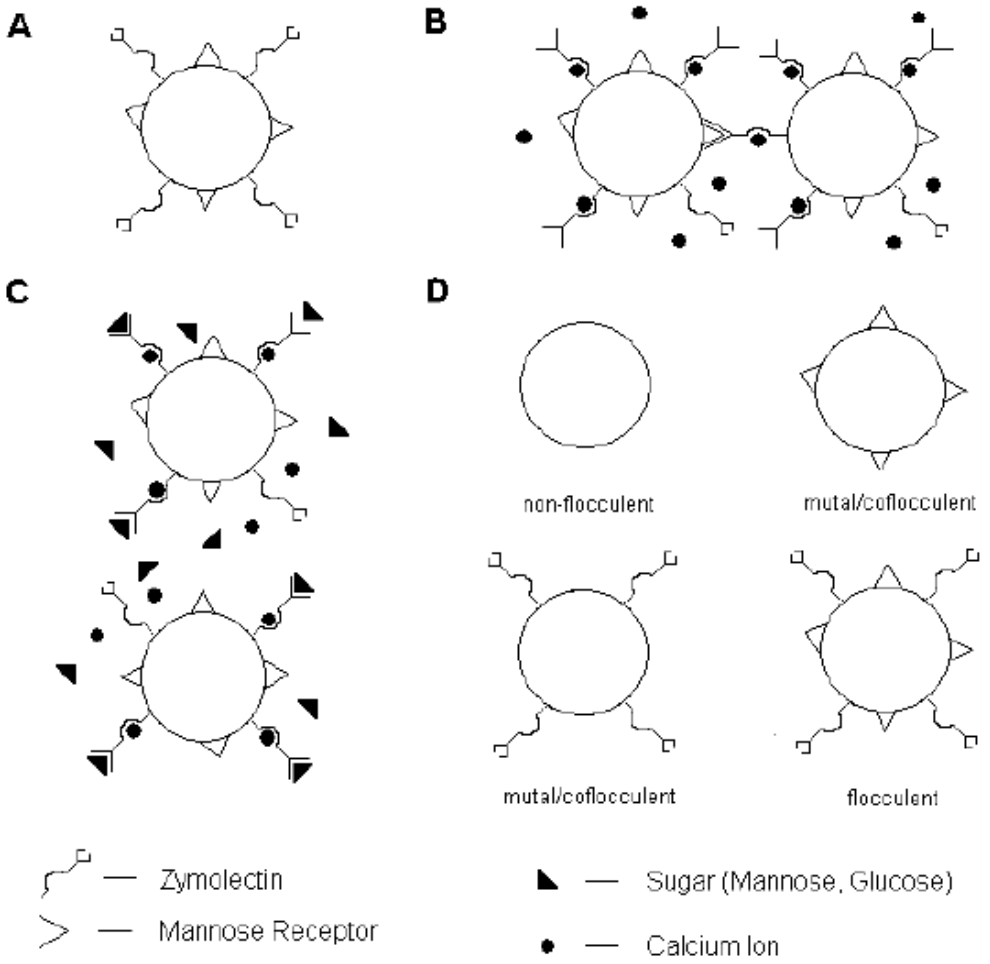



Figure 2.5 Zymolectin interaction theory of yeast flocculation: A) Potentially flocculent yeast B) Flocculent yeast in Ca2+ media. C) Flocculent yeast inhibited by specific sugars. D) Potential mutual and coflocculating yeast with non-flocculating and regular flocculating yeast as comparisons. Note: scale of zymolectins exaggerated to display mechanism of flocculation. (Lake , 2008)

### *2.2.3 Factors Affecting Flocculation*

While the zymolectin interaction theory does provide the most credible explanation to the mechanism of flocculation, it does not explain why certain yeast strains do not exhibit flocculation or why flocculent yeast do not flocculate in certain conditions. The reason for this is because flocculation is affected by a multitude of parameters displayed in Table 2.1. These factors have been divided into three categories, and these three categories are agreed upon in the associated literature to affect flocculation (Speers, 2012; Kihn et al, 1988; Quain et al., 1981);

I. Genetic
II. Environmental
III. Physical

Table 2.1 Factors affecting flocculation and their associated categories (Lake, 2008)

| Factor | Category |
|---|---|
| Phenotypic Expression | Genetic |
| Yeast viability/vitality | Genetic/Environmental |
| pH | Environmental/Physical |
| Temperature | Environmental |
| Nutrient content | Environmental/Physical |
| Alcohol | Environmental |
| Oxygenation level | Environmental |
| Cell-Surface Hydrophobicity | Physical |

While no genetic analysis was conducted throughout the work in this report, understanding the information presented in this portion is pertinent to understand the complexities and uncertainties of flocculation. In the early 1980's it was proposed that flocculation was under genetic control and therefore a heritable trait passed on in successive generations. This proposal was strongly supported when three phenotypes from S. cerevisiae were discovered. These phenotypes include three categories of genes; FLO, NewFlo and MI (Kihn et al., 1988; Powell, et al., 2003).

While all three phenotypic categories have been shown to affect flocculation, conflicting results from laboratory testing have not yet conclusively determined the extent of how these genes are expressed in industrial settings (Jin et al., 2000; Speers et al., 1992). The three categories involve 1) encoding for molecules required for flocculation, 2) encoding for activators of the molecules and 3) genes that encode for changes in yeast physiology. While the industrial expression of these genes is not fully understood, it is agreed that the FLO family of genes are the main contributors to flocculation as they code for the production of zymolectins. Further research into the FLO family of genes has confirmed that FLO1 and FLO10 are essential to flocculation in some environments. FLO1 genes encode for cell wall proteins, which when degraded by proteases will cause permanent non-flocculation and knocking out the FLO10 gene left yeast with the inability to flocculate in the presence of mannose (Li et al., 2013).

It should be noted that the genes associated with flocculation have been found to be the most variable property of brewing yeast (Seong et al., 2006). The reason for this variation is due in part to FLO gene's abundance of serine/threonine repeats. These repeats are prone to mutations and these mutations lead to strains with variable flocculation characteristics after only a small amount of successive generations (5-15 generations) (Powell et al., 2003). As brewers will repitch yeast for economic reasons, it is important to analyze the yeast and/or routinely propagate new yeast to avoid clarification/flocculation issues.

While many of the factors listed in Table 2.1 are environmental, not all of these factors will have a noticeable effect in relation to industrial practises. The temperature, pH, dissolved oxygen, and nutrient levels have all been shown to affect flocculation either negatively or positively (with conflicting findings) however, in industry the ranges analyzed in laboratory are not in-line with industrial realities. In practise, brewing has a fairly consistent range within these parameters. This

range generally (with the exception of some beer styles) creates an ideal situation for the presence of flocculation if other parameters are met (Speers et al., 2003).

An interesting environmental factor that has been met with drastically different findings throughout the literature is the level of ethanol's effect on flocculation. Generally in brewing ethanol levels are below 7% ABV (exempting high gravity brewing). The effect of ethanol has shown to have; no effect, negative effects and positive effects (Seong et al., 2006). The divergent findings probably have more to do with the strains of yeasts used for analysis and how the fermentations were conducted. While ethanol's variable effect on yeast flocculation in laboratory testing is most likely linked to strain dependence and intrinsic factors (genetics, generation number) of the yeast itself, speculating how ethanol may affect flocculation is important due to multiple suggestions that yeast flocculate to preserve survival in inhospitable environments. Ethanol is toxic to yeasts, and some researchers believe that the development of flocculation was for survival when in extreme conditions. It has also been hypothesized that ethanol's presence at gradually increasing levels will alter the state of the cell walls causing physical changes which are generally advantageous to form aggregates (Jin et al., 2000).

There are two main physical factors associated with flocculation. The first of these factors are orthokinetic forces. This force forms the supported mechanism driving the collisions caused by agitation during fermentation. These forces are driven by the turbulent flow created during fermentation via $C0_2$ evolution and they lead to electrostatic repulsions and attractions (Hsu et al., 2001).

Cell-surface hydrophobicity (CSH) has also been noted to be important in cell interactions. Most of the pertaining literature suggests a correlation between increased CSH and the formation of

floccs (Smit et al., 1992). The associated findings are supported by the zymolectin-interaction theory, as high CSH is linked to high protein concentration in the yeasts cell walls. CSH also increases throughout the fermentation, which correlates well with the multitude of studies that show flocculation after greater than 90% attenuation (Jin et al, 2000). CSH has been suggested to be one of the most crucial parameters, but does not drive flocculation alone.

## 2.3 Premature Yeast Flocculation

In section 1.3 PYF was briefly introduced. This section delves deeper into the topic and forms a thorough background into the issue and the driving forces behind it.

### *2.3.1 Defining Premature Yeast Flocculation*

Similarly to flocculation, the understanding of PYF's cause(s) and mechanisms are still an area of active research. The topic is not fully understood and many times the research conducted does not thoroughly define the interpretation of what PYF actually entails. As the exact mechanisms driving it are unknown, and no single component (and level of the component) has been isolated to be present in PYF, industry and academia generally focus on fermentation performance for how PYF is defined and Figure 2.6 displays a visual representation of this. Utilizing a combination of various definitions acquired from literature the "basic" definition of PYF is '*the faster than typical flocculation leading to a quality defect*' (Bamforth, 2011; Adler et al., 2012). Because of the complexities of the extrinsic and intrinsic variables affecting flocculation (discussed in section 2.2), generalizing a definition of PYF via fermentation performance is not ideal. This "basic" definition used by both industry and some academic studies does not draw a distinction between 'true PYF' and general fermentations issues (MacIntosh et al., 2012).

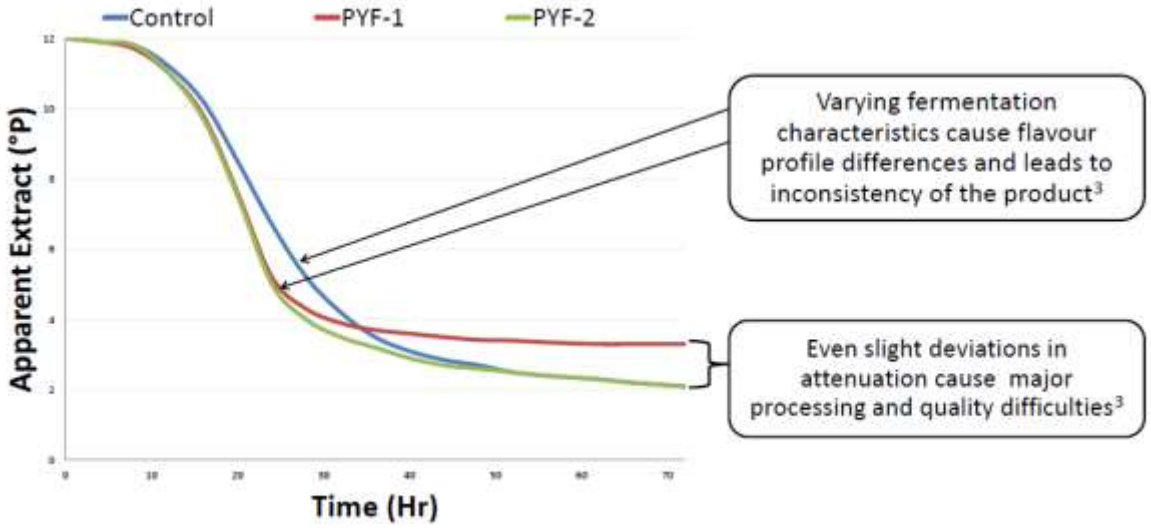



Figure 2.6 Visual Overview of how PYF affects fermentation (Adler et al, 2012).

The basic definition of PYF stated above groups together production issues leading to PYF exhibiting fermentations and 'true PYF'. One of these issues that can yield fermentations that mimic 'true PYF' include poor oxygenation. This issue may yield the exact same fermentation characteristics as 'true PYF', however, these issues could be solved with stricter quality assurance programs and yeast population maintenance (Bamforth, 2011). 'True PYF' fermentations are related to a specific factor (compound or group of compounds) that would only yield characteristic fermentations if it was removed. Therefore, for the purposes of this study 'true PYF' and PYF will be linked to the presence of this factor and negate production issues leading to PYF characteristics.

Another difficulty with using the basic PYF definition is the general trend in studies that define PYF to yield "Premature" or faster/early flocculation. Using this definition somewhat different flocculation characteristics during fermentation may not be recognized. Therefore, if the presence of these 'PYF factors' could slow down or alter flocculation patterns (causing quality defects), they may not fit into what is typically regarded as PYF. For this reason, this study will associate

any significant change in flocculation characteristics caused by the presence of a 'PYF factor' will be termed PYF. Changing the term to 'Factor Induced Altered Flocculation' (FIAF) could potentially clarify the definition, however, PYF is very much engrained in the scientific literature and many of the studies do show early or premature flocculation characteristics when the factor is present. For these reasons this work refers group PYF and FIAF together.

### *2.3.2 Industrial Issues Arising from PYF*

The brewing industry prides itself on quality. Whether discussing quality of raw materials, to the products head retention (List of other quality parameters in Table 1.1), quality and beer are very much intertwined. The ramifications of creating a product that does not fall into brand parameters can be detrimental to the company's reputation and could have a drastic effect on sales (Bamforth, 2011). For these reasons research has been ongoing into PYF in the brewing industry for over half a century.

PYF is an issue that creates an assortment of quality issues in the brewery. Occurrences of PYF have been linked to a variety of issues and these issues rarely come singularly. The primary and most drastic issue is the loss of attenuation (reduced alcohol production and high residual sugars). This leaves the brewery with a product that does not fit into its brands attributes. In addition, it may not meet government regulations as it may not meet label requirements. The issue of attenuation always comes with flavour profile inaccuracies due to the lower alcohol and also variations in fermentation parameters (Speers et al., 2006). Therefore, if PYF occurs and affects ethanol production, it will also affect the residual sugar level of the product.

PYF has also been linked to a variety of secondary issues. These secondary issues include; poor yeast health, high diacetyl levels, higher susceptibility to microbial contaminations, increased $SO_2$

levels, and lower carbonation levels post fermentation (Speers et al., 2012). All of these quality issues are directly linked to yeast viability/vitality, and when yeast exhibit PYF, they tend to exhibit fermentation performance similar to stressed or mutated yeast. These secondary issues not only have direct effect on the batch where PYF arouse, but some of these issues will cause production difficulties as the yeast may not meet standards for re-pitching. Recent findings (displayed in Figure 2.7) by Panteloglou et al showed that repitching yeast that was present in a PYF fermentation yields similar results to the original occurrence of PYF from wort containing the PYF factor. Therefore the secondary affects can cause just as much financial and logistics issues as the primary issue itself.

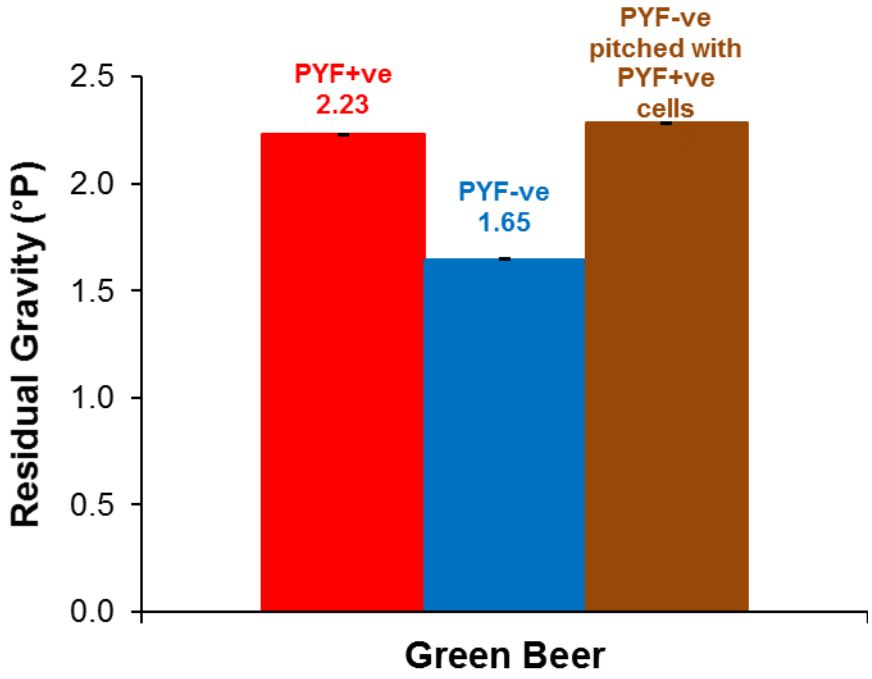



Figure 2.7 Residual gravity left after fermentations with a PYF positive malt (red), a PYF negative malt, and a PYF negative malt with yeast cells that have been exposed to a PYF positive malt in a previous fermentation (Panteloglou et al., 2012)

When yeast falls out of suspension early, a major problem beyond possible attenuation issues is the increase diacetyl rest time. Frequently brewers making lager style beers will increase the temperature of the fermentation near the last 5% of attenuation to speed up the process of Diacetyl reduction. The amount of YIS has been proven to be a large factor in the time it takes to reduce this compound and PYF can lead to this delay (Hsu et al, 2001).

### *2.3.3 The "PYF Factor"*

Using the description described above it becomes difficult to discuss the "factor" when researchers are not currently assured what it is. There have been over ten descriptions of factors that have been linked to PYF, some of which are drastically different in components and molecular weights. Focus has shifted to factors which include at least one or combines an arrangement of a large polysaccharide containing arabinoxylan, a protein/peptide fraction and acidic compounds (Herrera et al., 1991).

The factor is believed to be predominantly associated with the husks of malt, and research conducted removing the husks of PYF inducing malt prior to mashing displayed similar fermentation characters to control malt samples (Herrera et al., 1991). Beyond experimental data, the husk is predominantly composed of what is believed to be the constituents of the PYF factor (Van Nierop, 2004).

β-Glucan and Arabinoxylan make up most of the non-starch constituents of barley. β-glucans have been researched thoroughly and are linked to filtration issues, whereas the arabinoxylan polysaccharide found in the hemicelluloses of plants is far less understood (Speers, 2003). Arabinoxylan in barley is both soluble (30%) and insoluble (70%), and the insoluble portion forms a gel. It is acidic, of high molecular weight and makes up 3.5-11% of the barley grain (Egi, 2004).

Some areas of the grain have highly concentrated portions of arabinoxylan and some areas have for less. Figure 2.8 shows a microscopic section of a barley grain and the corresponding amounts of arabinoxylan and Figure 2.9 displays the chemical structure. As seen in Figure 2.8, the largest portion of arabinoxylan is present in the husk and aleurone. Figure 2.9 displays 5 enzymes which interact with arabinoxylan and these interactions alter the structure (Gamlath, 2008). As these enzymatic alterations are not very well understood, little information can be drawn as to how they directly associate to form PYF, however, research does conclude that this complex polysaccharide does survive the malting and brewing process and they make it into the finished product (Herrera, et al., 1991).

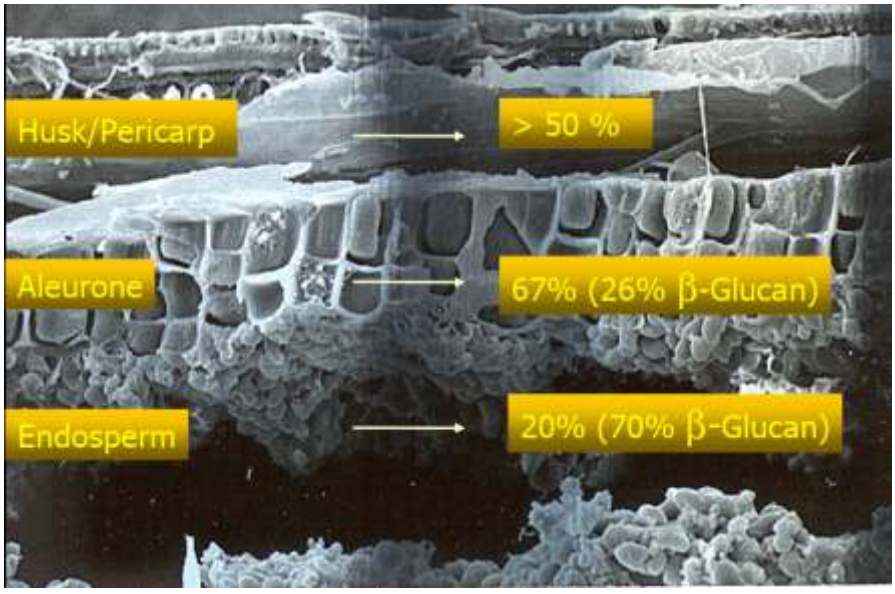



Figure 2.8 Composition of arabinoxylan and β-glucan within a barley grain (Gamlath, 2008)

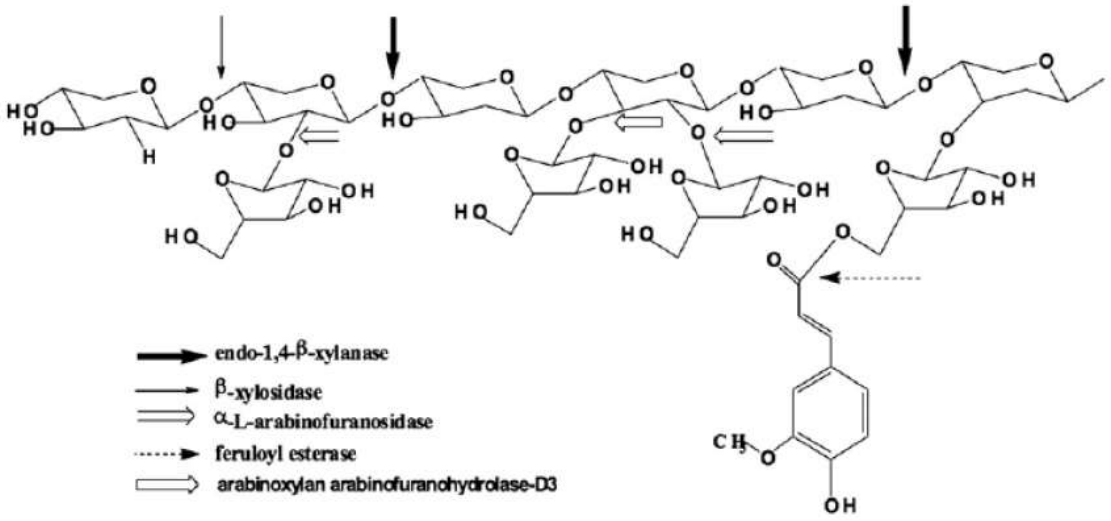



Figure 2.9 Structure of arabinoxylan and the sites of degradation enzymes (Lake, 2008)

The proteins and peptides associated with the PYF factor are linked to the plants defense mechanisms to external environmental factors. The barley plant produces high amounts of these peptides when infectious microbes are present, and these exhibit anti-microbial effects against the infection (Macintosh et al., 2014). The peptides are classified as cationic antimicrobial peptides (CAPs) and they can be found in many plants other than barley. In barley the CAP's tend to be small and numerous types have been found. With relation to brewing, lipid transfer proteins (LTPs) have been of particular interest because they are stable enough to withstand mashing. CAPs composition, amphipathicity, cationic charge and small size in barley allow them to enter cell membranes of microbial "invaders" and disrupt growth, inhibit attachment and lyse cells foreign cells (Panteloglou, 2013).

A final probable component of the PYF factor is a polymer-bound hydroxycinnamic acid derivative (PBHAD) (Panteloglou, 2012). The PBHAD commonly linked to the PYF factor is the natural phenol ferulic acid which has both an antioxidant and anti-carcinogenic compound due to its ability to scavenge free radicals. Displayed in Figure 2.10 is the structure of ferulic

acid, and if you reference Figure 2.9 you can clearly see the attachment location on arabinoxylan for the acid. The association of arabinoxylan and ferulic acid makes research into the area very complex. Beyond its relations to PYF, ferulic acid can be decarboxylated to form 4-vinyl guaiacol which forms medicinal/clove aromas in certain styles of beer (Vanbeneden, 2008).

Figure 2.10 Structure of Ferulic Acid

The components of PYF (arabinoxylan, proteins, acidic compounds) are present in all malt, however, an external influence is believed to alter levels and cause PYF fermentation characteristics when used to create wort for brewing. These external influences, such as fungal infections, are hypothesized to create higher than typical levels of PYF factor which alter fermentation performance (Macintosh et al, 2014).

### *2.3.4 PYF Mechanisms*

When looking into the mechanism of PYF, it is important to understand that the PYF factor being created at higher than typical levels is caused by external forces, and then this increased level of factor causes PYF to be exhibited in the fermentation by changing the physical and/or genetic forces driving flocculation. Therefore, the formation of a PYF exhibiting fermentation has two steps.

I. External influences increase the formation of PYF inducing factors that are otherwise not present in high quantities
II. The presence of high levels of PYF factor cause a physical change in flocculation by altering how cells interact with one another

The first part of the mechanism has been linked to fungal infections of malt which cause the barley to create more PYF inducing factors. This finding is supported by laboratory research manipulating infection levels as well as field studies which have linked the occurrence of PYF to high infection rates. Figure 2.10 illustrates the theory behind the formation of increased PYF inducing factors. The theory suggests that fungal infections lead to increased production of HMWPs and CAPs. Whether the infection occurs in the crop field or the malthouse is still debated, however, it is agreed upon in the literature that the factor's increased generation is triggered by fungal infections.

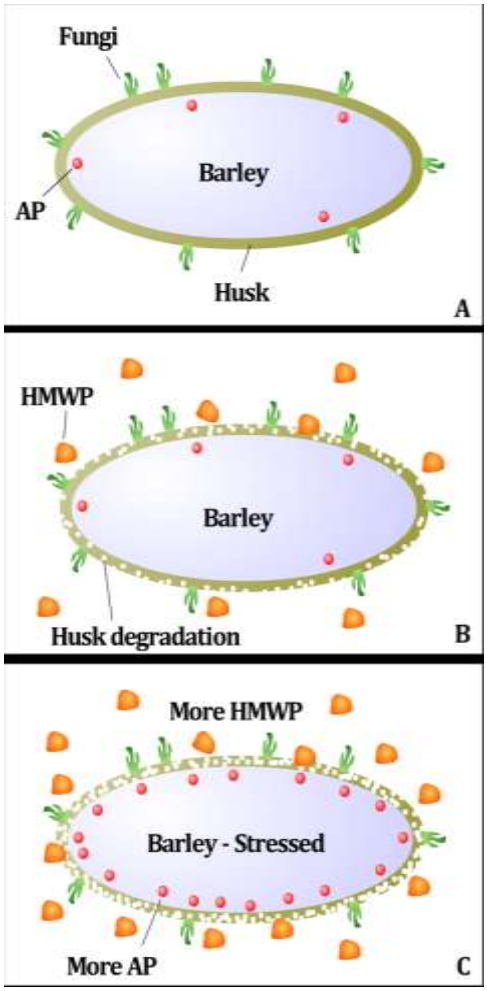



Figure 2.11 Theoretical mechanism behind increased PYF-inducing factor formation when barley experiences a fungal infection. A) fungus invades barley, B) fungus enzymatically degrades the husk of barley initiating the formation of high molecular weight polysaccharides (HMWP), C) Stressed barley also increases the formation of Antimicrobial peptides (AP) (Panteloglou, 2013)

Once the fungal infection has caused increased PYF inducing factor generation, the alteration of yeast interactions to form PYF can occur. This is proposed to occur via two varying pathways displayed in figure 2.11. In figure 2.11A, the links are caused by an acidic HMWP (generally accepted to be arabinoxylan) which cross-link the lectin-like surface proteins of yeast before all of

the available sugars are consumed. This is presumed to occur because the HMWP outcompete sugar due to their high affinity for lectin-proteins (Panteloglou, 2013).

In Figure 2.11B both the HMWP and the APs (CAPs) work together to inhibit the uptake of sugar and initiate flocculation between cells. CAPs ability to weaken yeast sugar uptake have been well documented (Panteloglou et al., 2012). This proposed mechanism has strong support because it is supported by numerous studies and links the genetic and physical (hydrophobic/electrostatic) linking that takes place in PYF. However, while it is supported by many researchers, the mechanism does not fully justify all research findings. Further research with standardized tests will likely confirm aspects of this mechanism, and a verifiable and unified test for PYF is essential for this purpose.

## 2.4 Threshold of Premature Yeast Flocculation

### *2.4.1 Evidence Supporting PYF Threshold Theory*

A small amount of work has been conducted to look into the effects of varying amounts of PYF(+) malt in conjunction with standard PYF(-) malt (Adler et al., 2012). While no direct percentage has been attributed to PYF exhibiting fermentations, it has been found that low amounts of PYF(+) malt wont exhibit PYF fermentations, whereas higher amounts will. It should be noted here that small/large percentages of PYF malts are relative as a malt with high amounts of the PYF factor would exhibit PYF fermentations at low percentages. The added complexity of varying PYF inducing malts and the associated yeast strain susceptibility to PYF make the threshold theory multivariable and only definitive when looking at a specific yeast strain with a specific crop of malt. For this reason, it is crucial to eliminate year to year variation in malt for the purposes of analysis.

## 2.5 Detection of Premature Yeast Flocculation

Due to the complex nature of PYF and the presence of PYF inducing factors in all malts, chemical and enzymatic tests have yielded little success. Simply testing for the presence of the factor is not sufficient, and levels of the factors causing PYF are not understood. Furthermore, the interactions between PYF inducing factors and how they interact with yeasts must be controlled for and add another dimension of uncertainty to tests of this nature. Both the malting and brewing industries have utilized small scale fermentations and monitored them using a variety of techniques to analyze malt for presence of PYF characteristics (Lake, 2008).

### *2.5.1 Small Scale Fermentation Assays*

To analyze malt samples for PYF potential the industry has relied heavily on monitoring small scale fermentations. To do this almost all PYF testing protocols rely on analyzing the turbidity (monitoring yeast in suspension) and sugar content throughout fermentation (Lake, 2008). The major differences between the tests employed regard the fermentor size and length of test. Some tests utilize tall glass tubes that require >1L of wort and take over 168 hours, others require as little as 50ml and can be completed in as little as 48 hr. The ideal test requires 1) small amounts of wort 2) is completed quickly, and 3) is easily fit into a regular work schedule. (Macintosh et al., 2014; Powell et al., 2003). A method developed at Dalhousie University was approved by the ASBC in 2012 and it successfully meets these three criteria. While standard methods do exist within the various brewing societies, no method is being used universally and methods utilized by many maltsters and brewers are not published.

### *2.5.2 Issues with Academic and Industrial Detection Methods*

An integral concern when analyzing fermentation tests to determine if malt samples exhibit PYF capability is the selection of control malt. Because malt is a natural product, it is essentially impossible to produce control malt to be utilized for these tests. Even small variations in enzyme content can yield significantly different results which will cause deviations from previous samples. This is of strict importance because all other areas; mashing protocol, boil temperature/length, fermentation size/length and type of analysis can be controlled for very closely using advanced techniques. The need for a test which can limit control malt variation to easily distinguish between PYF (+) and PYF (-) samples is vital. Also, the ability to determine if a fermentation test corresponds with production performance is of major industrial importance (MacIntosh et al., 2012).

### *2.5.3 Correlating Miniature Assays with Industrial Performance*

Even though industry relies on small scale fermentations for predicting malt fermentability and PYF characteristics, very little work has been done correlating the tests with large scale fermentations. This correlation is needed because poor correlations are reported between these tests and industrial performance. The ability to correlate these tests to industrial performance is of utmost importance because PYF analysis goes beyond the lab bench and affects a breweries production output and financial success. Therefore, any test utilized for malt analysis should be assessed for its ability to correlate with industry and judged accordingly.

To use the MFA developed at Dalhousie University for research into PYF, it was crucial to develop an understanding of how the assay correlates to industrial performance. For this reason an initial study was conducted over a four month period to determine how suitable the MFA assay is to monitor industrial fermentations (Variations between miniature fermentations and the correlation to industrial fermentations are discussed in section 4.5.3). This study adds to a combined effort between colleagues at Dalhousie and part of the work was published in the Journal of the American Society of Brewing Chemists. The work in this report overviews the findings of the published research (section 5.1.1) and expands on how the test shows promise for analyzing/researching PYF in section 5.1.2.

Once the suitability of the MFA was analyzed in the research entitled the "Suitability of the Miniature Fermentability Method to Monitor Industrial Fermentations", further trials were conducted. The other trials conducted are intended to enhance our understanding of PYF and the research utilized the MFA. The trials analyzing the potential threshold of PYF are analyzed in sections 5.1-5.2, whereas the stability of the PYF factor is analyzed in 5.4. Section 2.5.4 of the literature reviews research that supports the use of the MFA.

A MFA was run simultaneously with two sizes of industrial fermenters for a total of 6 trials (3 trials at 19.6HL and three trials at 8.5HL) using the parameters from section 4.5.3. The parameters of the logistic model (Equation 4.2) and the results of an F-test for these trials are shown in Table 5.1. The density attenuation is modeled from the parameters in Table 2.2 and displayed in Figure 5.1.

Table 2.2 Parameters from the correlation study fit to the logistic model for 6 trial fermentations

| Experiment | Fermentor Size (L) | $P_i$ (°P) | $P_e$ (°P) | B ($hr^{-1}$) | M (hr) | Coefficient of Determination ($r^2$) | F Test P-Value |
|---|---|---|---|---|---|---|---|
| Craft Brewery 1 | 1960. | 12.4 (0.39) | 2.17 (0.12) | -0.082 (0.007) | 25.6 (1.17) | 0.998 | <0.0001 |
| Assay | 0.015 | 13.7 (0.75) | 3.13 (0.14) | -0.072 (0.008) | 21.7 (2.26) | 0.988 | |
| Craft Brewery 2 | 1960. | 11.3 (0.08) | 2.31 (0.09) | -0.120 (0.004) | 35.5 (0.36) | 0.999 | <0.0001 |
| Assay | 0.015 | 11.7 (0.59) | 3.24 (0.36) | -0.088 (0.013) | 33.7 (2.11) | 0.978 | |
| Craft Brewery 3 | 1960. | 12.1 (0.05) | 2.90 (0.56) | -0.150 (0.006) | 25.1 (0.17) | 0.998 | <0.0001 |
| Assay | 0.015 | 12.2 (0.39) | 3.90 (0.24) | -0.090 (0.009) | 29.5 (1.39) | 0.981 | |
| Brew-Pub 1 | 850. | 10.7 (0.07) | 1.51 (0.18) | -0.070 (0.003) | 41.7 (0.63) | 0.999 | 0.4645 |
| Assay | 0.015 | 10.7 (0.43) | 1.51 (1.12) | -0.068 (0.012) | 43.7 (3.47) | 0.979 | |
| Brew-Pub 2 | 850. | 11.1 (0.33) | 2.90 (0.19) | -0.155 (0.04) | 27.5 (1.74) | 0.999 | 0.5851 |
| Assay | 0.015 | 11.1 (0.33) | 3.22 (0.14) | -0.152 (0.02) | 28.9 (1.17) | 0.991 | |
| Brew-Pub 3 | 850. | 13.7 (0.59) | 2.35 (0.29) | -0.086 (0.01) | 33.3 (1.63) | 0.997 | 0.0048 |
| Assay | 0.015 | 13.7 (0.53) | 2.30 (0.25) | -0.08 (0.01) | 38.6 (1.55) | 0.992 | |

† The asymptotic standard error of each parameter was determined by taking the square of the product of the Sy.x (the standard deviation of the residuals) and the parameter's diagonal element of the covariance matrix (the dispersion matrix) (MacIntosh et al., 2012).

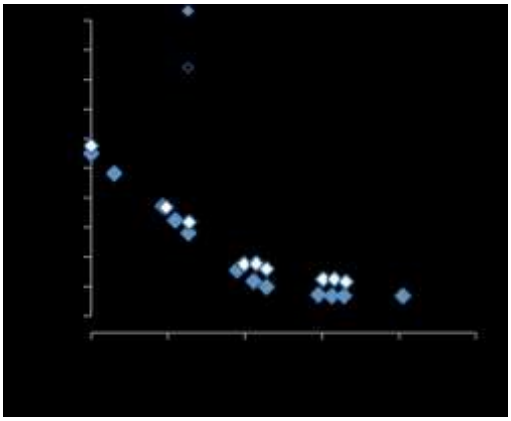
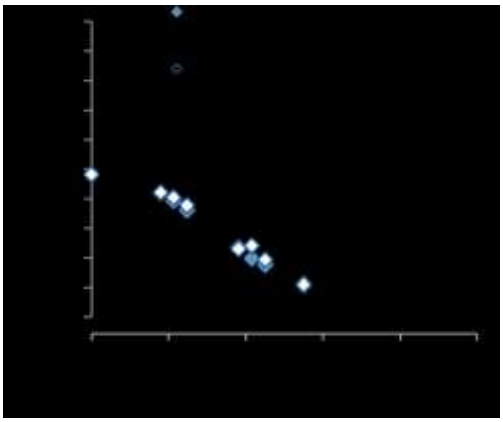
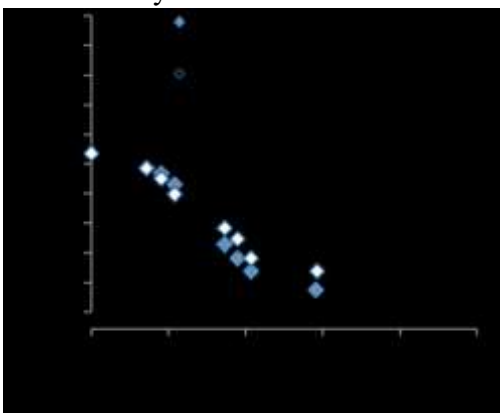
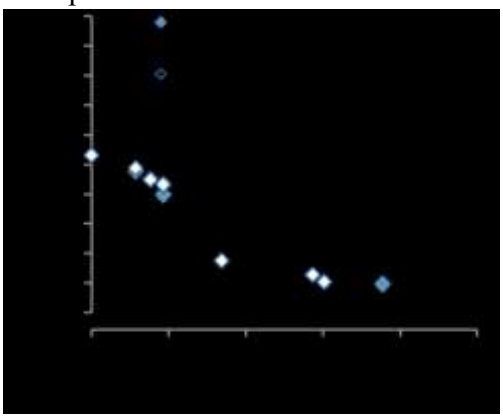
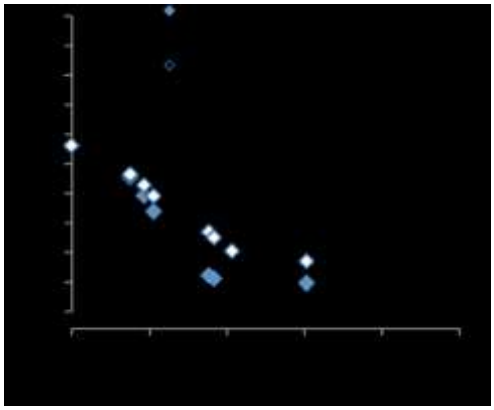
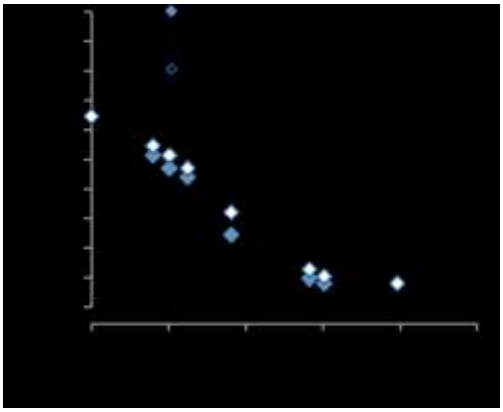
| Comparison of Craft Brewery scale (19.6 hL) fermentation to 15 mL assay | Comparison of Brew-pub scale (8.5 hL) fermentation to 15 mL assay |
|---|---|
| Craft Brewery 1 | Brew-pub Trial 1 |
|  |  |
| Craft Brewery 2 | Brew-pub Trial 2 |
|  |  |
| Craft Brewery 3 | Brew-pub Trial 3 |
|  |  |

Figure 2.12 Density attenuation for 6 trials modeled using Equation 4. (MacIntosh et al., 2012)

It is apparent from analyzing Table 2.2 and Figure 2.112 that the brew-pub and MFA fermentations displayed very similar fermentations. Two trials were statistically the same ($P > 0.05$) and one was nominally different ($P = 0.0048$). Conversely, the results between the craft brewery (19.6hL) and the MFA did not display very similar fermentations and they were significantly different ($P < 0.05$). Although significantly different, the craft brewery and the MFA differences were predictable and the MFA had higher densities after fermentation ($1.1 \pm 0.2$ °P). While the larger craft (19.6 hL) fermenter's yielded different fermentation parameters, it should be noted that the time to reach full attenuation was statistically the same.

By using the logistic models information displayed in Table 2.2, calculated shear rates were determined and they are shown in Figure 2.13. This variation in shear rates between industrial samples and the MFA are linked to the height of the fermenter. The reason for this variation is due to reduced energy imparted into the medium via rising $CO_2$.

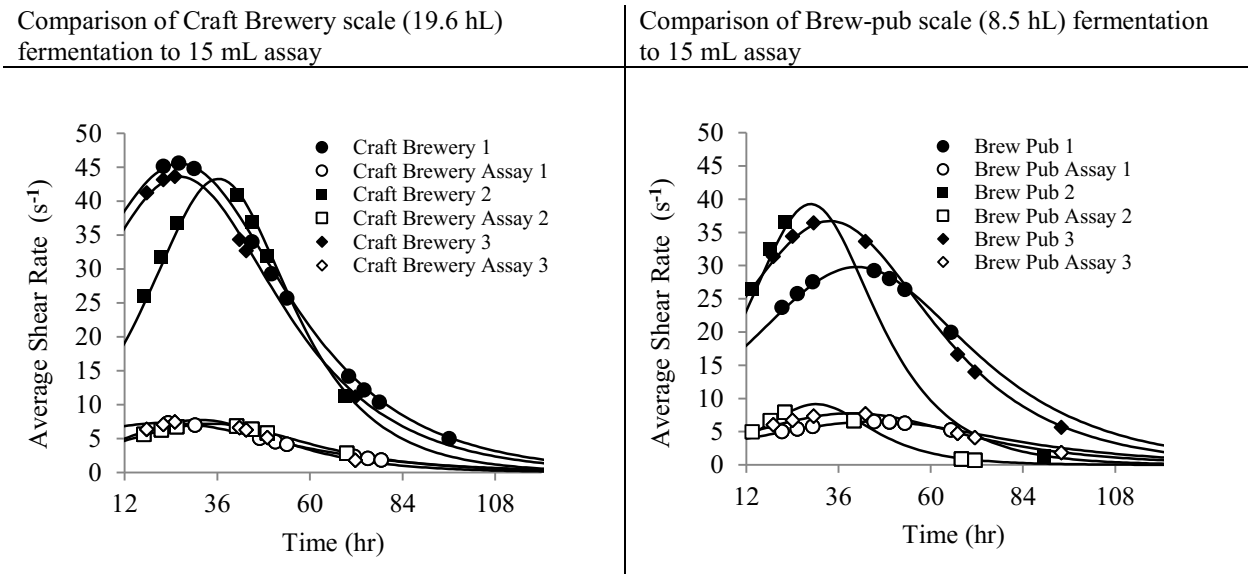



Figure 2.13 Comparison between 19.6 and 8.5 hL fermentors and the MFA assay (MacIntosh et al., 2012)

From this data collected it is apparent that the fermentor size plays a key role in the potential fermentability of wort. The findings in this study are most likely explained by the variation in energy imparted to the wort between the industrial and lab scale. The reduced shear rate in the MFA is hypothesized to be significant enough to decrease the number of YIS when compared to the larger fermentations. This lack of YIS within the MFA leads to a reduction in yeast activity which in turn causes higher densities once full attenuation is reached. The diminished level of shear did not appear to affect the brew-pub size fermentor's attenuation (8.5hL), therefore the MFA may only work directly if the fermenters are of certain dimensions.

While fermenter size is noted to vary the fermentability of wort between the MFA and large scale brewing, the results from the MFA appeared to create consistently different results and yield reproducible findings. Therefore, the MFA can be utilized in large scale brewing facilities and may prove to be a highly accurate tool to determine fermentation performance and identify if PYF positive malt.

### *2.5.4 MFA and PYF Prediction in Industrial Fermentations*

The results from the correlation study have shown that the MFA is a feasible method for consistently predicting wort fermentability. Looking beyond the scope of fermentability, the MFA must be able to identify control malt samples from PYF inducing malt samples to be an effective test for the determination of PYF potential. From the findings it is hypothesized that the MFA is able to be an effective method for identifying PYF potential for three main reasons.

The first reason supporting the MFA's ability to identify PYF is the tests proven ability to reliably and accurately determine the length of fermentation. Other researchers (Herrera et al., 1991; Lake et al., 2009; Speers et al., 1992) have noted that the length of fermentation has been reduced when PYF inducing factors are present. The cause of this reduction in fermentation time has been attributed to lowered yeast activity due to a loss of YIS.

The second area of support is the MFA's ability to create very robust fermentation models. Unlike other tests which rely on singular samples, this test utilizes destructive sampling which generates three points of measurement for all time intervals measured (12 times total per fermentation). This generates a model that is built on fundamental findings rather than perceived assumptions of YIS and statistical inferences as it is more robust than singular data points at given time intervals. Triplicate sampling is crucial for identifying minuet statistical differences. Due to the tests robust models, developing control or regular fermentation models is fairly simple. Once the test has been correlated (taking into account fermenter size and brewing techniques) to an industrial system it is easy to identify even small deviations from typical fermentations (assuming control malt is always within tight parameters).

The last reason that the MFA works as a suitable measure for determining PYF in malt, is due to the findings from recently published work that analyzed 45 trials of "control" malt to determine a strong average control band (Figure 2.14) which shows an acceptable range of absorbance characteristics for non-PYF inducing malt. PYF inducing malts did not fit within this band when assessed visually (Figure 2.15).

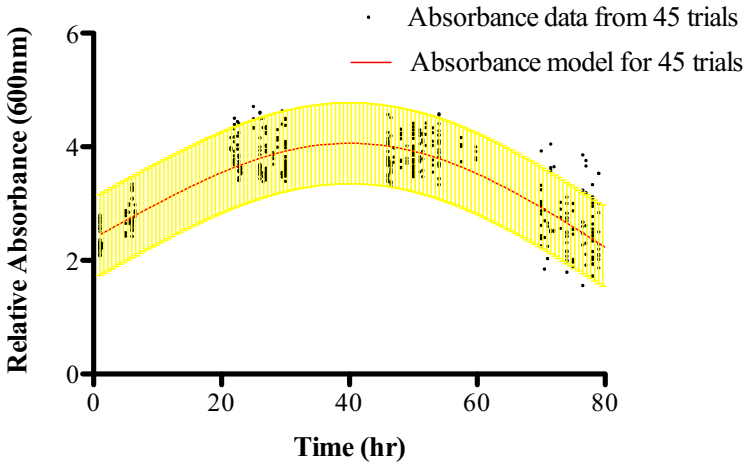



Figure 2.14 Absorbance data from 45 malt fermentations using ASBC Yeast-14 with the 80% "Control Band" Superimposed (Macintosh et al., 2014).

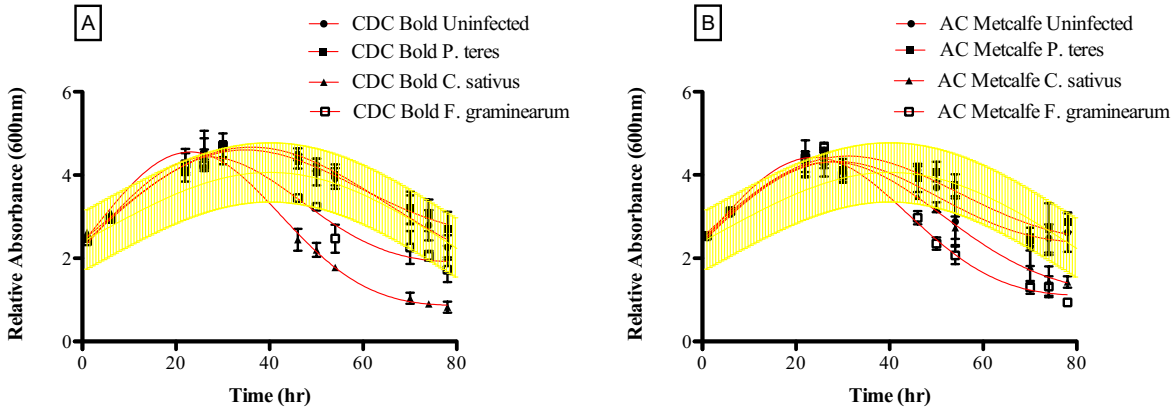



Figure 2.15 Relative absorbance of CDC Bold (A) and AC Metcalfe (B) fungally infected samples with 95% control band overlay generated from the 45 trial data (Macintosh et al., 2014).

The MFA has been proven to link well with industry for fermentability when the predictable variation from fermenter size is factored in. The presumptions acquired through analysis of the correlation study are further supported by the results of a collaborative study which found the MFA to be effective at determining a malts PYF potential. As the test has been shown to produce reliable results, further analysis into the threshold and stability of the PYF factor was analyzed using this method.

# Chapter 3. Objectives

Academic and industrial research focused on PYF is yet to fully understand the complexities of the topic. The work conducted in this thesis focuses on the following objectives to provide useful insights into PYF and aid in future research.

I. Understand the amount of PYF inducing malt needed to display PYF characteristics during fermentation
II. Remove the need for control malt when testing for PYF using the miniature fermentation assay by creating an alternative soaking method which can be used instead of mashing when testing for PYF
III. Inactivate or reduce the display of PYF characteristics via further processing post mash/ pre-fermentation

The hypotheses related to the three major objectives within the thesis research are:

I. Including small amounts of PYF inducing malt will not affect fermentations and a "threshold" must be reached before PYF characteristics are noticeable
II. Soaking suspected PYF-inducing malt will be sufficient for analysis when using the miniature fermentation assay
III. Further treatments of pre-fermented wort will inactivate/reduce the display of PYF characteristics due to PYF-factor degradation

# Chapter 4. Experimental Methods

Much of the experimental methods for this research followed the newly approved (2012) ASBCs Yeast-14 methods of analysis for miniature fermentation assays. This assay has proven to be very effective in analyzing PYF inducing malt samples in a collaborative study between 3 laboratories and within Macintosh et al. in 2012. Any deviations from Yeast-14 are clearly stated and all treatments are introduced and defined (Macintosh et al., 2014; Yeast-14, 2012).

## 4.1 Yeast Culture, Propagation and Counting

YEPD broth was prepared by dissolving 10 g/L yeast extract, 20 g/L bacteriological peptone, and 20 g/L D-glucose in 500 ml of distilled water. Once the broth was prepared it was autoclaved for 20 min and then cooled at room temprature. SMA yeast acquired from Wyeast laboratories (Odell, OR) was removed from an agar slant using a sterile loop and aseptically transferred into a 125-ml flask containing 50 ml of the YEPD broth. An autoclaved foam stopper is then placed on the flask to avoid contamination and the flask is then placed in an orbital shaker at 100 rpm for 24 hr at 30 °C.

The resulting slurry is then aseptically transferred into sterile 50 ml tubes, capped and centrifuged (3,000 rpm x g for 30 min). The resulting supernatant is discarded and replaced with a 50 ml of sterile distilled water (SDW) wash which is vortexed to re-suspend the yeast. The centrifugation step is then repeated twice for a total of three SDW washes. After the final wash the yeast pellet is re-suspended in SDW and vortexed.

The resulting yeast slurry is counted using methods from Yeast-4 and pitched at a rate of 1.5 x $10^7$ cells/ml into two 250 ml flasks which contain 100 ml of YEPD broth each. The flask is then

capped with a sterile foam stopper and incubated with the same parameters as the initial propagation. The resulting slurry is then washed using the same methods three times, re-suspended, and counted to prepare for pitching.

## 4.2 Malt and Malt Extracts

### *4.2.1 Malt Selection and Milling*

The control malt used was two-row barley malt which exhibits excellent fermentation performance (proprietary supplier information). 150 g samples of the malt are ground in a laboratory mill to adhere to ASBC's "fine" standard (Malt-4, 1975).

The PYF malt was supplied from a proprietary source and the predicted fermentation performance was characterized as PYF. Between grinding different samples, sufficient cleaning was provided on holding containers and contact points of the mill.

### *4.2.2 Malt Extract*

Malt extract was prepared using Difco$^{TM}$ malt extract broth supplied by Becton, Dickinson and company (Franklin Lakes, NJ). The contents of the broth (Table 4.1) are mixed with 150 ml of distilled water at 20 °C on a stir plate until a gravity of 14.4 °P is reached. As there was high variability between extracts used in the study, additions were made slowly to assure that 14.4 °P was reached by using a digital density meter (DMA Anton Parr, Richmond, NY) and routinely checking gravity.

Table 4.1 Approximate Formula* Per Liter of Difco Malt Extract Broth

| Ingredient | Weight (g) |
|---|---|
| **Malt Extract** | 6.0 |
| **Maltose, Technical** | 1.8 |
| **Dextrose** | 6.0 |
| **Yeast Extract** | 1.2 |

*Adjusted and/or supplemented as required to meet performance criteria of 14.4 °P.

## 4.3 Wort Preparation

### *4.3.1 Mashing*

Mashing milled samples was conducted in accordance with the ASBC standard mashing regime. The mash bath used had magnetic stirrers for agitation. The 150 g malt sample yields over 450 ml of high gravity (~13.9 °P) wort which is sufficient for analysis of the malt. Once the mash cycle is complete, the wort is filtered through fluted filter paper and deposited into a 1 L Erlenmeyer flask. The wort then goes through an autoclave cycle for 20 min at 121 °C. The now sterile wort is chilled overnight (never exceeding 12 hr) at 4 °C. Following this chilling stage, the wort sample is centrifuged at 3.31 x $10^3$ x g for 15min to remove the trub. The wort is then diluted to 12.6 °P with SDW and 410 ml of wort is measured out. 18 g of lab grade D-glucose is added to form a final wort density of 16.1 °P. The wort is then aerated with medical grade oxygen for 5 min at 20 °C.

The wort prepared from the malt extract broth did not undergo mashing and was prepared for the autoclave cycle once the steps in section 4.2.2 were followed.

### *4.3.2 Soaking Technique – Creating PYF Solution*

The soaking technique used in this experiment is a modified and down-sized version of work conducted by Herrara and Axcell in 2004 as well as Jibiki in 2006 (Jibiki et al., 2006). This modified technique involves soaking 150g of un-milled malt in 640ml of 20C DW for 4 hours while being stirred in a 3L Erlenmeyer flask with a 75mm stir bar.

After 4 hours of soaking and stirring, the grain/water solution is passed through a funnel with a plastic mesh (1 mm pore size) affixed to the outlet and passed into a flask. The remaining wet grain is wrapped in the plastic mesh and tightly pressed in a manual laboratory hand press (hand pressure 'squeezing' is also sufficient). The resulting turbid malt soak solution (MSS) is passed into a 500 ml erlenmyer flask through coarse fluted filter paper. The turbid PYF solution is then placed in the autoclave cycle ($3.31 \times 10^3 \times g$ for 15 min). The resulting MSS replaces fractions of the DW that are used in section 4.2.2 to create synthetic wort.

### *4.3.3 High PYF Sample Solution*

Creating samples that display high PYF are essential in determining the ability of a PYF screening method's validity. To create a high PYF sample solution, the steps in section 4.3.2 are followed in double quantities until the clarified MSS step is created from PYF inducing malt (alternatively control malt is used in the same fashion to create controls for these trials). Once the clarified MSS is formed, the sample is slowly boiled at 100 °C until half of the initial volume of the MSS has been boiled away. The resulting MSS container is rapidly chilled in an ice bath to reach 20 °C and synthetic wort is created from solution following the guidelines from section 4.3.2.

Once the synthetic wort is created from the High PYF solution it follows the same pathway as pre-autoclaved malt and malt extracts.

The added boiling step to reduce and add more PYF solution was developed to address the need for a very strong PYF inducing sample for analysis in this work.

## 4.4 Pre-Fermentation Treatments

The purpose of performing temperature treatments to the pre-fermented wort is to further our understanding of the stability of the PYF factor(s). As the PYF factor can undergo the rigours of brewing, these treatments attempt to diminish the factor's ability to eventually create PYF fermentations.

### *4.4.1 Boiling*

Three allocates of MSS formed from using the methods within 4.3.2 were placed in three separate beakers at 100 °C. At three separate time intervals (60, 90 and 120 min) the samples were taken off the hot plate and cooled at room temperature. The samples were then prepared for fermentation by using the methods in section 4.3.2. Distilled water was added to the samples after boiling to have enough solution for running the fermentations

### *4.4.2 Chilling*

Fully prepared synthetic wort created using DW and MSS (Sections 4.2.2/4.3.2) was subjected to chilling (5 °C) for 24 hours directly prior to fermentation in a 500 ml Erlenmeyer flask, stir bar and capped with aluminum foil. After the 24 hour period the samples were placed on a low heat plate until 20 °C was reached. The samples were then ready to be used in fermentation trials.

### *4.4.3 Freezing*

Fully prepared synthetic wort created using DW and MSS (Sections 4.2.2/4.3.2) was subjected to freezing (-30 °C) for 72 hours directly prior to fermentation in a 500 ml Erlenmeyer flask capped with aluminum foil. The samples were thawed prior to fermentation by placing the flask within a larger water filled beaker on a hot plate set to 65 °C. Once the sample was thoroughly liquefied, it was allowed to reach equilibrium at room temperature before being prepped for fermentation.

## 4.5 Fermentations and Monitoring

Once ready to be pitched with yeast all of the controls and treatments undergo the same handling and monitoring throughout the fermentations.

### *4.5.1 Test-Tube Fermentations*

Wort samples were inoculated at 1.5 x $10^7$ cells/ml with yeast slurry propagated using methods in section 4.1. The volume of slurry needed for inoculation is calculated and the corresponding amount is placed in the prepared wort.

Once inoculated, the pitched wort samples are transferred into 30 sterile fermentation tubes (agitating the sample by hand to assure even distribution of yeast) with a sterile PTFE boiling stone. The tubes are then capped with sterile sponges and placed on a rack within a water bath set to 21 °C. It is essential that all contact materials are fully autoclaved to assure that wild yeast and bacteria do not interfere with the experiment.

### *4.5.2 Destructive Sampling*

Sampling the fermentation tubes took place at 12 intervals (0, 1, 6, 22, 26, 30, 46, 50, 54, 70, 74, and 78 hr). A density measurement at time 0 hr just prior to pitching is taken from the wort flask using the digital density meter. The remaining samples are taken in triplicate and each individual sample is analyzed for density and absorbance.

To assess the samples for absorbance readings, the top portion (3.5 ml) of the samples are pipetted into a cuvette and placed in the absorbance meter. The absorbance meter is operated at 600 nm (measuring yeast in suspension) and a blank of DW is used for calibration.

Density measurements are performed by filtering the remaining wort into a clean test tube from the fermentation tube through a Whatman #4 filter. Once an adequate amount of wort has been filtered for measurement with the digital density meter, the samples are taken and the results logged.

### *4.5.3 Variations When Comparing to Industrial Fermentations*

Successful correlation was shown in Macintosh et al. between the MFA and industrial fermentations, some key factors were adjusted to more accurately isolate the fermenter size as the key variable being analyzed. This was accomplished by taking a wort sample (450 ml) directly from a fermenter (8.5 HL or 19.6 HL) once filled and pitched with yeast (Time 0 hr). The method of yeast pitching at the associated breweries caused significant agitation, and it is assumed that the contents were homogeneous. Because the samples were identical (same mashing regime, aeration, yeast strain, pitch rate, fermentation temperature) the only difference between the two fermentations is the size of the vessel. When the samples were pulled, limited headspace was left in the transfer flask to reduce the loss/pickup of dissolved gases. The samples were then quickly brought to the laboratory (~15 min travel), transferred into 30 X 15 ml test tubes with boiling chips,

foam bungs and then incubated in a water bath at 21 °C. The samples were then treated as if they were at time zero (beginning of monitoring) of a typical MFA sample. The samples taken from the MFA and the industrial fermentor's were taken at the same intervals and the industrial fermenter samples were brought into the laboratory for analysis at each time interval.

## 4.6 Calculations and Statistical Analysis

### *4.6.1 Absorbance Modeling*

The data collected from absorbance measurements (indication of YIS) during the MFA is analyzed via nonlinear regression which is a method of modeling that fits data by successive approximations. The modeling is performed within a variety of statistical software packages using a "tilted Gausian" fit (Equation 4.1). The parameters within Equation 4.1 are displayed in Table 4.1.

Equation 4.1 $$Abs(t) = Rt + Ae^{-\frac{1}{2}\left(\frac{t-\mu}{\sigma}\right)^2}$$

Table 4.2 Definitions of the Parameters for the absorbance non-linear regression

| Parameter | Definition |
|---|---|
| **Abs** | Absorbance obtained at a specific time |
| **R** | Rotation of the Gaussian curve |
| **A** | The Absolute amplitude (AU) |
| **μ** | Mid-point in hours |
| **σ** | Standard deviation of the curve |

Malt types and treatment variation can be compared quantitatively by using a global F test of equation parameters that was preformed within the Prism statistical software package (Graphpad Software, La Jolla, CA). A visual depiction displaying all of the parameters of Equation 4.1 are shown in Figure 4.1.

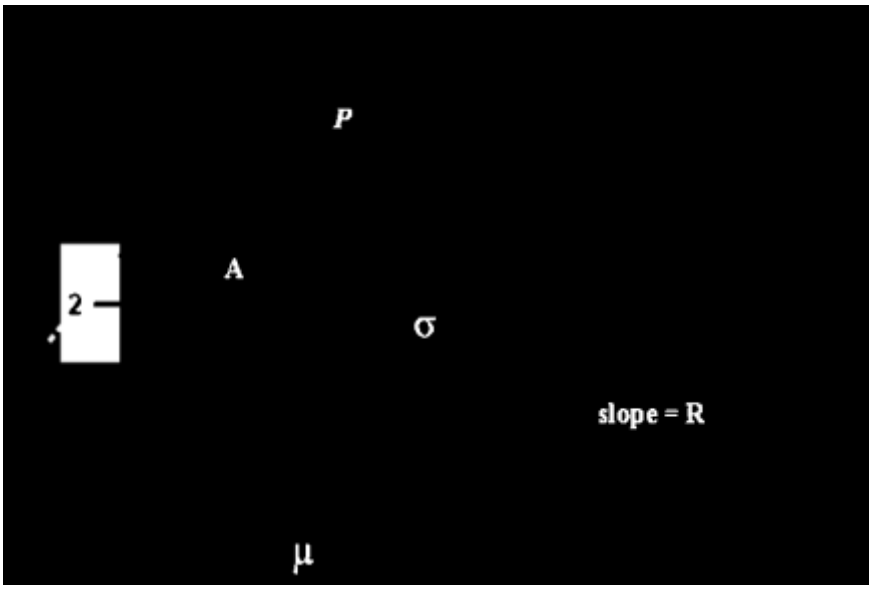



Figure 4.1 The non-linear regression model used to fit to the change in absorbance with time.

### *4.6.2 Density Modeling*

The density measurements that are obtained in the MFA are also analyzed using a logistic model by utilizing Equation 4.2 with statistical software Prism™. The equations parameters are displayed in Table 4.3 and a visual depiction of the model is shown in Figure 4.2. This logistic model has been used successfully to analyze and predict the change in density throughout a fermentation (Lake, 2008).

Equation 4.2

$$P_{(t)} = P_e + \frac{P_i - P_e}{1 + e^{-B(t-M)}}$$

Table 4.3 Definitions of the parameters in the Logistic Model for Density

| Parameter | Definition |
|---|---|
| $P_t$ | Particular density at a specific time |
| $P_e$ | The Equilibrium asymptotic density value |
| $P_i$ | The initial asymptotic density value for the density attenuation regression |
| B | Function of the slope at the inflection point |
| M | Time for a specific B value |

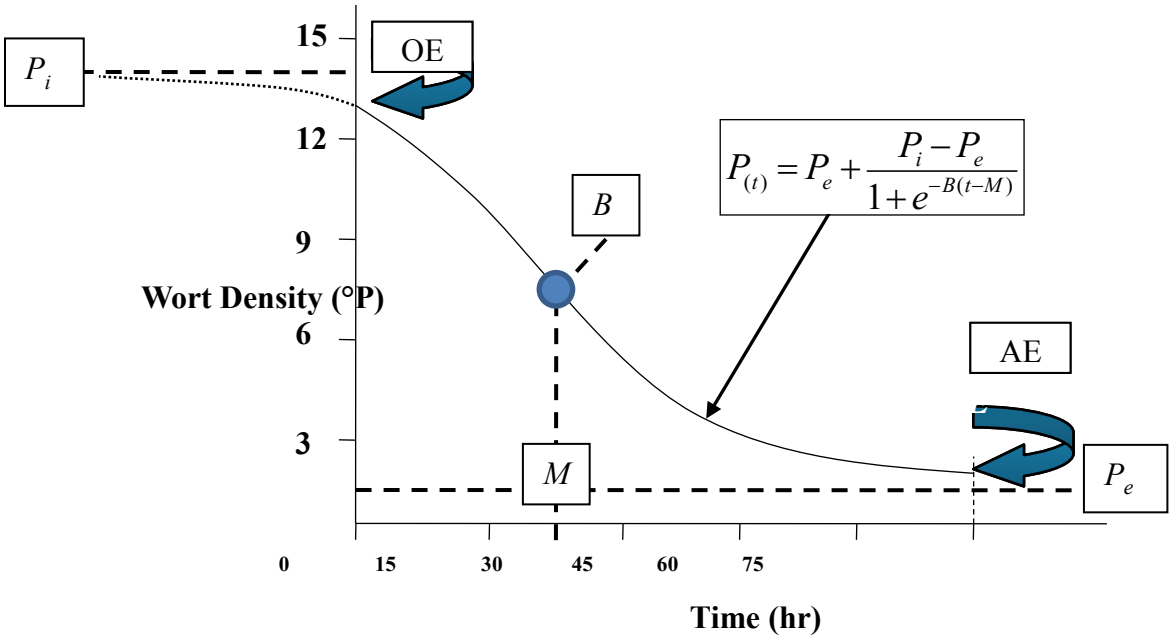



Figure 4.2 Visual depiction of the calculation used for non-linear regression analysis of fermentation using the logistic model from equation 4.2

Using values from equation 4.2 the original Extract (OE) and apparent extract (AE) can be calculated and the apparent degree of fermentation (ADF) can be determined by using Equation 4.3. The parameters for Equation 4.3 are displayed in Table 4.3. Treatments and different varieties of malt samples or MSS are compared using a global F test.

### *4.6.3 Shear Rate Modeling*

To understand and quantify the differences between the MFA and industrial fermentations, the shear rate was calculated. The shear rate of a fermentation can be calculated using work described by Delente et al. First, the amount of power produced from $CO_2$ generation is calculated using Equation 4.3. Once this power per unit volume is calculated, the shear rate calculation (Equation 4.4) can be utilized (Delente, 1969; Lake, 2008; MacIntosh et al., 2012).

Equation 4.3 $$P = B_{vol} Q_{CO2} P_a \left( \frac{P_a + P_b}{P_b} ln \frac{P_a + P_b}{P_a} - 1 \right)$$

Equation 4.4 $$\dot{\gamma} = \left( \frac{P}{\eta B_{vol}} \right)^{1/2}$$

Equation 3 determines the power released (P) using the atmospheric pressure ($P_a$), the hydrostatic pressure ($P_b$), $CO_2$ evolution rate ($Q_{CO2}$) and the wort volume ($B_{vol}$). Once P is determined at different times throughout fermentation, the values obtained can be used in Equation 4 to determine the average shear rate ($\dot{\gamma}$) and these values are plotted for varying fermenter sizes (MacIntosh et al., 2012).

# Chapter 5. Results and Discussion

## 5.1 PYF Ratio Effect on Fermentation

Quality assurance testing as a preventative measure to assure that PYF inducing malt is not used in processing is currently the best method to prevent occurrences of PYF. Using this knowledge to procure unaffected barley is the most proactive solution, however, this knowledge may not be economically feasible to implement because large contracted barley crops may be affected. The potential costs of changing distribution or suppliers may yield higher expenses than the alternative of changing the production to control for PYF related quality defects until unaffected barley can be obtained. This aspect of the study intends to enhance testing procedures and identify the amount of PYF inducing malt that can be used in conjunction with "control" malt to yield typical non-PYF fermentations.

### *5.1.1 Milled Malt Ratios*

Samples containing ≤ 60 % PYF inducing malt all produced apparent extract fermentation characteristic's that were not significantly different from the control (Appendix). When 80% or greater amounts of PYF inducing malt were used, absorbance measurements yielded significantly different values (Appendix). Both density and absorbance measurements yielded significantly different values ($P < 0.05$) when 80% and greater amounts of PYF inducing malt were used in fermentations (Table 5.5).

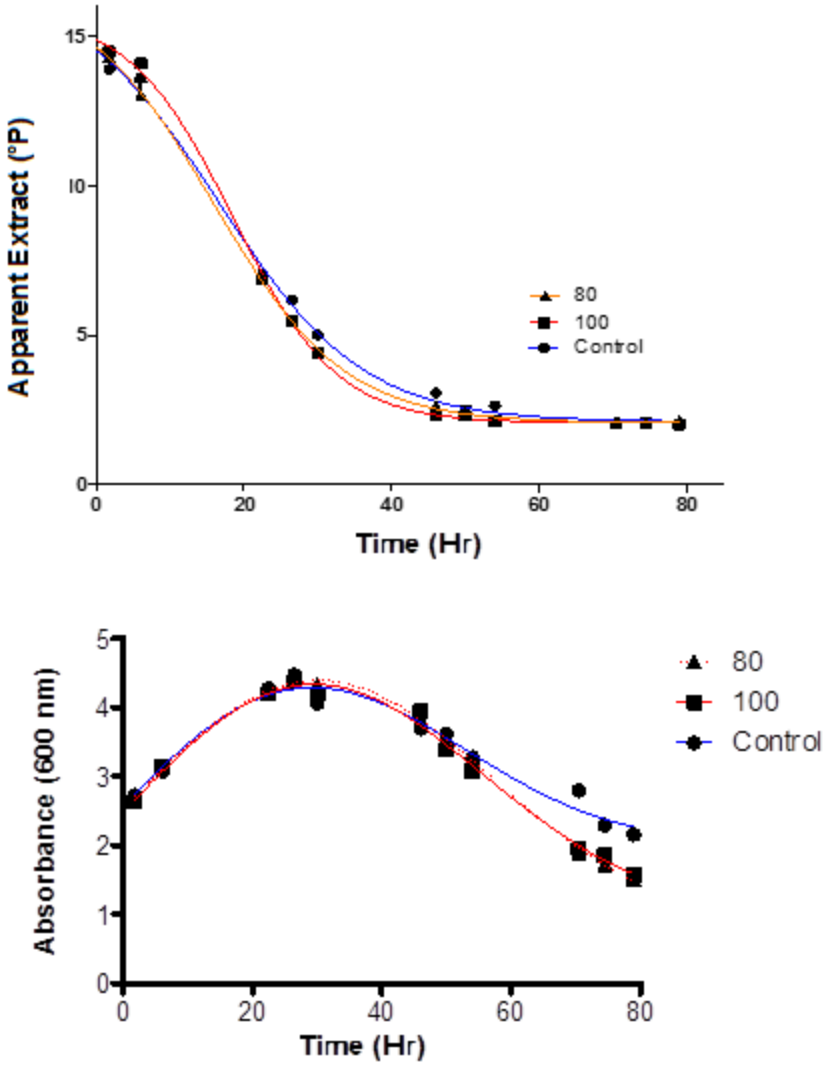



Figure 5.5 Comparison of absorbance (Top) and apparent extract (Bottom) profiles of control malt and malt with 80% and 100% PYF inducing malt [Regression Lines Shown]

Figure 5.5 does display a visual divergence as well as a statistical difference between the control and ratio treated samples ($P<0.05$). This finding supports the hypothesis that PYF inducing malt must reach a threshold before PYF fermentation characteristics can be noticed via statistical analysis. The need for at least 80% PYF inducing malt suggests that this particular malt being analyzed could easily be blended at fairly high levels to avoid the need to procure other malts. In addition, this finding shows that the MFA is robust enough to properly identify small variations.

While the divergence was significantly different for 80% and 100% PYF malt, these did not fall out of the "control band" created from 45 different malt varieties (Figure 5.3). Fitting within this band does lead to a notable point that the PYF malt being tested does not create the strength of PYF as other malts used in the Macintosh et al research published in 2014. Therefore the PYF inducing malt used in this study is fairly weak when compared to other PYF inducing malts. It also shows that using the "control band" alone as a measure of PYF capability is flawed. This weak PYF inducing malt may still lead to industrial issues, as these very small variations extrapolated on the large scale can cause quality defects. For these reasons the "control band" and other statistical models need to be stricter with regard to the variation in their controls and a more rigid control standard is needed for malt comparisons.

### *5.1.2 Malt Soak Solution Ratios*

The malt soak solution produced similar results to that of those found in the malt ratio treatments. When samples contained >80% PYF MSS, they produced fermentations that yielded statistical differences for both absorbance and apparent extract (Figure 5.6). No differences were found in samples that contained 60% or less of the MSS (Appendix).

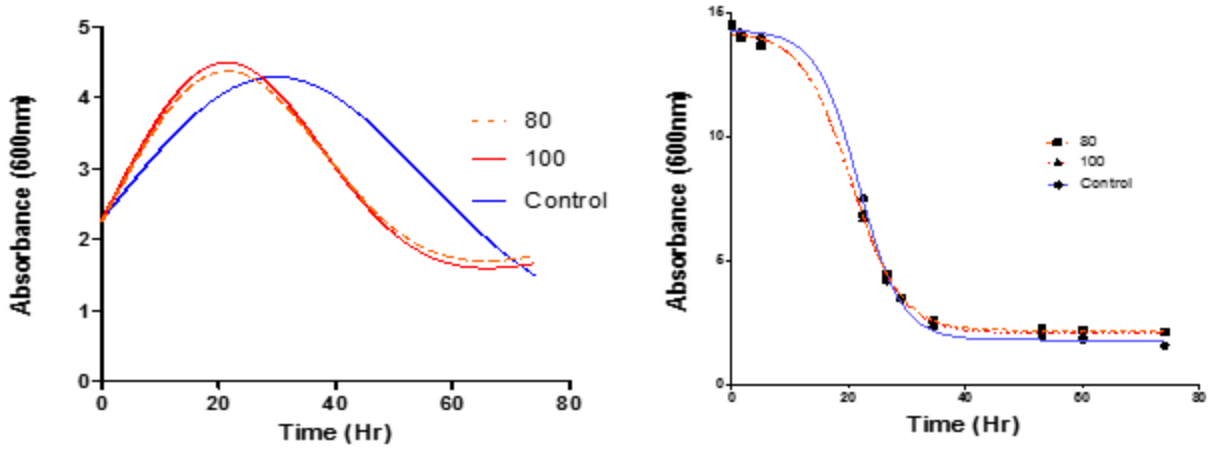



Figure 5.6 Comparison of absorbance (left) and apparent extract (right) profiles of control malt wash water and PYF inducing wash water mixed at 80% and 100% with wort extract [Regression Lines Shown]. *Note: Control displayed is the shared global curve for MSS and DW

The fermentation characteristics of malt with >80% PYF inducing MSS yielded very apparent visual differences in both absorbance and apparent extract. Unlike the slight statistical and visual differences observed in the malt ratio samples, these differences yielded results that fall out of the "control bands" (Figure 2.14) produced from 45 trials (Macintosh et al, 2012).

Figure 5.7 displays the absorbance "control bands" produced from all of the control MSS trials. Throughout the study five MSS controls were utilized and a combination of them is used to compare the treatments performed. While differences in FG were noted, the variation was high and it did not provide the same type of statistical difference that the Absorbance (correlated with YIS) provided. The vessels used in the MFA are very small and likely do not see the same effect on FG that an industrial fermentation would display. For this reason, Absorbance values were focused on throughout the research conducted. By using this method we can conclude that any global curve that falls outside of the control band would be "out of control".

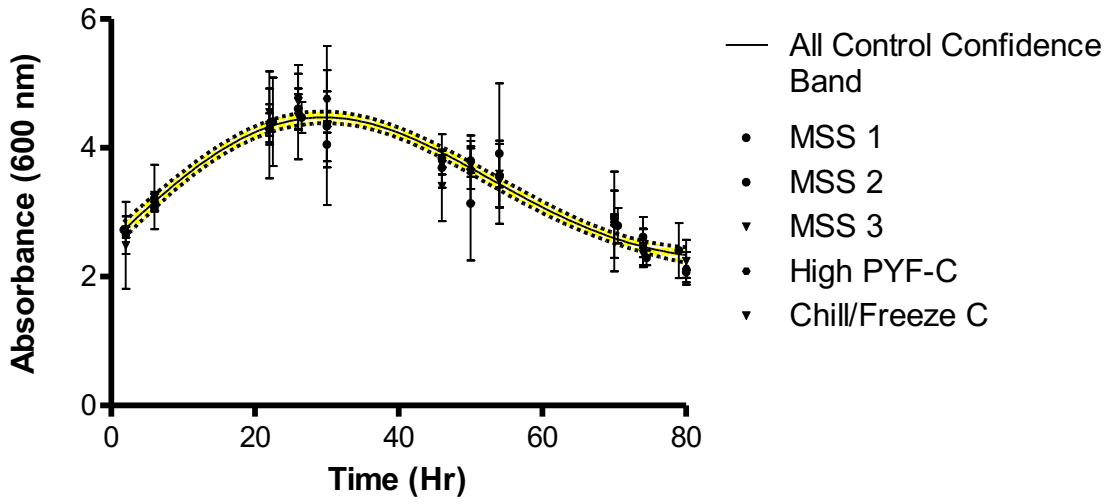



Figure 5.7 Absorbance values for a 5 separate MFA's using a control malt soak solution and malt extract with the average curve and 95% control band

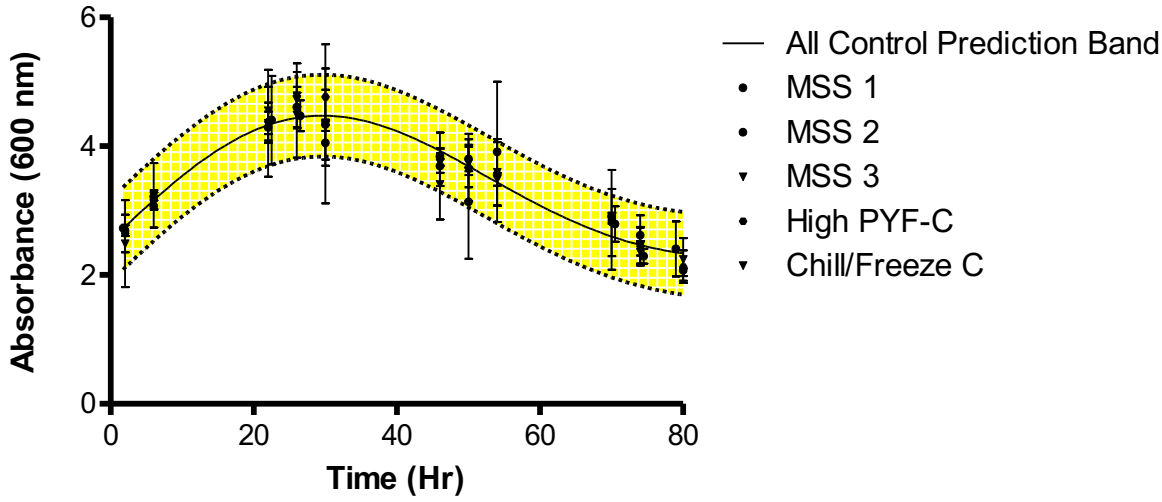



Figure 5.8 Absorbance values for a Five MFA's using a control MSS with the average curve and 80% Prediction band

By using the MSS method for creation of control wort, the sensitivity of the study was increased by making a more reliable control and therefore statistical differences of various treatments would be more easily identified. The main benefit of using malt extract with MSS over conventional

mashing is the ability to easily create a strong and reproducible control that does not change with varietal variation. These findings here support the threshold of PYF theory and further strengthen the hypothesis that soaking samples is sufficient for analysis when using the MFA.

## 5.2 High PYF Solution

By using the method discussed in section 4.3.3, a solution derived by concentrating PYF inducing MSS was created. Figure 5.9 displays the findings from the MSS that was created using three times the amount of malt compared to the standard MSS method (4.3.2). While the "high PYF" solution did generate a fermentation consistent with PYF, falling out of the control band and being statistically different ($P < 0.05$), it did not generate a stronger PYF response by the yeast during fermentation as they were statistically the same ($P > 0.05$). This finding suggests that the increased presence of the PYF factor does not increase the amount of PYF displayed once a maximum level is reached.

## 5.3 Pre-Fermentation Treatments

The purpose of fermentation trials with various different treatments was to identify how and if the PYF factor can be rendered inactive. This portion of the study focused on methods that manipulate prepared wort. The malt itself can undergo treatments, however, these treatments were not performed as the MSS-MFA uses synthetic wort and any pre-treatment to the malt would have an effect on the malt and confound specifically targeting the PYF factor itself. The treatments analyzed were boiling (60, 90 and 120 min), chilling (5 °C for 24 Hours) and Freezing (-30 °C for 160 hours).

### *5.3.1 Analysis of Singular Treatments*

*Boiling*

Figure 5.10 displays the results from the pre-treatments of boiling PYF inducing MSS for 60, 90 and 120 min. The treatments had no significant difference from the control ($P > 0.05$). It should be noted that the boiling did slightly increase the initial and final absorbance value, this is attributed to a slight change in colour between the samples. This result was expected as typical brewing practises include boiling and this itself does not inhibit PYF factor's ability to cause variation in fermentations (Herrera et al., 1991).

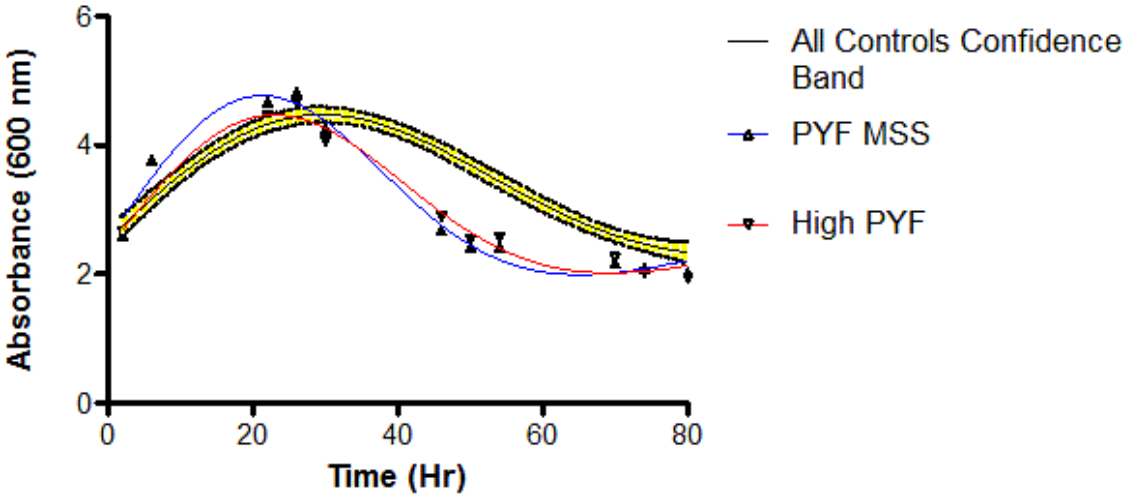



Figure 5.9 Absorbance comparison of PYF (+) MSS treatments and the control band for PYF (-) MSS

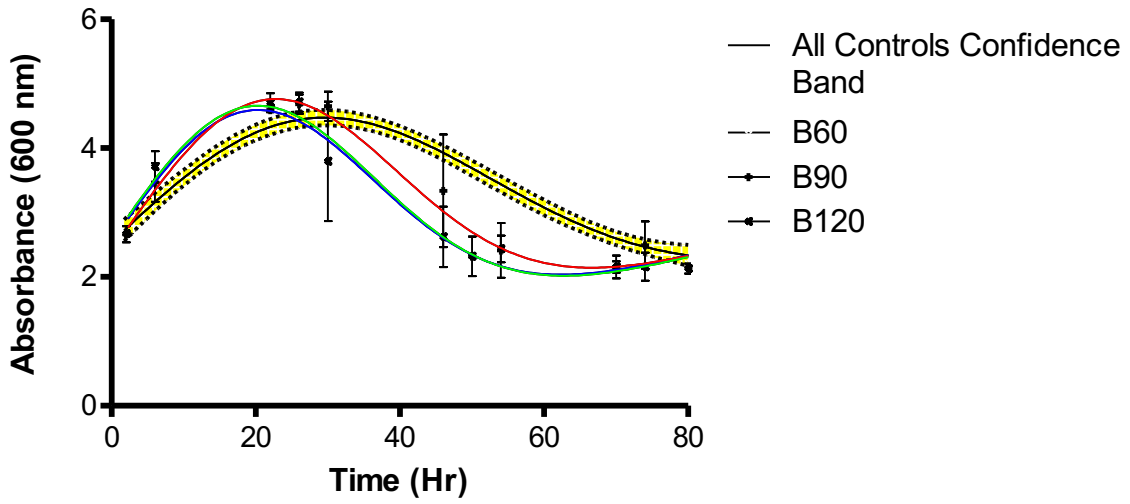



Figure 5.10 MSS boiling trials at 60, 90 and 120 minutes compared to 3 control MSS'

*Chilling*

Refrigerated samples at 5 °C for 24 hours maintained a significant difference from PYF control samples ($P < 0.05$) (Figure 5.11). Chilling wort before fermentation is not standard practise, and this treatment does not show promise as an option for breweries exhibiting PYF fermentations as the Chilled PYF MSS did not produce a curve similar to control.

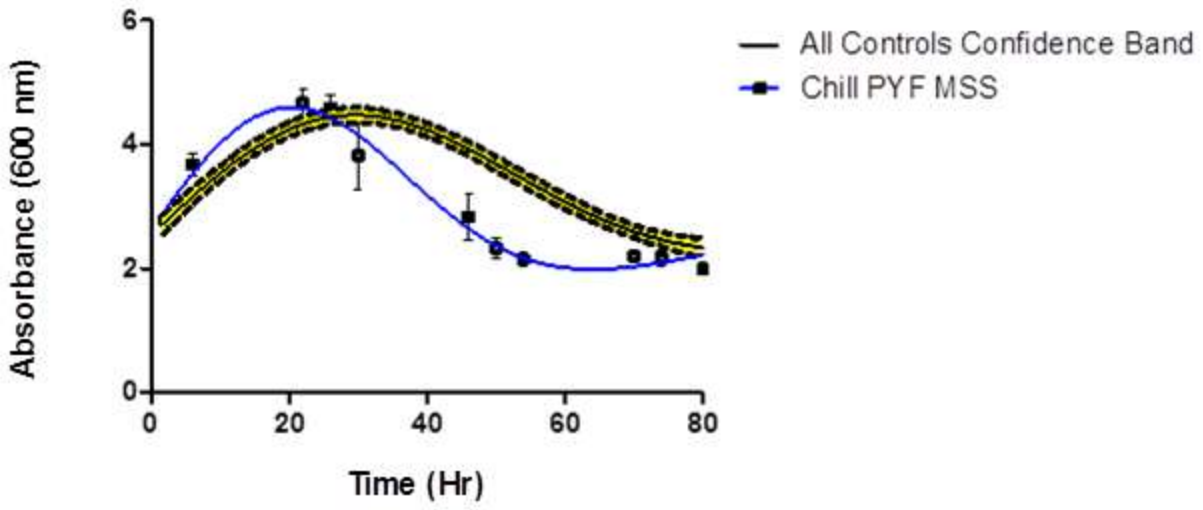



Figure 5.11 PYF (+) MSS chilling trials compared to 3 control MSS'

*Freezing*

Freezing can have a radical effect on the structure of polysaccharides (Nathdanai, 2014) and therefore can possibly effect PYF-factor's ability to exhibit variations in fermentations. For this study, the freezing process was used in conjunction with thawing, which can also have an effect on proteins, carbohydrates and their structure (Tao, 2015). Figure 5.12 displays the findings from two trials of the freezing treatment. Variations from the control samples and the treated samples were found ($P < 0.05$).

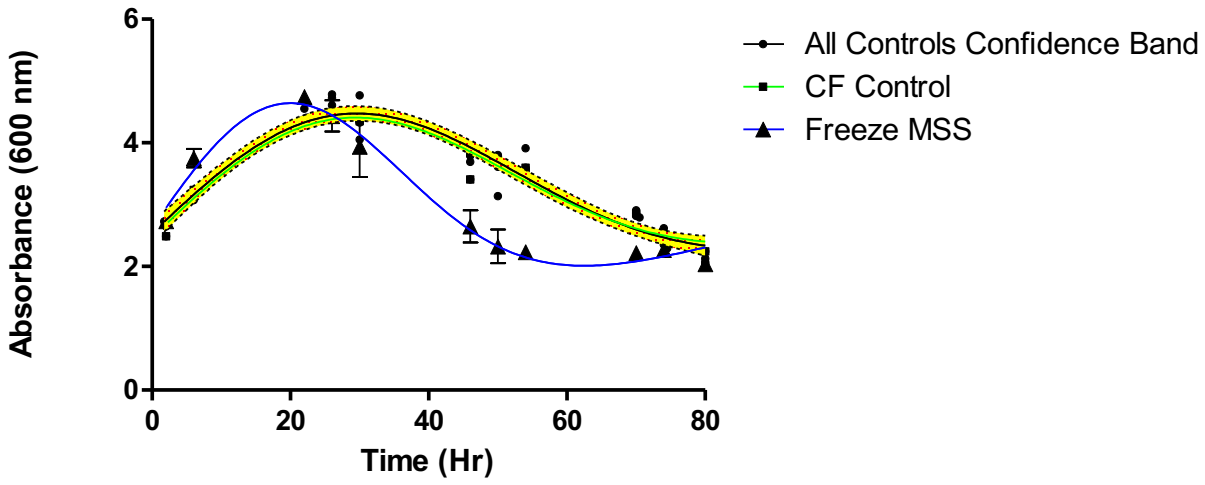



Figure 5.12 PYF (+) MSS freezing trial compared to 3 control MSS'

### *5.3.2 Analysis of Multiple Treatments*

The pre-fermentation treatment of boiling at 120 minutes and freezing (-30 °C) followed by subsequent thawing was chosen to be tested in conjunction as these were the two most extreme pre-fermentation treatments used in the study.

Figure 5.12 displays the results from boiling (120 min) and Freezing (168 hours) the MSS prior to fermentation. This treatment appears to have diminished the effect of the PYF factor as findings maintain within the prediction band for control MSS.

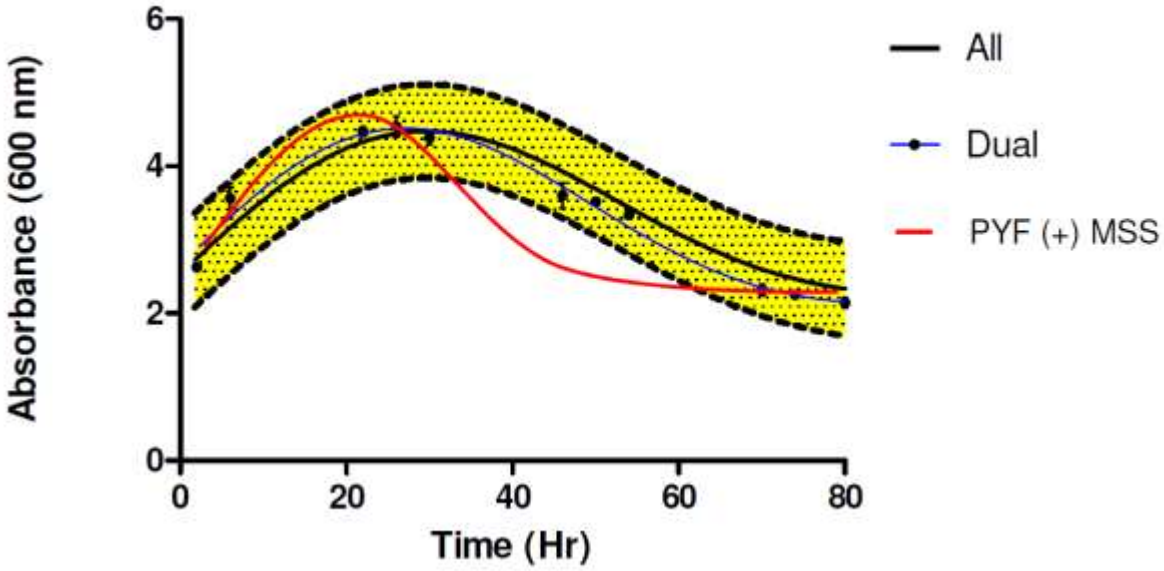



Figure 5.13 Dual-Treatment PYF (+) MSS with an 80% prediction band for control MSS. A untreated PYF (+) MSS average curve is shown for comparison.

While not the most practical industrial solution, this finding does show how the PYF factor can be rendered inactive. This finding can help brewing scientists understand the factor better and possibly create protocols that can mimic this to stop PYF from occurring.

### *5.3.3 Understanding Inactivity of PYF Solution*

The action of boiling a solution carries with it a large effect on the physical properties of that solution and can render the solution permanently molecularly changed (Vaclavik, 2014). Beyond boiling, the act of freezing (and subsequent thawing for the study) can cause various structural and degenerative effects on foods and beverages. The treatment of boiling and freezing separately did not result in degeneration of the effect of the PYF factor. However, when these methods were used in conjunction, the effect of PYF was inhibited.

Therefore, this study concludes that the PYF factor's structure and capability to effect fermentations is lost when it is boiled at 100 °C, frozen at -30 °C for 168 hours and then subsequently thawed at room temperature. These combined treatments are likely effective at rendering the factor inactive because of permanent molecular changes that are likely occurring to the PYF inducing compounds (Arabinoxylan, CAPs and Lipid transfer proteins). While these compounds are known to go through the brewing process and survive mashing and boiling, the added dimension of freezing is likely causing a more thorough degeneration and rendering the factor inactive. Understanding what happens to these compounds during the boiling and freezing is key to determining how to avoid PYF in the brewing process.

# Chapter 6. Conclusion

The main objectives (Section 3) of this work were all accomplished. The first objective was to find the threshold of PYF malt which will display PYF fermentation characteristics. This work found that levels greater than 80% of PYF malt induced PYF fermentation characteristics and also strengthened the validity of the MFA used.

The second objective of this work was to minimize the need for control malt in the MFA as it is highly variable depending on year and area produced. The use of malt extract was utilized with a novel malt soaking technique and it proved to be a highly reproducible way of testing for PYF characteristics of malt. This method proved to create high statistical differences between control malt and PYF malts, leading to a strong case for the methods validity. Having a wort that exhibits strong PYF fermentation characteristics is a highly valuable tool for developing a more thorough understanding of PYF.

The final objective of this work was to inactivate the PYF factor. This work was successful at de-activating the factor via the combination of multiple treatments. While these methods are not practical to be implemented in an industrial setting, they do provide insights into future research that can lead to work that will create methods to not only identify PYF malt successfully, but treat it to develop methods for its removal from the malt and brewing industries. The action of freezing is known to cause changes to the molecular structure of molecules, this could be why this treatment affected the PYF factor.

Future research into PYF can utilize the finding of PYF inactivity to further understand how brewers and maltsters can avoid this production difficulty. The brewing industry has recently

focused on enzymatic solutions to complex issues, and it is possible that an additive can cause the same effect on wort that freezing and boiling has displayed in this research.

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# Appendix

Data from the Percent PYF Malt Trials

| Trial | Time | Abs 1 | Abs 2 | Abs 3 | Plato 1 | Plato 2 | Plato 3 |
|---|---|---|---|---|---|---|---|
| 0 | 1.75 | 2.7171 | 2.7415 | 2.7218 | 13.9 | 13.9 | 13.9 |
| 0 | 6 | 3.0311 | 3.1108 | | 13.6 | 13.6 | |
| 0 | 22.5 | 4.6807 | 3.6238 | 4.5273 | 7 | 7 | 7.1 |
| 0 | 26.5 | 4.348 | 4.748 | 4.3192 | 6.2 | 5.9 | 6.4 |
| 0 | 30 | 4.1248 | 4.0994 | 3.9522 | 4.9 | 5 | 5.1 |
| 0 | 46 | 3.753 | 3.7588 | 3.5688 | 3.1 | 3 | 3.1 |
| 0 | 50 | 3.4253 | 3.8211 | 3.6028 | 2.8 | 2.3 | 2.1 |
| 0 | 54 | 2.687 | 3.689 | 3.4386 | 2.5 | 2.6 | 2.8 |
| 0 | 70.5 | 2.7346 | 3.0893 | 2.5518 | 2 | 2.1 | 2.1 |
| 0 | 74.5 | 2.2089 | 2.2428 | 2.4257 | 2.1 | 2 | 2 |
| 0 | 79 | 2.383 | 2.0914 | 1.9921 | 2 | 2 | 2.1 |
| 20 | 1.75 | 2.5719 | 2.6121 | 2.5959 | 14.5 | 14.5 | 14.5 |
| 20 | 6 | 3.0602 | 3.0676 | | 13.9 | 14.1 | |
| 20 | 22.5 | 4.0886 | 3.6238 | 4.3164 | 7 | 7 | 7.3 |
| 20 | 26.5 | 4.7058 | 4.4924 | 4.6701 | 5.9 | 6 | 6 |
| 20 | 30 | 4.3795 | 4.5779 | 4.3732 | 4.8 | 4.9 | 5 |
| 20 | 46 | 3.811 | 3.498 | 3.6203 | 2.3 | 3 | 3.4 |
| 20 | 50 | 3.49 | 3.7875 | 3.5593 | 2.6 | 2.5 | 2.5 |
| 20 | 54 | 3.3053 | 2.8172 | 3.0894 | 2.6 | 2.8 | 2.5 |
| 20 | 70.5 | 2.3054 | 2.8457 | 2.2148 | 2.3 | 2.3 | 2.3 |
| 20 | 74.5 | 2.4328 | 1.908 | 1.7993 | 2.2 | 2.3 | 2.3 |
| 20 | 79 | 1.4445 | 1.511 | 1.9216 | 2.2 | 2.4 | 2.4 |
| 40 | 1.75 | 2.6004 | 2.5942 | 2.5959 | 14.5 | 14.5 | 14.5 |
| 40 | 6 | 2.9889 | 3.0087 | | 13.7 | 14 | |
| 40 | 22.5 | 3.8722 | 4.1149 | 4.329 | 7.3 | 7.6 | 7.1 |
| 40 | 26.5 | 3.955 | 4.3289 | 4.8809 | 6.2 | 6.2 | 6.3 |
| 40 | 30 | 4.0944 | 3.8273 | 3.8739 | 5 | 5.2 | 5 |
| 40 | 46 | 3.7151 | 3.8889 | 3.9002 | 2.9 | 2.9 | 2.9 |
| 40 | 50 | 3.5746 | 3.4205 | 3.5309 | 2.4 | 2.8 | 2.6 |
| 40 | 54 | 3.053 | 3.1603 | 3.3732 | 2.5 | 2.5 | 2.5 |
| 40 | 70.5 | 2.5509 | 2.759 | 2.4937 | 2.2 | 2.2 | 2.2 |
| 40 | 74.5 | 1.7918 | 2.149 | 2.1305 | 2.1 | 2.3 | 2.2 |
| 40 | 79 | 1.925 | 1.9821 | 1.7219 | 1.9 | 2 | 2 |
| 60 | 1.75 | 2.6725 | 2.6635 | 2.6588 | 14.5 | 14.5 | 14.5 |
| 60 | 6 | 3.093 | 3.08 | | 13.6 | 13.8 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 60 | 22.5 | 4.0563 | 4.2885 | 4.3012 | 6.7 | 7.2 | 7.4 |
| 60 | 26.5 | 4.6218 | 4.2361 | 4.3438 | 5.6 | 5.8 | 5.9 |
| 60 | 30 | 4.5184 | 4.3394 | 4.3957 | 4.6 | 4.7 | 4.7 |
| 60 | 46 | 3.8796 | 3.7709 | 3.6645 | 2.8 | 2.7 | 2.8 |
| 60 | 50 | 3.4573 | 3.4869 | 3.3829 | 2.3 | 2.6 | 2.4 |
| 60 | 54 | 3.3315 | 3.1534 | 3.3002 | 2.3 | 2.2 | 2.3 |
| 60 | 70.5 | 2.6096 | 1.8301 | 2.4075 | 2.3 | 2.2 | 2.3 |
| 60 | 74.5 | 1.8772 | 1.6541 | 1.7613 | 2.2 | 2.2 | 2.2 |
| 60 | 79 | 1.3306 | 1.529 | 1.3621 | 2.1 | 2.2 | 2.2 |
| 80 | 1.75 | 2.6988 | 2.6897 | 2.7135 | 14.3 | 14.3 | 14.3 |
| 80 | 6 | 3.162 | 3.083 | | 13.5 | 12.6 | |
| 80 | 22.5 | 4.8467 | 4.1134 | 3.8278 | 6.7 | 6.9 | 6.9 |
| 80 | 26.5 | 4.2544 | 4.6619 | 4.2174 | 5.5 | 5.6 | 5.4 |
| 80 | 30 | 4.5086 | 3.9279 | 4.5688 | 4.4 | 4.5 | 4.4 |
| 80 | 46 | 3.5621 | 3.8495 | 4.0549 | 2.7 | 2.5 | 2.7 |
| 80 | 50 | 3.4955 | 3.6028 | 3.4893 | 2.4 | 2.4 | 2.4 |
| 80 | 54 | 3.2729 | 3.3419 | 3.2564 | 2.1 | 2.1 | 2.1 |
| 80 | 70.5 | 2.0831 | 1.9637 | 1.6105 | 2.1 | 2.1 | 2.1 |
| 80 | 74.5 | 1.7613 | 1.6542 | 1.7613 | 2 | 2 | 2.2 |
| 80 | 79 | 1.4413 | 1.5696 | 1.5177 | 2 | 2.1 | 2.3 |
| 100 | 1.75 | 2.6243 | 2.6402 | 2.629 | 14.5 | 14.5 | 14.5 |
| 100 | 6 | 3.1528 | 3.1269 | | 14.1 | 14.1 | |
| 100 | 22.5 | 4.2723 | 4.1986 | 4.1593 | 7 | 6.9 | 6.8 |
| 100 | 26.5 | 4.1187 | 4.4646 | 4.5303 | 5.4 | 5.6 | 5.4 |
| 100 | 30 | 4.3339 | 4.1098 | 4.1384 | 4.4 | 4.4 | 4.4 |
| 100 | 46 | 3.8882 | 4.0377 | 3.9033 | 2.3 | 2.3 | 2.3 |
| 100 | 50 | 3.3508 | 3.4398 | 3.378 | 2.3 | 2.3 | 2.3 |
| 100 | 54 | 3.0765 | 2.8616 | 3.3063 | 2.1 | 2.1 | 2.2 |
| 100 | 70.5 | 1.8784 | 1.9703 | 1.9998 | 2 | 2.1 | 2 |
| 100 | 74.5 | 2.0321 | 1.7746 | 1.7383 | 2 | 2.1 | 2.1 |
| 100 | 79 | 1.5286 | 1.6432 | 1.5443 | 2 | 2 | 2 |

Malt Soak Solution Control Data

| Trial | Time | Abs 1 | Abs 2 | Abs 3 | Plato 1 | Plato 2 | Plato 3 |
|---|---|---|---|---|---|---|---|
| 0 | 1.75 | 2.7171 | 2.7415 | 2.7218 | 13.9 | 13.9 | 13.9 |
| 0 | 6 | 3.0311 | 3.1108 | | 13.6 | 13.6 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0 | 22.5 | 4.6807 | 3.6238 | 4.912 | 7 | 7 | 7.1 |
| 0 | 26.5 | 4.348 | 4.748 | 4.3192 | 6.2 | 5.9 | 6.4 |
| 0 | 30 | 4.9543 | 4.0994 | 3.9522 | 4.9 | 5 | 5.1 |
| 0 | 46 | 3.753 | 3.7588 | 3.5688 | 3.1 | 3 | 3.1 |
| 0 | 50 | 3.9814 | 3.8211 | 3.6028 | 2.8 | 2.3 | 2.1 |
| 0 | 54 | 2.687 | 4.2816 | 4.7692 | 2.5 | 2.6 | 2.8 |
| 0 | 70.5 | 2.7346 | 3.0893 | 2.5518 | 2 | 2.1 | 2.1 |
| 0 | 74.5 | 2.2089 | 2.2428 | 2.4257 | 2.1 | 2 | 2 |
| 0 | 79 | 2.383 | 2.846 | 1.9921 | 2 | 2 | 2.1 |
| | | | | | | | |
| 0 | 2 | 2.6398 | 2.7845 | 2.6471 | | | |
| 0 | 6 | 3.1584 | 3.2146 | 3.2847 | | | |
| 0 | 22 | 4.013 | 4.4181 | 4.4522 | | | |
| 0 | 26 | 4.2547 | 4.8639 | 4.6981 | | | |
| 0 | 30 | 3.6519 | 4.1873 | 4.3112 | | | |
| 0 | 46 | 3.8674 | 3.9557 | 3.6895 | | | |
| 0 | 50 | 3.5971 | 2.1153 | 3.6984 | | | |
| 0 | 54 | 3.5618 | 3.5574 | 3.5578 | | | |
| 0 | 70 | 2.8875 | 2.9766 | 2.8594 | | | |
| 0 | 74 | 2.3964 | 2.4781 | 2.9731 | | | |
| 0 | 80 | 2.1082 | 2.008 | 2.097 | | *February 19th trials no plato taken | |
| | | | | | | | |
| 0 | 2 | 2.7442 | 2.7649 | 2.6873 | | | |
| 0 | 6 | 3.1684 | 3.2199 | 3.1147 | | | |
| 0 | 22 | 4.9761 | 4.3168 | 4.3684 | | | |
| 0 | 26 | 4.7911 | 4.7844 | 4.5968 | | | |
| 0 | 30 | 4.3535 | 4.3964 | 4.2284 | | | |
| 0 | 46 | 3.877 | 3.8102 | 3.6847 | | | |
| 0 | 50 | 3.6471 | 3.5447 | 3.6481 | | | |
| 0 | 54 | 3.4157 | 3.6003 | 3.4478 | | | |
| 0 | 70 | 2.8829 | 2.7948 | 2.9615 | | | |
| 0 | 74 | 2.2669 | 2.554 | 2.2364 | | | |
| 0 | 80 | 2.0125 | 2.0036 | 2.1129 | | *February 19th trials no plato taken | |

Various Treatments and Corresponding Control Data

| **Trial** | **Time** | **Abs 1** | **Abs 2** | **Abs 3** |
|---|---|---|---|---|
| 0-MSS | 2 | 2.5489 | 2.7762 | 2.6124 |
| 0-MSS | 6 | 3.1521 | 3.2366 | 3.1254 |

| | | | | |
|---|---|---|---|---|
| 0-MSS | 22 | 4.2946 | 4.5167 | 4.3369 |
| 0-MSS | 26 | 4.5482 | 4.919 | 4.8783 |
| 0-MSS | 30 | 4.9175 | 4.5746 | 4.8141 |
| 0-MSS | 46 | 3.7958 | 3.6322 | 3.966 |
| 0-MSS | 50 | 3.4561 | 3.6724 | 3.8763 |
| 0-MSS | 54 | 3.5698 | 3.766 | 3.3675 |
| 0-MSS | 70 | 2.587 | 3.0017 | 2.8594 |
| 0-MSS | 74 | 2.3364 | 2.5681 | 2.444 |
| 0-MSS | 80 | 2.1178 | 2.0338 | 2.2372 |
| | | | | |
| PYF | | | | |
| MSS | 2 | 2.3468 | 2.6471 | 2.8173 |
| PYF | | | | |
| MSS | 6 | 3.4861 | 3.9762 | 3.8462 |
| PYF | | | | |
| MSS | 22 | 4.4589 | 4.6823 | 4.8844 |
| PYF | | | | |
| MSS | 26 | 4.8174 | 4.7443 | 4.8736 |
| PYF | | | | |
| MSS | 30 | 4.4996 | 4.7651 | 3.456 |
| PYF | | | | |
| MSS | 46 | 2.4871 | 2.647 | 2.8769 |
| PYF | | | | |
| MSS | 50 | 2.184 | 2.6381 | 2.4581 |
| PYF | | | | |
| MSS | 54 | 2.3469 | 2.4428 | 2.4816 |
| PYF | | | | |
| MSS | 70 | 2.1003 | 2.007 | 2.3871 |
| PYF | | | | |
| MSS | 74 | 1.9517 | 1.8462 | 2.4872 |
| PYF | | | | |
| MSS | 80 | 1.8937 | 1.7982 | 2.3487 |
| | | | | |
| HI | | | | |
| PYF | 2 | 2.6148 | 2.8921 | 2.4846 |
| HI | | | | |
| PYF | 6 | 3.2461 | 3.3648 | 3.0237 |
| HI | | | | |
| PYF | 22 | 4.649 | 4.2117 | 4.4976 |
| HI | | | | |
| PYF | 26 | 4.7273 | 4.5529 | 4.6856 |
| HI | | | | |
| PYF | 30 | 4.4615 | 4.2988 | 3.456 |
| HI | | | | |
| PYF | 46 | 2.8762 | 2.9431 | 2.8769 |

| | | | | |
|---|---|---|---|---|
| HI | | | | |
| PYF | 50 | 2.6891 | 2.7229 | 2.223 |
| HI | | | | |
| PYF | 54 | 2.347 | 2.6657 | 2.6681 |
| HI | | | | |
| PYF | 70 | 2.1003 | 2.2842 | 2.3746 |
| HI | | | | |
| PYF | 74 | 2.1648 | 1.8847 | 2.006 |
| HI | | | | |
| PYF | 80 | 1.968 | 1.8941 | 1.967 |
| | | | | |
| B60 | 2 | 2.6847 | 2.643 | 2.7471 |
| B60 | 6 | 3.5681 | 3.6873 | 3.5682 |
| B60 | 22 | 4.5482 | 4.7682 | 4.5287 |
| B60 | 26 | 4.8647 | 4.6877 | 4.5532 |
| B60 | 30 | 4.6829 | 3.1028 | 3.3681 |
| B60 | 46 | 2.3415 | 2.8083 | 2.6839 |
| B60 | 50 | 2.1864 | 2.5573 | 2.4588 |
| B60 | 54 | 2.3547 | 2.0686 | 2.497 |
| B60 | 70 | 2.1543 | 2.589 | 2.0683 |
| B60 | 74 | 2.1513 | 1.9868 | 2.0074 |
| B60 | 80 | 2.0576 | 1.976 | 2.6863 |
| | | | | |
| B90 | 2 | 2.6483 | 2.5473 | 2.7924 |
| B90 | 6 | 3.5872 | 3.1247 | 3.6874 |
| B90 | 22 | 4.5683 | 4.5572 | 4.6583 |
| B90 | 26 | 4.6248 | 4.5684 | 4.8554 |
| B90 | 30 | 4.419 | 4.6584 | 4.876 |
| B90 | 46 | 4.296 | 3.125 | 2.5842 |
| B90 | 50 | 2.3684 | 2.2965 | 2.2834 |
| B90 | 54 | 2.068 | 2.2769 | 2.8864 |
| B90 | 70 | 2.0365 | 2.3596 | 2.0687 |
| B90 | 74 | 2.1873 | 2.3918 | 2.9024 |
| B90 | 80 | 2.1551 | 2.1748 | 2.1083 |
| | | | | |
| B120 | 2 | 2.6184 | 2.7184 | 2.6734 |
| B120 | 6 | 3.5617 | 3.5864 | 3.9872 |
| B120 | 22 | 4.5682 | 4.6816 | 4.8653 |
| B120 | 26 | 4.8849 | 4.605 | 4.6582 |
| B120 | 30 | 4.8602 | 3.3586 | 3.167 |
| B120 | 46 | 3.1055 | 2.1654 | 2.5915 |
| B120 | 50 | 2.2479 | 2.6589 | 2.0559 |
| B120 | 54 | 2.2791 | 2.6721 | 2.3573 |
| B120 | 70 | 2.2421 | 2.0689 | 2.1573 |

| | | | | |
|---|---|---|---|---|
| B120 | 74 | 2.3609 | 1.9272 | 2.1878 |
| B120 | 80 | 2.148 | 2.1986 | 2.0473 |
| | | | | |
| Boil Control | 2 | 2.493 | 2.4973 | 2.2734 |
| Boil Control | 6 | 3.6842 | 3.4811 | 3.5186 |
| Boil Control | 22 | 4.1776 | 4.562 | 4.2584 |
| Boil Control | 26 | 4.8621 | 4.6087 | 4.8106 |
| Boil Control | 30 | 4.3661 | 4.3807 | 4.8568 |
| Boil Control | 46 | 3.9973 | 3.4879 | 3.3671 |
| Boil Control | 50 | 3.038 | 3.3628 | 3.685 |
| Boil Control | 54 | 3.2411 | 3.8807 | 3.0072 |
| Boil Control | 70 | 2.1569 | 2.439 | 2.0652 |
| Boil Control | 74 | 2.1776 | 2.0683 | 2.3516 |
| Boil Control | 80 | 2.0137 | 2.927 | 2.1189 |
| | | | | |
| Chill | 2 | 2.7849 | 2.8921 | 2.6472 |
| Chill | 6 | 3.98723 | 3.3648 | 3.6847 |
| Chill | 22 | 4.895 | 4.2117 | 4.8854 |
| Chill | 26 | 4.2583 | 4.5529 | 4.9581 |
| Chill | 30 | 3.4891 | 4.8972 | 3.065 |
| Chill | 46 | 2.4604 | 2.4618 | 3.5681 |
| Chill | 50 | 2.2879 | 2.6251 | 2.0654 |
| Chill | 54 | 2.3681 | 2.0486 | 2.0371 |
| Chill | 70 | 2.0658 | 2.2478 | 2.281 |
| Chill | 74 | 2.1743 | 1.9586 | 2.3748 |
| Chill | 80 | 2.0042 | 1.9843 | 1.9961 |
| | | | | |
| CF-Control | 2 | 2.6739 | 2.1769 | 2.6124 |
| CF-Control | 6 | 3.1147 | 3.4685 | 3.1254 |
| CF-Control | 22 | 4.3684 | 4.0176 | 4.6847 |

| | | | | |
|---|---|---|---|---|
| CF-Control | 26 | 4.6187 | 4.1817 | 4.6671 |
| CF-Control | 30 | 4.9201 | 4.0879 | 4.033 |
| CF-Control | 46 | 3.5517 | 3.5193 | 3.1547 |
| CF-Control | 50 | 3.9069 | 3.6283 | 3.6719 |
| CF-Control | 54 | 3.6649 | 3.766 | 3.3675 |
| CF-Control | 70 | 2.5019 | 3.083 | 2.987 |
| CF-Control | 74 | 2.3972 | 2.5831 | 2.3576 |
| CF-Control | 80 | 2.3749 | 2.244 | 2.1085 |
| | | | | |
| CF-Control | 2 | 2.6739 | 2.1769 | 2.6124 |
| CF-Control | 6 | 3.1147 | 3.4685 | 3.1254 |
| CF-Control | 22 | 4.3684 | 4.0176 | 4.6847 |
| CF-Control | 26 | 4.6187 | 4.1817 | 4.6671 |
| CF-Control | 30 | 4.9201 | 4.0879 | 4.033 |
| CF-Control | 46 | 3.5517 | 3.5193 | 3.1547 |
| CF-Control | 50 | 3.9069 | 3.6283 | 3.6719 |
| CF-Control | 54 | 3.6649 | 3.766 | 3.3675 |
| CF-Control | 70 | 2.5019 | 3.083 | 2.987 |
| CF-Control | 74 | 2.3972 | 2.5831 | 2.3576 |
| CF-Control | 80 | 2.3749 | 2.244 | 2.1085 |
| | | | | |
| Freeze | 2 | 2.84762 | 2.6841 | 2.6591 |
| Freeze | 6 | 3.9557 | 3.8476 | 3.4575 |
| Freeze | 22 | 4.68571 | 4.8637 | 4.6854 |
| Freeze | 26 | 3.9517 | 4.7941 | 4.5582 |
| Freeze | 30 | 4.8631 | 3.7532 | 3.1951 |
| Freeze | 46 | 2.415 | 3.1682 | 2.3647 |

| | | | | |
|---|---|---|---|---|
| Freeze | 50 | 2.0547 | 2.0476 | 2.8694 |
| Freeze | 54 | 2.3569 | 2.0684 | 2.2743 |
| Freeze | 70 | 2.1475 | 2.1648 | 2.3369 |
| Freeze | 74 | 2.1541 | 2.486 | 2.1574 |
| Freeze | 80 | 1.9578 | 2.1548 | 1.9972 |