THE EFFECT OF WORT BOIL TIME AND TRUB ON BARLEY MALT FERMENTABILITY THROUGH THE MINIATURE FERMENTATION METHOD

by

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ABSTRACT

The basic aim of barley malt breeders and maltsters is to produce malt with optimum fermentability levels. The purpose of this research was to understand and evaluate the effect of wort boiling and autoclaving at varying time periods (30, 45, 60, 90, and 120 min) on malt fermentability. The effect of trub content was also analyzed. Small-scale fermentations were carried out using a 'Control malt' and a standard SMA yeast strain. The Apparent Degree of Fermentability (ADF), Turbidity (absorbance at 600nm), and Density (^{o}Plato) were measured at specific time intervals over three day fermentation period and non-linearly modeled. From the results obtained, we suggest that heat treating wort with or without trub, (at 100°C, 121.1°C) for a range of times results in significant decline in the ADF ($p<0.05$). Free amino nitrogen levels of wort declined significantly with respect to both increase in wort boiling temperature and intervals ($p<0.001$).

List of Abbreviations and Symbols Used

A	amplitude of absorbance curve
Abs_{600}	absorbance at 600 nm at time t
ADF	apparent degree of fermentation
ADH	alcohol dehydrogenase
AE	apparent extract
ALDH	aldehyde dehydrogenase
$AREA_{DENSITY}$	area under density curve
$AREA_{TURBID}$	area under turbidity curve
ASBC	American Society of Brewing Chemists
ASE	asymptotic standard error
Atm	standard atmosphere
ATP	adenosine triphosphate
AMP	adenosine monophosphate
B	slope at point of inflection of "density" curve
$BOIL_{TIME}$	Period of wort heat treatment
dH_20	de-ionized water
EDTA	ethylenediaminetetraacetic acid
FAN	free-amino nitrogen/α-amino nitrogen
FLO	flocculation zymolectins encoding genes
hr	hour
$HEAT_{TYPE}$	Type of wort heat treatment
HMF	hydroxymethyl furfural
HMP	high molecular weight protein
HPLC	high pressure liquid chromatography
M	inflection point of the curve
OE	original extract
PDH	pyruvate dehydrogenase
PTFE	Poly-tetra flouro ethylene

$^{o}P_e$	final asymptotic extract value
$^{o}P_i$	initial asymptotic extract value
kPa	kiloPascal
$^{o}P_t$	Plato at time t
R	slope of turbidity curve
r^2	coefficient of determination
r	correlation coefficient
TSN	total soluble nitrogen
VDK	vicinal diketones
YEPD	yeast extract peptone dextrose
σ	standard deviation from mean
°C	degrees Celsius
°P	degrees Plato
μ	absorbance curve mean

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CHAPTER 1 INTRODUCTION

1.1 Overview of the Malting and Brewing Process

Malting initially involves hydrating barley grains by immersing them in warm water for 24-48 h which is termed as steeping (52). At this stage, enzymes in the endosperm hydrolyze and the grain starts to swell. Air rests are provided so that sufficient oxygen is available for germination to occur. In the next stage germination, the grain is incubated at a suitable temperature and humidity level for physical modification of the grain to begin. During the incubation period, enzymatic breakdown of complex starch and polypeptides into simpler, fermentable forms takes place (63). Kilning is the third essential step for producing colored malts, wherein the germinating grain is subjected to heating at high temperatures to deactivate the enzymatic hydrolysis as well as to provide necessary flavor and color to the malt (52). Once the grains are kilned, they are milled and the resulting ("grist") can be stored for long periods under controlled environmental conditions.

Mashing involves mixing the milled grain or "grist" with liquor (water) and subjecting it to specific time-temperature regimes to obtain optimum enzymatic breakdown of complex molecules prior to fermentation. The fermentable sugars, amino acids, simpler peptides, nutrients, and other minerals leach into the mash at this stage. An adjunct which serves as a rich source of starch may be added during mashing to increase the fermentable sugar content of the mash. Once mashing is complete, the mash is filtered to remove the spent grains and the resulting "wort" is subjected to boiling.

Wort boiling is one of the most energy-consuming steps in beer production, and can consume up to 50% of the total brew house energy requirements (52). Wort is boiled in a kettle or copper with hops for 60-120 min (45). Hops provide a characteristic aroma and bitter flavor to the beer. Due to high temperature during boiling, denaturation and precipitation of protein molecules take place. The precipitated wort solids are collectively referred to as trub (7). After boiling, the wort is cooled and then often clarified with the help of a whirlpool. The clarified bright wort is oxygenated and pitched with healthy yeast cells and fermented.

Yeast cells metabolize extract available in the wort to grow and multiply three to five times during fermentation (52). Fermentation produces ethyl alcohol, carbon dioxide, flavor compounds and heat. Fermentation is normally carried out for three to seven days, depending on the desired alcohol levels, yeast strain, flavors desired, etc. (52). By the end of fermentation, the yeast flocculates and beer can be easily separated. Finally, the beer is stored cold or lagered in conditioning tanks to enhance and refine its clarity and flavor. An overall brewing process outline is presented in figure 1.1.

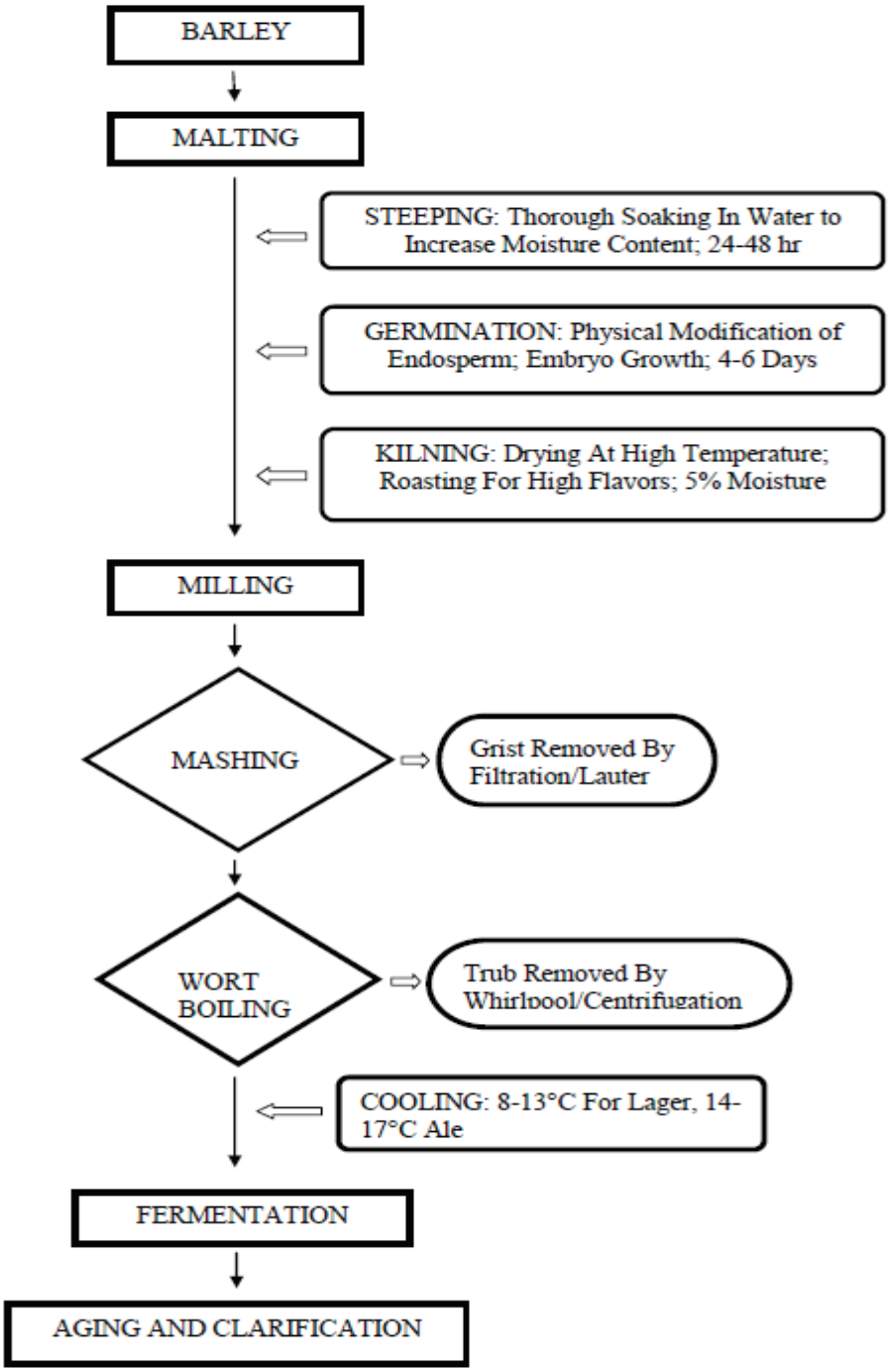

Figure 1.1 Outline of brewing process (Adapted from 9)

1.2 Wort Boiling and Miniature Fermentation

Beer has evolved as one of the most popular beverages over the years. Although, the basic principles behind brewing remain the same, there have been numerous advancements and additions to the process with time. Brewing primarily involves malting and milling of grains (barley), which are then mashed to produce wort. The wort is boiled at this stage with adjuncts (hops) and finally fermented to make beer.

Wort boiling is an important, energy-consuming but poorly understood, stage in the brewing process. Boiling not only sterilizes the wort but also brings required biochemical and physical changes in wort color, flavor, and composition (7). It leads to volatile stripping by evaporation, thereby causing substantial reduction in wort volume and flavor volatiles. Studies show that with prolonged boiling, flavor volatiles accumulate due to amino-carbonyl reaction that decreases both the amino acid and fermentable sugar concentrations (20). Consequently, the optimum wort boil time and temperature have been previously determined on the basis of flavor volatile concentration and thermal load reduction (46). However, an elaborate effect of different boiling schedules on malt fermentability has not previously been examined in detail. Boiling can bring significant biochemical changes to wort composition. It is by far most crucial step for wort stabilization, and can directly affect fermentation.

Boiling also brings out both qualitative and quantitative changes in the protein content of the wort. The denaturation and coagulation of proteins taking place during boiling cause precipitation of "trub". Formation of trub leads to significant losses in the total soluble nitrogen (TSN), high-molecular proteins (HMPs) and α-amino nitrogen (or fermentable amino, FAN) levels which can affect wort fermentability (7). Micro-nutrient deficiency can also arise when ions such as Ca^{2+}, and Mg^{2+} get entrapped in the trub and are removed thus potentially causing a decline in wort fermentability. Moreover, some studies have shown that yeast growth is enhanced when the wort is fermented with trub, as the trub acts as the site of

nucleation and carbon dioxide (CO_2) release (60). Due to the complex nature of the trub, its effect on malt fermentability is difficult to ascertain.

Boiling provides a complex environment resulting in the formation of several volatile compounds such as di-methyl sulphide (DMS), and hydrogen sulphide (H_2S), etc. (7). These volatile compounds if not allowed to evaporate, can continue to persist in the wort at high concentrations. However, the effect of boiling and volatile evaporation on yeast growth and fermentation has not yet been studied. With prolonged boiling and lack of evaporation, the fermentation performance could be compromised due to the persistence of volatile compounds or the creation of new volatile compounds.

Miniaturized fermentation assays were used to monitor fermentability in this study. It allows for the assessment of the effect of wort boiling and trub formation on the overall fermentability while keeping other fermentation variables constant. A limited amount of control barley is required, and the fermentation is carried out in small amounts (31). These small scale assays help in determining the rate of sugar consumption over a short period of time, which distinguishes and characterizes the given wort fermentability. Scaling down of fermentation assay can enable better monitoring and evaluation of parameters being studied.

CHAPTER 2 HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Biochemical and physical modification of wort caused by variations in kettle boil and trub levels affect malt fermentability.

This is possibly due to multiple, correlated factors such as nitrogen levels, browning reactions and products and hydrogen ion activity.

2.2 Objectives

This study was designed to understand and quantify the effect of (I) heat treatment time and temperature, and (II) trub levels on malt fermentability. The wort was subjected to heat treatments of different temperatures and pressures: boiling (at 100 ^{o}C, 1 atm), and autoclaving (at 100^{o}C, 1 atm), and finally high-temperature autoclaving (at 121.1^{o}C, 2 atm) for increasing time intervals of 30, 45, 60, 90, and 120 min. Any changes in wort fermentability based on the treatment temperature, condition, or intervals were then substantiated and reasoned. The physical and nutritional effects of the trub were determined by carrying out fermentations (I) with the trub and (II) without the trub. The amount of trub formed after each heat treatment was quantified and fermentations were compared.

CHAPTER 3 LITERATURE REVIEW

3.1 Wort Boiling

Wort kettles in early times were referred to as “coppers” as they were made of copper metal. Owing to modern advancements, coppers are now made with stainless steel, but can still be referred to as coppers. Traditionally, kettle boil time was between 90 and 120 min with 10% evaporation rate (18). However, typical modern kettles boil wort for 60-90 min with evaporation rate of 5-9% (56). High temperature boiling is also widely employed in Europe, where a temperature range of 118-122°C and holding time of 8-10 min can be selected (41). Steam at 148°C and 400 kPa (3.95 atm) absolute pressure is used as the heating agent in heat exchangers (8). Boiling alone consumes 40% of the total brewhouse energy requirements (56). Hops are added during boiling to provide bitter flavor, aroma, and antimicrobial attributes to beer (52). During kettle boil hop acids isomerize enhancing the bitter taste of beer.

A vigorous boil is essential to bring about the required changes in the wort. Vigor is denoted by wort circulation or agitation and the rate of evaporation (52). Insufficient vigor leads to turbidity in wort, which signifies an incomplete or improper coagulation of HMPs (56). However, excessive shear can break the trub into fine flocs, not allowing them to coagulate properly. It has been suggested that with adequate turbulence during the boil, the removal of HMP fractions is a function of time and vigor, and is independent of the evaporation rate (56).

Low-pressure wort boiling involves increasing the pressure in the copper to a small extent that provides an absolute pressure of up to 200 kPa (1.97 atm) (8). With boiling at these relatively low-pressures, the boil temperature can be increased sufficiently while the duration of boiling is decreased. At a high boil temperature, the rate of reaction increases. Therefore, the changes required in the wort can be brought in lesser time. When boiling is done in pressurized containers, minimal evaporation takes place. This causes inadequate release of volatiles from the wort. However, in modern day systems "dynamic low pressure boiling" can be employed where wort is boiled at 100–103°C under series of pressure build-up and the pressure is released at the end of boiling. This causes the stripping of volatiles with a lower evaporation rate of 3.5-5% (40). Holding time at high temperature boiling (<140°C) is substantially reduced so as to minimize volatile formation (41).

3.2 Changes during Wort Boiling

3.2.1 Thermal Destruction of Microbial Load

Wort production involves many processes, and this can make contamination by microbial sources highly probable. Boiling at 100°C destroys microbial vegetative cells in the initial 10-15 min (52). Spores, however, may persist. Although the beer environment due to its high alcohol, low pH, and low oxygen content makes it almost impossible for the spores to germinate, the growth of contaminating microbes from the wort is plausible.

3.2.2 Inactivation of Malt Enzymes

It is essential to denature and deactivate residual malt enzymes that passed from the mash to the wort. With active malt enzymes, there will be a continuous breakdown of already simplified sugar and protein units, which will alter the fermentability of the wort (56).

Therefore, with a boil temperature of 100°C, thermal denaturation of all malt enzymes passed from the mash is achieved.

3.2.3 Evaporation of Volatile Compounds and Concentration of Wort

Wort contains numerous undesirable flavors and aromatic compounds that upon boiling get volatilized and evaporate. The Maillard reaction itself produces numerous volatile aromatic compounds, which if not volatilized, can easily be detected in beer due to their low flavor thresholds (8). Therefore, evaporation is considered crucial for maintaining beer flavor consistency. Sufficient evaporation during the kettle boil normally result in a 7-10% decrease in wort volume (8). While it is desirable to obtain concentrated wort with less volatile composition after the boil, however, the rate of evaporation has been reduced to 4-8% over the last 30 years to optimize yields (56).

Boiling reduces levels of inorganic sulphur compounds such as hydrogen sulphide and sulphur dioxide which can deliver off-flavors in the final beer (52). One of the major sulphur flavor volatiles from malt is DMS. It has a low flavor threshold of 40-60 µg/l and gives a sweet corn smell to the beer (8). DMS is produced during wort boiling and cooling stages from the thermal decomposition of precursor compound, S-methylmethionine. Evaporation during boiling causes a significant loss of DMS. However, S-methylmethionine continues to breakdown during wort cooling, eventually leading to the presence of free DMS in the wort (56). It is noteworthy that due to the major loss of this volatile during evaporation, the final concentration of DMS in the beer is usually below the detection limit.

An increase in boiling time can also result in serious flavor defects as the rate of formation of volatiles can exceed their evaporation rates. The levels of certain aging relevant carbonyl compounds, such as Strecker aldehydes (2-methylpropanal, 2-methybutanal and 3-methylbutanal) and other flavor volatiles (4-vinylguaiacol and 3-

methyl-2-butene-1-thiol), in aged beer showed an increase with increase in boil times (46). Thermal heat treatment of wort has also been associated with the formation of diacetyl and 2,3-pentanedione, which can cause serious flavor defects at high concentrations (11).

3.2.4 Proteins

Proteins are degraded during the malting and mashing stages, wherein proteinases break down the protein reserve of a barley endosperm. Boiling causes changes in the physiochemical properties and structure of wort proteins (26). The level of coagulable nitrogen in unboiled wort was found to be 35-70 mg/l while it was reduced to 15-25 mg/l after boiling (8, 41). High-molecular weight proteins (HMPs) either get removed by coagulation during boiling or get transferred to the final beer, where they can interfere with filtration and colloidal stability (52). Rourke (2002) stated that boiling causes 96% removal of polypeptides and proteins >10,000 Da. A one hour boil can result in 6% loss of the wort`s proteinaceous material (52).

Osman (2003) reported that only 20% of barley proteins are water soluble and remain unaffected during boiling and fermentation stages. Gel electrophoresis of wort before and after the boil showed high concentration of low molecular weight proteins (<14,000 Da) but no fraction greater than 14,000 Da. This signifies that boiling has no effect on the protein content (47). It was suggested that only small, soluble proteins formed during the malting and mashing stages are present in the wort and which partially get removed during boiling and precipitation (47). It should be noted that his report stating that no HMW proteins exist in wort is at odds with most brewing texts and literature (8, 47, 52).

3.2.5 Trub Formation

Wort boiling causes protein molecules to denature and coagulate. Upon cooling, these protein molecules coagulate to form protein-protein and protein-polyphenol complexes, termed as trub. It comprises precipitated protein-protein (50%), protein-polyphenol complexes (15±25%), lipids, and wort carbohydrates (20±30%) (8).

Proteins are composed of peptide chains folded together in a way that renders their stability and functionality. Boiling causes disruption in the tertiary structures of HMPs, exposing their hydrophobic areas. The protein molecules get destabilized and interact with the hydrophobic groups on other protein surfaces. This causes insolubility and coagulation of proteins leading to formation of large flocs. Protein-protein complexes formed during boiling weigh 40-70 g dry wt./hl (8). With more vigor and longer boiling durations comparatively higher protein coagulation is obtained, leading to added trub formation. High temperature and pressure have also been found to increase protein coagulation (52). Removal of large protein molecules from the wort is favored as they tend to form non-biological haze during the maturation of beer as well as affect foam stability (8).

Wort also contains high level of polyphenols. They are highly reactive and combine with sulfhydryl and amino groups of proteins, eventually leading to the formation of complexes that constitute the trub (15). However, due to the nature of these complexes, they are found to be unstable at boiling temperatures. Protein-polyphenol and polyphenol-polyphenol complexes become stable and coagulate upon cooling the wort by around 80°C (8).

Undesirable fatty acids derived from malt, can interfere with foam stability. Around 99% of wort lipids also get deposited with the trub, while some get volatilized during evaporation (8).

3.2.6 Color and Flavor Compounds

One of the major changes incurred by wort boiling is the Maillard reaction/non-enzymatic browning. During non-enzymatic browning, reduced sugar moieties react with amino acids in a complex series of reactions, leading to the formation of insoluble brown colored products, melanoidins (15). These reactions are discussed in detail in section 3.3. Melanoidins are majorly responsible for wort color and flavor. The Maillard reaction also produces many aliphatic and aromatic compounds that are responsible for providing a unique flavor and aroma (such as caramel flavor) to the beer. Boiling also causes trans-2-nonenal (ageing flavor) to react with amino groups to form imine compounds which are responsible for cardboard-like flavors in beer (59). Phenolic and polyphenolic compounds also contribute to wort color (47).

3.2.7 Changes in pH

Towards the end of mashing, optimum mash pH is usually maintained at 5.4-5.6. During a wort boil, the pH drops by 0.2-0.3 units to reach a final wort pH of 5.2-5.3 units (52). Drop in the pH can be attributed to the loss of Ca^{2+} ions due to protein-protein and protein-polyphenol precipitation in the trub. Binding of Ca^{2+} ions causes release of H^{+} ions from the complex formed, thereby increasing the acidity of the solution (52). It has been found that even with a slight increase in the level of calcium from 50 mg/l to 350 mg/l, the wort`s pH decreases substantially from 5.51 to 5.1 (4, 70). The pH has a significant effect on the clarity of the wort; it has been found that wort with a pH of below 4.5 completely fails to achieve clarity (4, 33).

3.3 Maillard Browning

Formation of the Maillard reaction products greatly depend on pH, time and temperature of the reaction. It has been reported that with an increase of 1°C correspondingly increased the Maillard reaction products by 10%, from a range of 60 to 100°C (3). Therefore, with an increase in the temperature and the duration of the reaction, more advanced Maillard products will be formed with greater or darker colors (15). Strecker aldehydes and furans which are intermediate browning compounds have been found to develop linearly with time in a pseudo-zero order relation and first-order relation, respectively (42). The extent of browning can be estimated by measuring absorbance at a wavelength of 420-460 nm (14). However, due to a complicated set of reactions along with several intermediate complex compounds, a kinetic study of the Maillard reaction is difficult. Also, the presence of multiple reducing sugar and amino acids, which show different reactivity at each stage, does not allow reaction products to be easily quantified (6).

3.3.1 Chemistry

Maillard reaction comprises of numerous steps leading to the formation of Melanoidins (3, 8). Primarily, reducing sugars (ketoses and aldoses) react with amine to form Shiff's base (imine compound), which isomerizes to a more stable "Amadori compound". Degradation of the Amadori compounds continues throughout the mashing and boiling stages and some are even passed on to the beer (3, 8). Under acidic conditions, Amadori compounds forms deoxysomes and then decomposes to 5-hydroxymethylfurfural (HMF). Further breakdown of deoxysome intermediates can give rise to α-carbonyl compounds, pyruvaldehyde, 2,3-butanedione (diacetyl), furfurals, and other related compounds (3, 8). The α-carbonyl compounds formed react with amine through Strecker reactions to form aldehydes and α-aminoketone, which condense together to form pyrazines. These

compounds have strong odors. Complex set of reactions involving aldol-condensation and carboyl-amine take place further and finally lead to the formation of melanoidins (3, 8). Other end products also include oxygen, nitrogen, and sulphur heterocyclic compounds (8).

3.3.2 Intermediate Browning Compounds

Browning reaction products have been known to affect growth and metabolism of many micro-organisms (5, 19, 69). Furfurals, maltol and 5-hydroxymethyl furfurals (HMF) are the most common intermediates in the formation of complex browning compounds. They are chemically related compounds (contain a furan ring and an aldehyde group), and their concentration can be used to determine the extent of a browning reaction in a given system (8).

HMF, maltol, and furfurals affect growth shortly after coming in contact with yeast cells (5). They affect the growth rate of yeast cells by interfering with their CO_2 producing ability. At concentrations as low as 1-5 mg/mL the fermentation rate was considerably affected (5, 69). HMF and furfurals are found to be assimilated by yeast cells during both aerobic and anaerobic fermentations resulting in the formation of corresponding alcohols, 5-hydroxymethylfurfural alcohol and furfural alcohol, as well as other intermediate compounds (69). The reduction reactions are carried out by enzyme alcohol dehydrogenase. The presence of oxygen does not have any effect on the conversion or uptake rate of these compounds or on their inhibitory effects (68, 69).

The addition of HMF at low levels (4 g/L) to anaerobic batch fermentations supplemented with glucose (50 g/L) showed a decrease (32%) of the carbon dioxide evolution rate compared to the controls (69). HMF is all taken up at constant rate from the medium and stops when wort sugars are completely depleted. Upon the addition of furfurals (2 g/L) along with HMF (2 g/L), furfurals were converted initially and no

substantial yeast growth took place till both furfural and HMF were present in the medium (69). When added singularly, furfurals showed high conversion and reduction in CO_2 evolution rates, signifying a stronger effect on yeast metabolism than that of HMF (68). Furfural degradation rate was found to be higher in young yeast cells than that in stationary cells (68). Taherzadeh et al. (1999) also suggested that low conversion rates of HMF can cause it to last comparatively longer in the media, which can lead to more serious effects than those of the furfurals. Therefore, even though browning compounds are formed in minor amounts during wort boiling, their effects can last during fermentation.

The basic mechanism by which HMF and furfurals affect yeast growth is unclear. It is believed that the reduction of HMF and furfurals interferes with the reduction of acetaldehyde and can compete with cell's glycolytic capacity (69). It has been found that the presence of furfurals leads to inhibition of the following enzymes: alcohol dehydrogenase, aldehyde dehydrogenase, pyruvate dehydrogenase and hexokinase. These enzymes are vital for yeast metabolism (68, 42). The activity of PDH and ALDH was reduced by more than 90%, while ADH activity was reduced by around 20%, when furfurals were present in a concentration less than 2 mM (42).

Inhibitory effect of these compounds gets partially reversed upon supplementing the media with small peptides, amino acids or vitamins. However, the effect of furfurals present in a concentration of 2 mg/mL was found to be too high to get reversed (5).

3.3.3 Melanoidins

Melanoidin pigments are a wide group of heterogeneous compounds which are differentiated based on the basis of their sugars and amino acid constituents, ratios, pH, and temperature of the reaction (8). They have high molecular weight polymeric structures of the order of 10,000-30,000 Daltons (8). They have been associated with

oxidative stability of beer and the production of flavor active volatiles. This makes melanoidin concentration a considerable factor for establishing malt quality (4, 24, 61). Melanoidins also improve foam stability and provide better mouth feel (11). Low levels of melanoidins are preferred in beer as at high concentrations they inhibit yeast cell growth by interfering with CO_2 production (5, 11). Reduced fermentability was observed upon fermenting with dark malts, as they have considerably higher levels of Maillard browning compounds, i.e., melanoidins (11).

3.4 Yeast Flocculation

Brewing yeast are off-white, single, oval eukaryotic cells of 5-10 µm in length and 5-7 µm in breadth (52). The brewing yeast can be differentiated into two groups: Ale and Lager yeast. Ales are top fermenting yeast belonging to the species *Saccharomyces cerevisiae*. They flocculate towards the end of fermentation and rise to the top of the fermenting vessel. On the contrary, lager yeast is bottom fermenting which are also classified as *Saccharomyces cerevisiae,* although previously they were considered separate species *Saccharomyces uvarum*, which is characterized by flocculating and settling to the bottom of the fermenter (52). Ale and lager yeasts show optimum fermentation at different temperatures of 15-20°C and 10-15°C respectively (8). However, the maximum growth temperatures can vary for the ale and lager strains and range between 37- 40°C and 31.5- 34°C, respectively (8).

3.5 Factors Affecting Yeast Flocculation

3.5.1 Cation Concentration

The flocculation process is strongly dependent on cell-cell proximity as well as Ca^{2+} ion concentration in the wort (62). Cells must be in close contact with each other for flocs to be formed which means flocculation requires high cell concentration. The mannoproteins on a yeast cell wall are cross-linked to lectin like proteins, termed zymolectins, on an adjacent cell by the help of Ca^{2+} ions (62). Soares et al. (2000) found that Ca^{2+} at least 10^{-8}-10^{-4} mol/L concentration is necessary to induce flocculation, while Ernandes et al (1993) reported that 50 mg/L of Ca^{2+} ions is required for good flocculation.

Presence of magnesium (Mg^{2+}) and zinc (Zn^{2+}) is also crucial for flocculation. Yeast cells do not show flocculation in medium lacking Mg^{2+} ion (61, 72). With Zn^{2+} ion supplementation, yeast flocculation improves while the average size of flocs decreases (54, 72, 73).

3.5.2 Wort pH

Wort pH is also found to influence yeast cell flocculation by interfering with surface charge distribution. Flocculation occurs toward the end of fermentation when the pH has declined from 5.2 to 4.5-4.0 (29). Wort pH declines during fermentation, resulting in a decrease in the electrostatic repulsion between cells. The effect of wort pH changes is not considered substantial as cells have been found to flocculate from pH 2.5-5.5 (62). However, at extreme pH values, cells tend to show dispersion. This is probably because of structural changes caused by the decline in pH on the ionization of amino-acids that constitute the zymolectin structure (29).

3.5.3 Cell Surface Hydrophobicity and Surface Charge

Other factor that influences flocculation is the phosphate content of brewing yeast cell wall. It determines the surface charge (zeta potential) and cell surface hydrophobicity (8). Yeast cells after 24-48 h of fermentation show increased surface hydrophobicity compared to that at the start of fermentation. The increased surface hydrophobicity may cause a decrease in the cell surface charge, allowing yeast cells to easily come in contact with each other (65).

3.5.4 Nitrogen

Nitrogen is considered as a growth limiting factor; therefore, it plays a role in the onset of flocculation (57, 72). Smit et al. (1992) found that the addition of glutamate (free amino nitrogen) or a standard mixture of various amino acids results in the dispersion of cells, thereby delaying flocculation of the yeast strain. It was also found that decline in amino acids resulted in less cell numbers at the stationary growth phase (61). Interestingly, the triggering of flocculation was observed at a high level of glucose (>10 g/L) but without nitrogen (57). However, in some cases, supplementing the wort with amino-acids did not change the flocculation behavior of the tested lager strains (57, 72).

3.5.5 Other Factors

Other factors that affect flocculation include fermentation conditions such as bio-availability of sugars, growth temperature, ethanol concentration and genetic make-up (62). Presence of sugar disrupts flocs and increases dispersion as zymolectin molecules on yeast cells bind to sugar molecules instead of acting as flocculation receptors. The onset of flocculation is, therefore, highly correlated to the presence of the lowermost

amount of fermentable carbon (57, 72). Under extreme fermentable carbon starved conditions, flocculation seems to be inhibited or reversed only by the presence of a carbon source (64). Presence of ethanol increases flocculation character as it is associated with decreasing cell-cell electrostatic repulsion and increasing cell surface hydrophobicity (27). Growth temperature is another influencing factor as it relates to the rate of metabolic reactions by yeast cells. Lower fermentation temperature will reduce yeast metabolism, resulting in slower release of CO_2. This will decrease shear or turbulence, thereby aiding in flocculation (65).

Flocculation is found to be triggered in lager strains if there is depletion of sterols or fatty acids from the culture medium (67, 62). Sterols and fatty acids play an important role in the delivery and stabilization of flocculins or zymolectins to the yeast cell surface (73). In terms of genetic factors, a majority of brewing lager strains belong to the NewFlo phenotype, which signifies that the expression of FLO genes (which encode for flocculation zymolectins) is dependent on and controlled by nutrient availability (71).

3.6 Yeast Metabolism

The normal aim of beer fermentation is to derive a low wort specific gravity by the conversion of sugar into ethanol and carbon dioxide. As yeast converts wort dissolved solids (extract) into alcohol, there is a continuous decline in specific gravity from start till the end of fermentation. The cell density increases with time while the pH of the medium declines. A typical fermentation profile of lager yeast is shown in Fig. 2, which includes decline in specific gravity, pH, ethanol, CO_2 release, and cells in suspension with time.

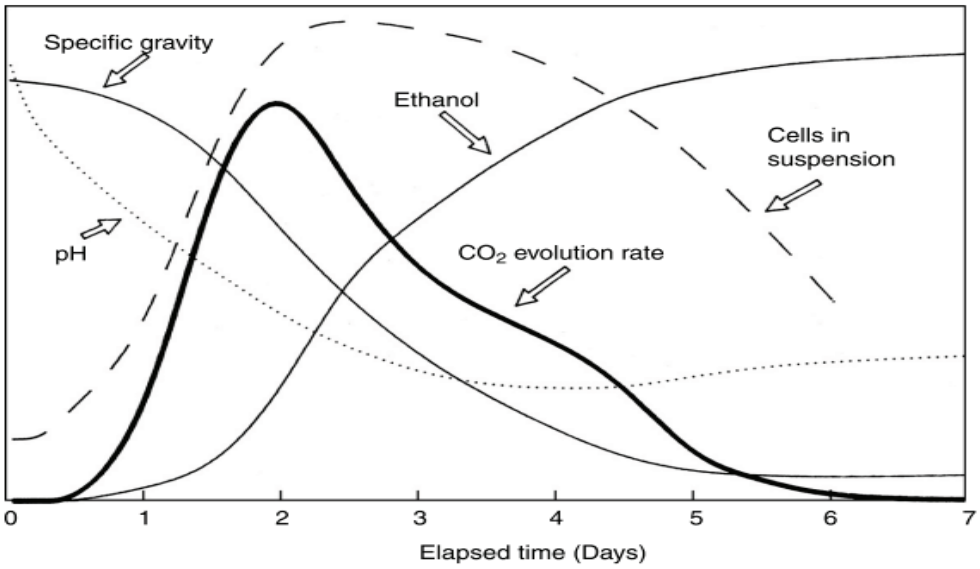

Figure 3.1 Typical fermentation profile of lager yeast (52).

3.6.1 Carbohydrates

Once yeast is inoculated in a fermentation vessel, it starts using up sugar, oxygen and other nutrient sources to grow and multiply. The types of fermentable sugars present in the wort are sucrose, glucose, fructose, maltose, and maltotriose. Some unfermentable sugars such as dextrins also exist. The yeast prefers to consume sugars in the sequence of glucose, sucrose, and fructose followed by maltose and then maltotriose (52). It is worth noting that recent findings by the Dalhousie brewing group have indicated that the yeast begins consumption of the sucrose before all of the wort glucose is metabolized.

3.6.2 Oxygen

Although fermentation is an anaerobic process, oxygen is required by the yeast at the time of pitching to grow efficiently. Yeast cells require oxygen for the bio-synthesis of unsaturated fatty acids and sterols which are incorporated in the cell membrane for cell fluidity and function (7). With cell division during the course of fermentation, the

amounts of unsaturated fatty acids and sterols decrease in all yeast cells and can reach low growth limiting levels (52). Therefore, it is required to properly aerate the fermentation media before yeast inoculation so that sufficient unsaturated fatty acids are synthesized in the beginning of the fermentation. The yeast is reported to consume all available oxygen in the initial 6-10 h of fermentation (52).

3.6.3 Wort pH

A constant decline in pH is observed as fermentation progresses. The fall in pH is due to a number of factors such as the decline in FAN levels, the production and release of organic acids, and the direct excretion of H^+ ions by yeast (52).

The decline in FAN levels can impact the pH to a great extent. Wort buffer systems depend on the level of amino acids such as glutamate and aspartate, which constitute the most of the amino nitrogen content of wort. Therefore, FAN levels and wort pH show a high degree of correlation (70). FAN is responsible for providing a buffering action; however, it also enhances yeast growth causing an increase in H^+ ion excretion (52). At low wort FAN levels, an increase in FAN results in higher wort and beer pH due to increased buffering ability (70). Upon increasing the wort FAN levels beyond 120 mg/L, the final beer pH started has been reported to decline because of an increase in H^+ ion excretion during stimulated yeast growth (70). Other factors that improve yeast growth such as increase in oxygen and zinc levels can also cause decline in the final beer pH (4).

3.6.4 Proteins

Typical wort nitrogen content is made of 30-40% amino acids, 30-40% polypeptides, 20% proteins and 10% nucleotides (8). A majority of the HMPs and polypeptides precipitate during wort boiling and are lost. Moreover, yeast cells also do not have the

pathways essential to breakdown and assimilate the majority of proteins and polypeptide molecules. However, some wort proteins are broken down by extracellular proteolytic enzymes synthesized by yeast upon the depletion of wort amino acids (35). Maria et al. (2009) reported a 35% decrease in non-assimilable nitrogen in all-malt beer. On the contrary, Osman et al. (2003) found that the fermentation process is entirely independent of proteins and polypeptides suggesting that all the nutritional needs of the yeast are satisfied by free amino acids and small peptides.

3.6.5 FAN

Yeast cells are able to assimilate ammonium ions, small peptides usually some di- and tri-peptides and individual free amino acids, which together constitute the FAN content or assimilable nitrogen (34, 45). The FAN levels are believed to be a suitable index for potential yeast growth and fermentation efficiency (34, 45). FAN levels in the wort depend on the barley variety and malt and mash regimes, but the overall proportion of individual amino acids is similar among different worts (45). FANs are mostly formed during mashing by the action of proteases on proteins and polypeptides present in the barley endosperm (45). Any variations in duration and temperature of mashing result in changes in the total FAN content as well as the amino acid spectrum of the wort. The optimum temperature for protolytic activity during a mash is from 40-50 °C (52, 45). The relative amounts of individual amino acids show high variability in wort as alanine, valine, leucine, lysine and proline are major amino acids; while aspartic acid and proline are formed in minor amounts (45). Of all these, proline is the most abundant amino acid in all malt worts but it cannot participate in the Maillard reaction to form flavor and color producing beer compounds because of lack of the free amino group (45, 49, 50).

FAN includes α-amino nitrogen that constitutes all amino acid moieties except proline as it does not get assimilated by yeast cells under anaerobic conditions (34). However, under extreme nitrogen-limiting conditions, proline can be incorporated intact into yeast

proteins (45). Wort ammonia and ammonium ions are also consumed quickly in the initial 19 h of fermentation (34). Amino acids have been divided into four groups on the basis of the corresponding preferential absorption by the yeast. Group A amino acids (glutamine, serine, threonine, lysine, arginine, asparagine, aspartic and glutamic acid) are absorbed first and assimilated, while glycine is one of the last assimilated amino acids (34, 51).

Yeast growth increases linearly with an increase in α-amino nitrogen content up to a level of 100 mg/L. This level was considered as the minimum FAN level to support a healthy yeast growth and fermentation in a 12°P wort, while 142 mg/L was considered as the high amino nitrogen content (49). Ingledew et al (1991) found 220 mg/L FAN in a standard all-malt wort of 12°P and considered it as the optimum FAN level. Other researchers suggested the minimum FAN level for normal gravity worts to be 140-150 mg/L (25). A total of 203 mg/L of assimilable nitrogen was considered to be appropriate for the fermentation of 14° P wort (16). The fermentation performance of 14°P wort was found to be affected when FAN levels were below 144 mg/L; while high gravity wort of 18°P required at least 280 mg/L initial FAN concentration (7, 40).

The supplementation of slow fermenting worts made from malt extract with rapidly assimilable amino acid, L-glutamic acid (150-300 mg/L) stimulated the fermentation rate (48). The rate of fermentation was further enhanced when yeast extract was added in place of a single amino acid, while supplementation solely with minerals showed no change (48). This indicated that yeast growth is stimulated to greater extent when the wort is supplemented with a variety of amino acids instead of a single amino acid or mineral.

Supplementing the wort or growth media with lysine showed an increase in the speed of fermentation compared to control worts (34). With the stimulation in yeast growth, addition of FAN's also promote the formation of Vicinal diketones (VDKs) such as

diacetyl, 2,3-pentanedione, and their precursors (34). Addition of aspartate (0-1600 mg/L) resulted in increased synthesis of ethyl acetate and n-pentanol (21). On the contrary, fermentations supplemented with methionine resulted in incomplete uptake of amino acids present in the wort, resulting in long fermentations with less VDKs compared to the control (34). This proves that yeast cells preferentially need certain free amino groups to synthesize the required cellular proteins and other nitrogenous compounds; however, FAN levels also play important role in deciding other aspects of fermentation such as levels of esters, higher alcohols and VDKs (10, 34, 37, 45, 49, 52). It has been found that yeast cells tend to synthesize essential amino acids (such as valine) that they lack by alternative pathways. This results in formation of by-products which eventually accumulates and cause serious flavor defects in the final beer (45).

Initial FAN levels in the wort determine the rate of FAN uptake, sugar consumption and ethanol production by yeast cells (48). Excess of FAN levels has been associated with the stimulation of rate of fermentation and not yeast growth (45). FAN levels can also alter the sugar uptake capability of yeast cells. It has been found that aspartate at 500mg/L enhanced maltose uptake capability but caused inhibition of maltose uptake at much higher levels (21).

Lack of amino acids can also cause the yeast to uptake less useful amino acids which can further affect the rate of fermentation and growth. In case of nitrogen-limitation, an increase in the cellular AMP:ATP levels has been reported. The increased cellular AMP:ATP levels inhibit phosphorylation of glucose and its entry to glycolysis (12). Therefore, lack of FAN can also lead to yeast growth arrest and can result in “stuck” or “hung” fermentations. It has been found that low levels of nitrogen sources in adjunct worts have resulted in extended fermentation (45). Yeast nitrogen deficiency can also lead to an increase in peptide uptake (16). Under high concentration of amino acids (10-27 mM), yeast cells preferentially assimilate certain amino acids than the rest (23). However, under limiting conditions (2mM) they show di-peptide assimilation (23).

3.6.6 Inorganic Ions

Zinc is an essential nutrient and is required as a cofactor in several metabolic enzyme reactions and protein synthesis in a yeast cell. Optimum Zn^{2+} ion concentration has been found to be 0.1-0.15 mg/L for fermenting16°P wort (9). Also, Zn^{2+} has been found to increase the rate of fermentation up to a level of 1.07 mg/L, beyond which the process is, affected adversely (44). Zn^{2+} is used up by cells in the initial 24-48 h of fermentation, and it gets diluted into the progeny during subsequent cell divisions. Concentrations less than 0.1 mg/L are considered low which can lead to stuck or slow fermentations (44). Interestingly, it was found that zinc has a direct positive effect on the fermentation rate and has no correlation with cell growth or cell number (9). However, a majority of Zn^{2+} ions are lost during the precipitation of proteins and polyphenols for trub formation.

3.6.7 Trub

Fresh wort normally contains variable amounts of suspended solids. These solids are various biological components precipitated “trub”. Trub loss during boiling process causes a loss of wort turbidity (18). Heat induced denaturation and coagulation of proteins and polypeptides is also termed as hot break (18). Duration and vigor of the boil along with wort pH decide the quantity of trub formed. On an average trub levels vary around 200-400 g/hl wet weight comprising of 80-85% water (18). A boiling time of 1-2 h is considered essential for sufficient hot break. Wort pH between 5.2-5.5 results in optimum protein coagulation, while pH below 5.0 does not allow protein to coagulate (18). Brewers remove the hot break by centrifuge, sedimentation or whirlpool action to obtain highly clarified wort (18).

Upon cooling of clear wort to 60°C, protein-polyphenol complexes precipitate which are termed as cold break (18). Unlike hot break which is mostly proteinaceous, cold break contains more carbohydrates and polyphenols. Both hot and cold breaks are collectively considered as trub. The wort must be cooled to below 10°C to obtain optimum cold break

(52). The total dry weight of trub formed from cold break varies from 15 and 30 g/hl (52). Researchers and brewers have varied opinions about removal of cold break. Some suggest filtration of cold break improves wort clarity and fermentability, while others have found no benefits and rather slow fermentations on trub removal (52).

Studies have been conducted on trub for a long time. It has been previously reported that trub has stimulatory effect on yeast growth as it acts as nutritional factor and hence, promote fermentation (60). Interestingly, authors have suggested improvement in yeast growth is not through any nutritional effect, but because of solid particles acting as nucleation sites for CO_2 release, that was further supported by addition of activated carbon in wort in place of trub (36, 60). Yeast growth gets affected by levels of wort dissolved CO_2; therefore, release of CO_2 improves fermentation. Moreover, it was found that increased release of CO_2 bubbles in the fermentation media provides significant turbulence to keep the yeast in suspension for longer times (36).

Different factors associated with trub have stimulatory effect on yeast growth, and hence, they improve fermentations. Minerals such as zinc, calcium, magnesium and manganese may be found in trace amounts in worts but their requirement is very crucial for the yeast`s metabolic processes, including glycolysis (9). Trub particles can chelate metal ions causing mineral deficiencies in fermenter. Mineral deficient worts show poor yeast growth and delayed fermentations (9). Trace metals such as zinc play a vital role in wort fermentation and is required by the yeast for cellular protein synthesis and nucleic and carbohydrate metabolism. Zn^{2+}concentration in brewery wort can vary between 0.1 and 5.0 mg/L (2). During wort boiling process zinc binds with precipitated proteins, therefore, limiting its bio-availability to cells (44).

Calcium ion, Ca^{2+} is a very vital wort nutrient involved in maintaining cell membrane structure and function. Calcium levels in the wort vary from 15 to 35 mg/L (9). Calcium protects yeast cells from ethanol stress and provides tolerance (9, 44). Increase in calcium levels of the wort by supplementation increases the ethanol production (9, 17). Interestingly, its stimulatory effect has been contradicted in various studies. Some studies

have observed increase in cell growth and fermentation with increasing Ca^{2+} levels while others have not (17). In addition, calcium has been shown to play a critical role in cell flocculation by controlling zymolectin conformation (17).

Magnesium is another important nutrient which is adsorbed with trub and plays vital role in fermentations. It is required for cell metabolism and for enzymes mainly involved in glycolysis (17). In addition it provides protection to cells from stress conditions of temperature and osmotic pressure (9). The amount of magnesium in the wort varies from 50 to 90 mg/L and its supplementation (up to 500 mg/L) in both standard (12°P) and high gravity (20°P) worts results in higher fermentation rates, increased sugar uptake and high ethanol production (17). Besides the individual presence of magnesium and calcium, the ratio of magnesium to calcium is also equally important for fermentations. Rees et al. (1997) showed that the fermentation rate increases with increase in magnesium to calcium ratio (55). However, increase in the fermentation rate was not observed with the addition of calcium. Interestingly, addition of calcium during the initial hours of fermentation leads to a decrease in ethanol production and the maltotriose uptake rate (55). These results suggested that the stimulatory effect on fermentation rates, sugar uptake and ethanol production may be related to the Mg:Ca ratio rather than the effective concentrations of magnesium and calcium individually (55).

Trub also comprises wort lipids which are derived from barley grain. These lipids are vital components of cell membranes and are involved in structural and metabolic membrane activities (7). Presence of unsaturated fatty acids in trub provides yeast nutrition and shows better fermentability of turbid worts (60). The addition of lipids mainly ergosterol and unsaturated long chain fatty acids, has a positive effect on yeast growth and fermentation (30, 52, 58). It was found that high concentration of lipids in deoxygenated or low oxygenated wort, stimulated the fermentation process (30, 58). In addition, filtered wort with low oxygen fermented poorly, showing the vital role of lipids and lipid materials in yeast growth and fermentation rates (58).

Some researchers have found negative effect of suspended trub solids on yeast growth and activity. It has been reported that suspended solids can lead to an increased tendency for yeast autolysis and a decreased cell density in the fermenter (22, 60). Removal of trub minimizes the adsorption of protein-tannin complexes on yeast cell walls (67).Trub lipids have also been found binding to yeast cell wall inhibiting active nutrient uptake and cell growth (52).

CHAPTER 4 MATERIALS AND METHODS

4.1 Experimental Design

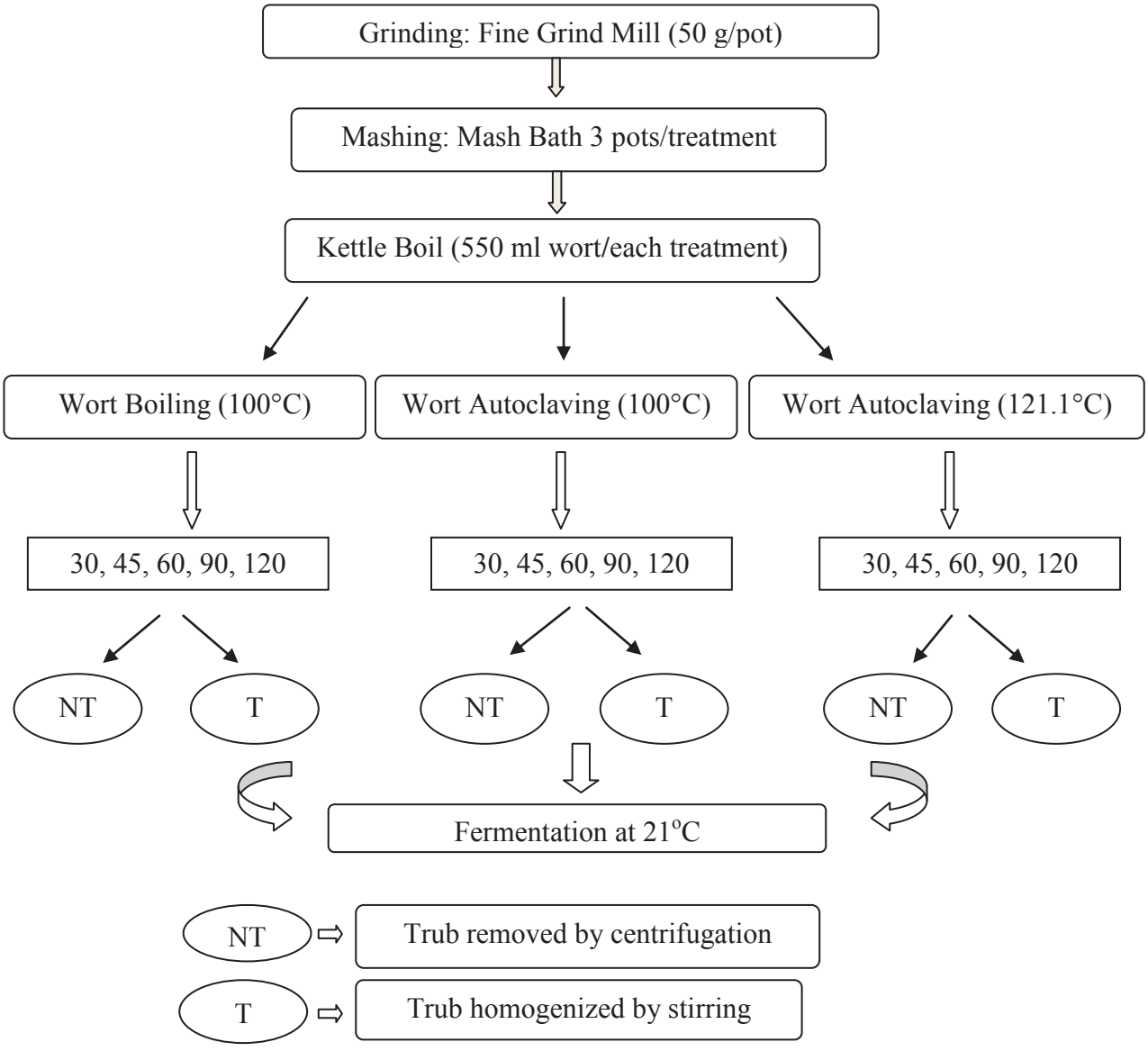

Figure 4.1 Schematic of experimental design.

4.2 Materials

Miniature fermentation assays were conducted according to ASBC-Miniature fermentation method (Yeast-14) (1).

4.2.1 Yeast Strain

Standard SMA Yeast strain (bottom fermenting yeast) was selected. It was obtained from VLB (Berlin, DEU).

4.2.2 Yeast Culture

4.2.2.1 *Primary Culturing On Yeast Slants*

Yeast cells were transferred onto the YEPD agar slants containing 20 g/L D-glucose (Bioshop, Burlington, ON), 20 g/L peptone (Bacto, Sparks, MD), and 10 g/L yeast extract (Bacto, Sparks, MD), as well as 25-30 g/L agar (Bacto, Sparks, MD) for preliminary colonial growth. The slants were then incubated aerobically at 30°C for 48 hr on a shaker. After the yeast grow to visible colonies, slants are sealed with screw caps and stored at 4 ^{o}C.

4.2.2.2 *Initial Yeast Growth*

A loopful of yeast from colonies growing on the YEPD slants was then aseptically transferred to 100 mL of autoclaved YEPD broth. The YEPD broth was made up of 20 g/L dextrose (Difco, Detroit, MI), 20 g/L peptone (Difco, Detroit, MI) and 10 g/L yeast extract (Difco, Detroit, MI). A single 250-mL Erlenmeyer flask containing 100 mL of YEPD broth will provide sufficient yeast count after the double grow-up stage to pitch a

single small- scale fermentation run. The culturing flasks were then plugged with foam plugs and aerobically incubated on a shaker for 24 hr at 30°C.

4.2.2.3 *Double Yeast Grow-Up*

After 24 hr, the YEPD broth was centrifuged at 3,000 x *g* for 3 min. The yeast pellet was suspended in 20 mL of sterile water and was again centrifuged. The yeast was subjected to three washes with 20 mL sterile distilled water. After washing, the pellet was suspended in 15 mL sterile water and inoculated in 200 mL YEPD broth at a rate $1.5x10^7$ cells/mL for secondary growth. The culturing flask were then sealed with foam plugs and aerobically incubated on a shaker for 24 hr at 30°C.

4.2.2.4 *Yeast Count*

The YEPD broth/yeast mixture was centrifuged at 3,000 x *g* for 3 min. The yeast pellet was then subjected to three washes with sterile water. After washing, the pellet was suspended in 15 mL sterile water to obtain a slurry. Yeast cells in the slurry were counted according to the ASBC Yeast-4 (microscopic yeast cell counting) using haemocytometer (Hausser Scientific Partnership, Horsham, PA) (1). A 1:200 dilution was made using 0.2*N* sodium acetate Buffer with 10 mM EDTA Buffer (pH 4.5). The yeast slurry volume (pitching volume) required to obtain an initial cell concentration of 1.5×10^7 cells/mL in 450 mL of wort was then calculated.

4.2.3 Wort Preparation

Standard barley variety was processed in a Standardized fine grinding mill according to ASBC Malt-4, Extract (1). A quality analysis report of 'Control malt' used has been provided in Appendix C. From 750 g of the fine grist, twelve aliquots of 50 g (±0.05) each were separated into containers of a bench top mashing per run. A single run yields sufficient wort to perform 4 small scale fermentations. A mash cycle was set up according to the temperature regime stated in ASBC methods of analysis (Malt-4, Extract). It was started by preheating grist containing containers to 45^{o}C. Two hundred mL of distilled water was added to each preheated container at 45^{o}C before the start of the stirring. The resultant mash was stirred steadily at 45^{o}C for 30 min at a speed of 80-100 rpm. Gradually the bath temperature after 30 min was raised by 1^{o}Celsius/min for 25 min. Another 100 mL of distilled water preheated at 70^{o}C was added to each individual mash when the final bath temperature reached 70^{o}C. The temperature was then held constant for another 60 min. An iodine conversion test was carried out at 10 min after mash bath reached 70^{o}C. After completion of the cycle, 3 mash containers were filtered using Whatman filter paper (Grade 802, 32 cm) to provide 500 mL of wort. Each 500 mL of wort collected was sealed with sponge buns and covered with aluminum foils for sterility. The wort was then subjected to heat treatments and Trub measurements as described in section 4.3.

4.2.4 Wort Adjustment

4.2.4.1 *Glucose Spiked Fermentation*

After the required trub treatment (Section 4.3.2), wort density was adjusted to 12.6^{o}P by adding calculated amounts of sterile water. Eighteen g of laboratory-grade D-(+)-glucose (4% w/v) was then added to 410 mL of adjusted wort. Even dispersion was assured by the magnetic stirring.

4.2.3.2 *Oxygenation*

Following glucose addition, medical grade oxygen was bubbled through wort by inserting tube having regulated oxygen flow from a compressed oxygen tank. To ensure proper oxygenation prior pitching, oxygen was bubbled for a period of 5 min through wort at 20^{o}C to achieve saturation of approximately 90%.

4.2.5 Fermentation

Wort at 21^{o}C was pitched with calculated amount of washed yeast slurry to give an initial concentration of 1.5×10^{7} cells/mL. Final wort volume and density after pitching was adjusted to 450 mL and 14.8^{o}P by adding sterile water. The ^{o}P of the pitched wort was measured immediately after this (Time, t= 0). Aliquots of 15 mL of wort were decanted into 20 mL sterile test tubes (12.5 ×1.3 cm diameter) containing one Poly-tetra flouro ethylene (PTFE) boiling stone. A total of 30 tubes were used for a single fermentation run. Test tubes were then sealed with sterilized foam bugs and fermented in a temperature controlled water bath at 21^{o}C for 78 hr.

4.2.6 Sampling

Destructive sampling was carried on triplicate tubes at time intervals of 0, 6, 22, 26, 30, 46, 50, 54, 70, 74 and 78 hr. Samples of 3.5 mL wort taken from the top of the fermentation tube was used for turbidity measurement while the rest was used for Density measurement.

4.3 Methods

4.3.1 Wort Treatments

After mashing and filtration, the wort obtained was subjected to heat treatments which were Boiling and Autoclaving as described in section 4.3.1.1 and 4.3.1.2. Twenty PTFE boiling stones were added to the flasks during each heat treatment to provide the required mixing and vigor. The size of boiling stones was found to cause undesirable variations in fermentations, therefore, its shape and weight was kept consistent. All heat treatment runs were done in duplicates to create a total of 2 sets. After the heat treatment, the wort was subjected to trub adjustments as described in section 4.3.2.

4.3.1.1 *Wort Boiling*

Setup

Wort was placed on a bench top heating blanket which was controlled by a rheostat. Each 550 mL of wort was taken in sterile 1 L round bottom flask and placed on the heating element. A condenser was attached to the flask with the help of a 2 way glass plug. Cooling water was circulated to condense the vapors during each boil. A digital

thermometer data logger (Extech Instruments- Easy view 15, Contoocook, NH) was inserted into the flask through 2nd opening of the plug, which was then tightly sealed with aluminum foil to avoid vapor escape during boil. The Data logger thermometer used a Type T thermocouple to measure and record temperature of wort all along the boil. Careful note was made to ensure the temperature sensitive end of data logger was completely suspended in the wort at all times.

Boiling

Five different time intervals of 30, 45, 60, 90 and 120 min were selected for boiling. Each 550 mL of wort was boiled at 100 ± 0.2°C under atmospheric pressure for respective time period. The start of boil was recorded when the wort reaches 100°C. After boiling, the flask was tightly sealed with sterile foam bungs and covered by aluminum foil.

4.3.1.2 *Wort Autoclaving at 100 °C*

Automated vertical laboratory autoclave (Steris, Amsco Century, Mentor, OH) was used for wort heat treatment. The specifications listed below were kept constant for each cycle:

Purge Time: 1:00 min
Sterilize Time: 30 min (Depending on the run)
Sterilize Temperature: 100°C
Overdrive: 1.5°C
Under Temperature: 1.8°C
Over Temperature: 6.0°C
Maximum Pressure: 2 atm
Minimum Pressure: 0.334 atm

In the event of an increase in autoclave temperature beyond the specified value, then the over temperature indicates the limit exceeding the required autoclave temperature beyond which the steam heater gets turned off while if the temperature is lower than the specified under temperature value, then the steam heater gets turned on automatically. Overdrive refers to the fluctuation limits allowed in the specified autoclave temperature during a sterilization programme. Autoclaving was carried at 100°C under pressure (1 atm) for the same periods of 30, 45, 60, 90 and 120 min. Each 550 mL of wort was placed in 1 L sterile conical flask and autoclaved for the particular time period. After autoclaving, the flask was tightly sealed with sterile foam bugs and covered by aluminum foil.

4.3.1.3 *Wort Autoclaving at 121.1°C*

Autoclaving was carried at 121.1°C under pressure (2 atm) for 30, 45, 60, 75 and 90 min. The procedure and specifications were same as described in section 4.3.1.2, although the temperature was changed to 121.1°C.

4.3.2 Trub Content

After each heat treatment, the wort was allowed to cool to room temperature and stored at 4^{o}C for 12 hr. The two sets of each treatment of specific time interval were then mixed together under sterile conditions to make up a total of 1.1 L. Magnetic stirring was carried out for 5 min to ensure homogenization of the trub in wort. An aliquot of 100 mL from each flask was transferred to sterile tubes to carry out sugar, wort color and FAN analysis (Section 4.3.6, 4.3.7, 4.3.8). The remaining 1000 mL wort was then separated equally into 2 sterile 1000 mL conical flasks termed, set I and set II.

4.3.2.1 *Measurement of Trub*

After the cold break, wort was subjected to centrifugation in triplicates by transferring 167 mL into centrifuge bottles (200-225 mL) and spinning at 3310 x *g* for 15 min for one complete set of heat treatments, set I. The clear, trub free supernatant was carefully transferred and subjected to wort adjustments (section 4.1.4). The trub cake was re-suspended and centrifuged with 50 mL of sterile, distilled water. Supernatant was discarded and the entire step was repeated twice more, making a total of 3 washes. To ensure complete removal of sugar, density of supernatant from the final wash was checked using DMA 35N digital density meter (Anton Paar GmbH, Graz, AUT) at 20^{o}C and found to be 0.0. The settled trub was then subjected to drying at 90^{o}C for two hours and subsequently weighed.

4.3.2.2 *Suspension of Trub*

For the second set of wort, trub was homogenized by magnetic stirring for 5 min at high speed and then wort was adjusted (section 4.2.4).

4.3.3 Wort pH Determination

Wort pH was measured according to ASBC (Beer-9). After each heat treatment, wort was cooled and pH was measured using standardized pH meter (Accumet, Fisher scientific, Model 15).

4.3.4 Turbidity Determination

Turbidity was determined according to ASBC (Yeast-14). An aliquot of 3.5 mL of wort was taken from the top and placed in a clean four sided 4.5 mL cuvette (12.5 x 12.5 x 45 mm). Setting up the spectrophotometer at 600 nm, blank was set up with distilled water. Following that absorbance was measured for wort containing suspended yeast.

4.3.5 Density Determination

Density was measured according to ASBC (Beer-2) (1). After turbidity measurement, the remaining wort was filtered using Whatman (Grade 4, 7 cm, Maidstone, GBR) filter paper in order to remove suspended yeast cells. The density of all fermentations was then measured in degrees Plato (^{o}P) using a DMA 35N digital density meter (Anton Paar GmbH, Graz, AUT) at 20 ^{o}C.

4.3.6 Sugar Analysis

Sugar analysis was performed from samples collected after cold break of heat treated wort. Fermentable sugar levels in wort after Benson Polymeric column for separation of carbohydrates, organic acids and alcohols was used (BP-100 H^{+} Carbohydrate column, Sparks, NV). For set I, 10 µl of wort samples which were filtered was fractioned by High pressure liquid chromatography while set II wort samples were introduced along with trub. The concentration of glucose, fructose, maltose and maltotriose was calculated in g/mL.

4.3.7 Wort Color Analysis

Wort color analysis was done according to ASBC method of analysis of beer color (Beer-10) (1). Fifty mL of cooled wort after each heat treatment was filtered using Whatman (grade 4, 7cm) filter paper to remove suspended solids and trub particles. Absorbance was then measured at 430 and 700 nm using spectrophotometer to check for turbidity. Once, wort was free of turbidity the absorbance at 430 nm was used to calculate color.

4.3.8 Free Amino Nitrogen Determination

FAN levels of the wort were checked according to ASBC method of analysis for free amino nitrogen (International method, Wort-12) (1). FAN levels were calculated for both wort containing trub and free of trub after each heat treatment. FAN levels were reported in mg/L.

4.3.9 Non-linear Data Modeling

Using the statistical software analysis package PRISM® Version 5 (Graphpad Software, La Jolla, CA), a logistic model was used to fit the decline in apparent extract data. The software performed a non-linear regression using least squares estimation to fit the model. The logistic model is given in Equation 1 and the sigmoidal fit is shown in figure 4.2. The fermentability parameters estimated using logistic equation are explained in Table 4.1. Any differences between treatments were determined using a global F-test of equation parameters which is available in Prism software (Graphpad Software, La Jolla, CA).

$$P_t = P_e + (P_i - P_e) / (1 + e^{-B*(t-M)}) \quad (1)$$

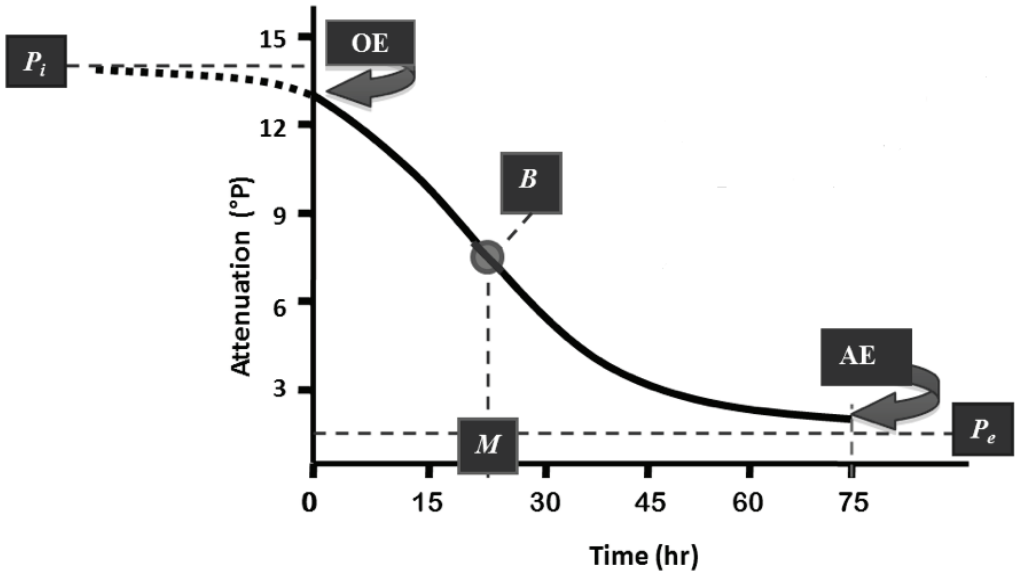

Figure 4.2 Apparent Extract Curve – Non-linear sigmoidal fit of decline in density (Yeast-14) (1).

Table 4.1 Calculated parameters for the "Logistic" model.

Variable	Variable Description	Effect on Curve
P_t	Extract at time (*t*)	Variable with respect to time (*t*)
P_e	Final extract at equilibrium (^oP)	Lower asymptote
P_i	Initial extract at time zero (^oP)	Upper asymptote
M	Time to point of inflection (h)	Inflection point of sigmoid
B	Slope at inflection point of sigmoid	Slope at inflection point
t	Time *t*	Progression of the curve

The Absorbance data was modeled using tilted Gaussian fit as reported in miniature fermentation method (1). The fit is depicted in figure 4.3. Modeling was done with Prism (Graphpad software, La Jolla, CA) which fits a tilted Gaussian curve along the data and provides the best fit values for the equation parameters described in Table 4.2. Any significant differences between the curve fits are checked by global F-test.

$$Abs_{600} = R^* t + A^* e^{\frac{1}{2}((t-\mu)/\sigma)^2} \qquad (2)$$

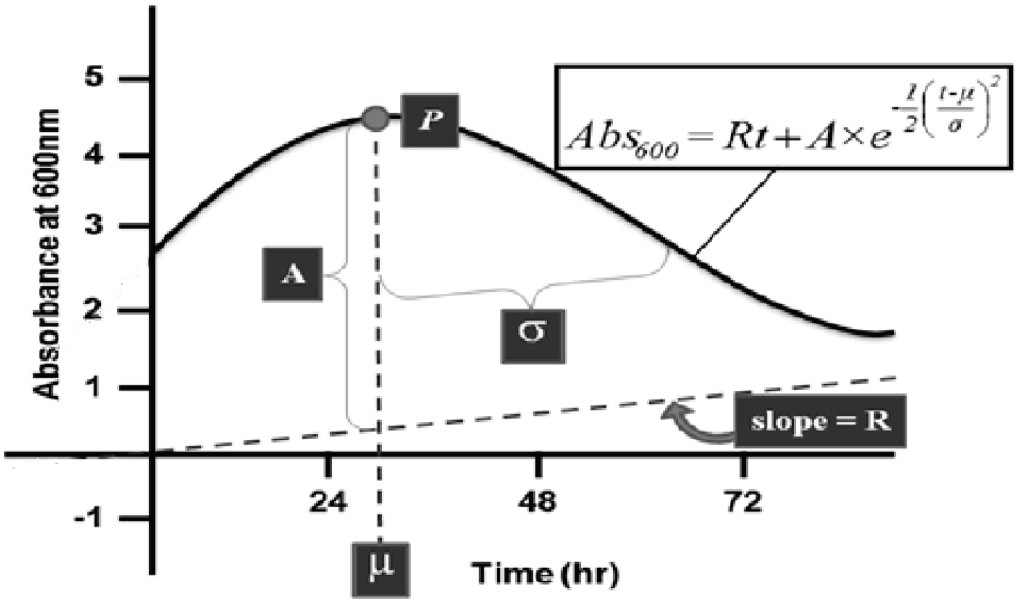

Figure 4.3 Absorbance Curve – Non-linear tilted-Gaussian fit (Yeast-14) (1).

Table 4.2 Calculated parameters for the "Tilted-Gaussian" model.

Variable	Variable Description	Effect on Curve
Abs_{600}	Absorbance at 600 nm at time (t)	Variable with respect to time (t)
σ	Width factor	Width of the curve from midpoint
μ	Midpoint	Time of highest amplitude
A	Absolute amplitude	Highest point of the curve
R	Slope	Tilt of the curve
t	Time t	Progression of the curve

CHAPTER 5 RESULTS

5.1 Fermentations

A total of 30 fermentations were undertaken using a set of five in 15 mL test tubes. Table 5.1 provides a summary of the treatments and conditions used for each fermentation run. The runs have been grouped into set of six depending on the type of wort heat treatment and presence/absence of trub. Each group included five heat treatment periods, (i.e. 30, 45, 60, 90 and 120 min). The decline in apparent extract and turbidity was measured over 78 h for all fermentation runs and data collected was analyzed by non-linear modeling. The predicted parameter values were then subjected to a global F-test analysis to check if the runs are each significantly different from one another. F-tests report the probabilities whether means or variances of a given parameter, calculated from each run, are different from one another or not, therefore, allowing multiple comparisons. No errors were introduced as mashing was done same day, same cycle was followed and same batch of malt was used. Similarly, no errors were introduced during boiling stages as wort for both with and without trub runs was heat treated same time and following same autoclave cycle.

Table 5.1 Summary of conditions used for the wort heat treatment experiments. Each type of treatment includes the runs carried by varying the respective treatment period.

Conditions	Boiling (at 100^{o}C, room pressure)	Autoclaving (at 100^{o}C, 1 atm)	Autoclaving (at 121.1^{o}C, 2 atm)
Fermentation without Trub	Group I	Group III	Group V
Fermentation with trub	Group II	Group IV	Group VI

5.1.1 Density

Density (Gravity) denotes the relative amount of fermentable sugars (Extract) present in the wort at the given time. It was measured in degrees Plato (^{o}P) at specific time intervals after start of a fermentation run. The highest density of the wort, which had been adjusted to 14.8 ^{o}P, was recorded at the start of a run. Once, the wort was pitched, the fermentable sugars were being used up by the yeast cells to convert into alcohol and carbon-dioxide. This marks the start of decline in apparent extract with respect to time.

The decline in density curves obtained during fermentation of boiled wort samples (at 100^{o}C, 1 atm), without trub of Group I are shown in Fig 5.1. The heat treatment of different duration, (i.e., 30, 45, 60, 90 and 120 min) on wort resulted in significantly different curves ($p<0.05$) as compared by global F-test. Differences in the density curves indicate different fermentation pattern because of variability in the rate of sugar consumption. Consumption of sugar was more rapid when wort was heat treated for less time at boiling temperature as marked by the rapid decline in density.

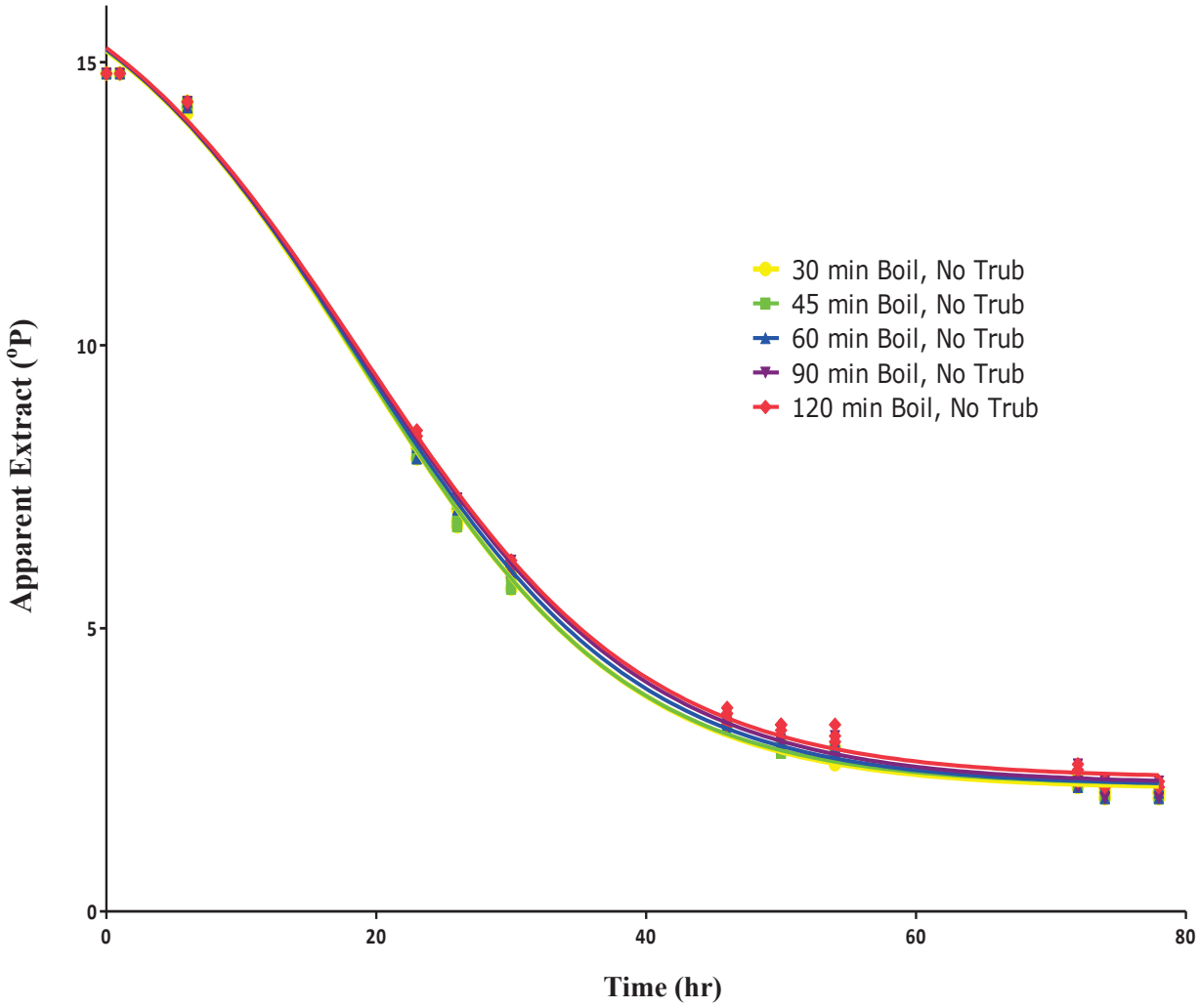

Figure 5.1 Comparison of decline in apparent extract during fermentation of wort prepared by boiling (at 100°C) under room pressure for different time periods and fermented without trub. The curves represent non-linear logistic fits of the density data collected. Error associated with each curve is reported in Table 5.2 (n=32).

Figure 5.2 shows the global fit of decline in apparent extract during 78 h of fermentation of boiled wort runs (at 100^{o}C, room pressure) containing trub (Group II). No significant differences in fermentation runs were obtained with respect to different boiling time intervals, (30, 45, 60, 90 and 120 min), when trub was present ($p>0.05$). Therefore, a single regression global curve representing all data sets was produced after analysis. This suggests that presence of trub in wort interferes with the ability of yeast cells to uptake sugar in the miniature fermentation assay. Some authors have suggested that presence of trub increases the rate of fermentation by acting as a source of nutrients such as amino acids while others consider it as a factor reducing yeast sugar uptake ability by membrane blocking (46, 63). Due to the multiple aspects by which trub influences the rate of sugar consumption, it is hard to elucidate whether trub increases or decreases the rate of sugar consumption in boiled wort.

The best fit values for fermentability parameters for Boiled runs (100^{o}C), with and without trub, along with associated asymptotic standard errors are provided in Tables 5.2. The coefficient of determination (r^2) value obtained for each run was around 0.998 signifying a good fit of logistic model to all data points. Final extract, P_e was found to significantly increase with increasing duration of the wort boil in fermentations carried both with and without trub (F-test, $p<0.05$). This suggests that when wort is heat treated for longer intervals, the sugar uptake is decreased resulting in high unfermented final sugar levels.

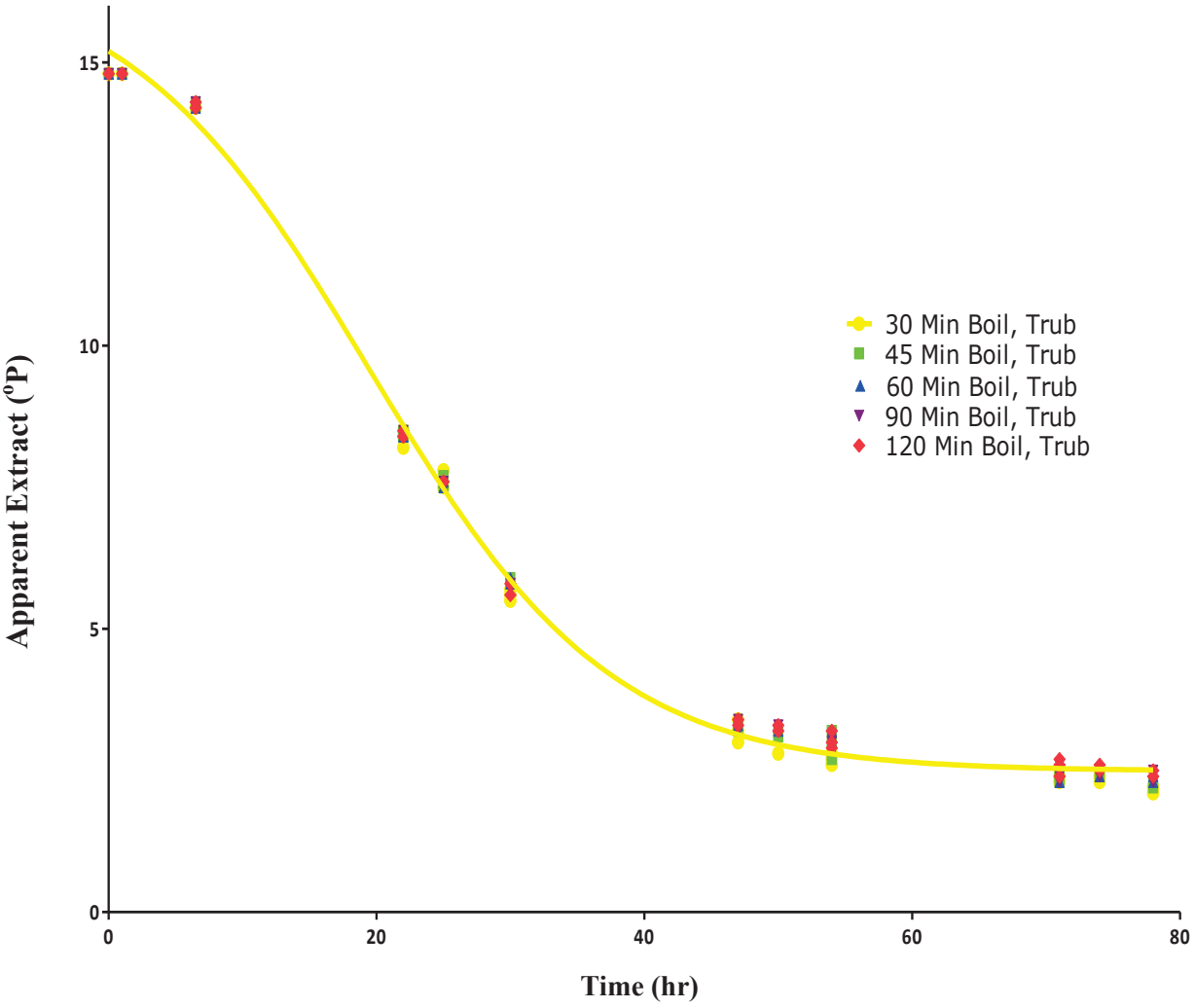

Figure 5.2 Comparison of decline in apparent extract during fermentation of wort prepared by boiling (at 100°C) under room pressure for different time periods and fermented along with trub. The curves represent non-linear logistic fits of the density data collected. Error associated with global curve is reported in Table 5.2 (n=32).

Figure 5.3 shows decline in apparent extract during fermentation of autoclaved wort runs (at 100^{o}C, 1 atm) without trub (Group III). Wort was subjected to autoclaving for periods of 30, 45, 60, 90 and 120 min and after trub removal, was fermented for 78 h. Decline in density of wort subjected to autoclaving (at 100^{o}C, 1 atm), without trub, showed significant differences from one another for all heat treatment time periods ($p<0.05$). The curve fits depicted more rapid decline in gravity with less heat treated wort runs (i.e., 30 and 45 min) autoclaved wort. Moreover, when wort was subjected to autoclaving (at 100^{o}C, 1 atm) for same periods but fermented with trub (Group IV), each run was again found to be significantly different ($p<0.05$) (Figure 5.4). The decline in density was found to be faster with less autoclaved wort, while 120 min autoclaved wort showed slowest decline.

The best fit values for fermentability parameters for autoclaved wort runs (100^{o}C, 1 atm), with and without trub, along with associated asymptotic standard errors are provided in Tables 5.2. An F-test analysis showed significant decline in slope and midpoint of the turbidity curve while higher final (P_e) extract values with increasing wort autoclaving duration ($p<0.05$). As previously mentioned a number of biochemical changes take place during wort boiling and can affect yeast sugar uptake as well as Maillard browning reactions, loss of wort nutrients by trub formation and formation of volatiles. Therefore, an increase in heat treatment duration will lead to corresponding increase in biochemical changes resulting in a slow and incomplete attenuation of wort density.

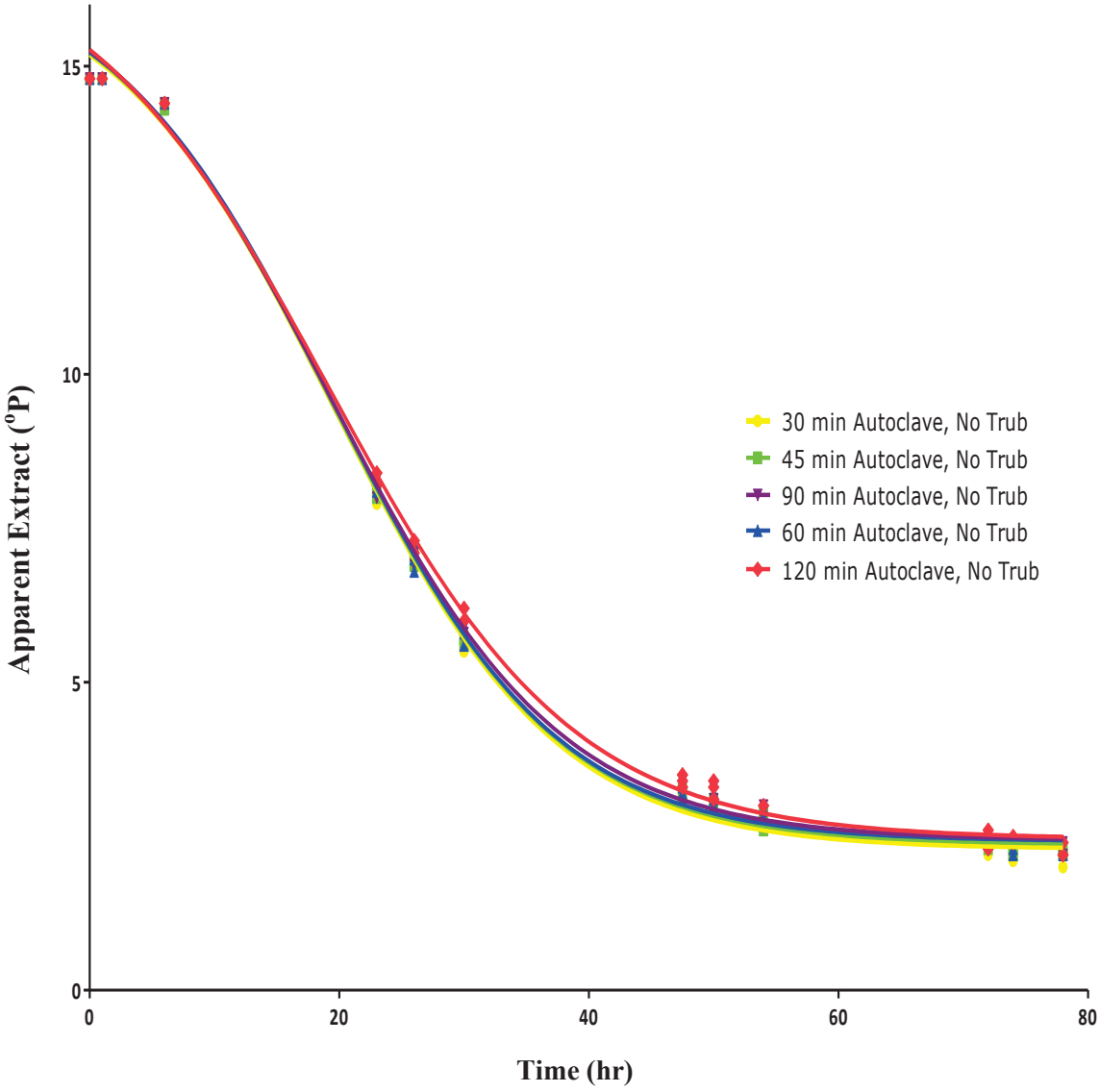

Figure 5.3 Comparison of decline in apparent extract during fermentation of wort prepared by autoclaving (at 100°C) at 1 atm pressure for different time periods and fermented without trub. The curves represent non-linear logistic fits of the density data collected. Error associated with each curve is reported in Table 5.2 (n=32).

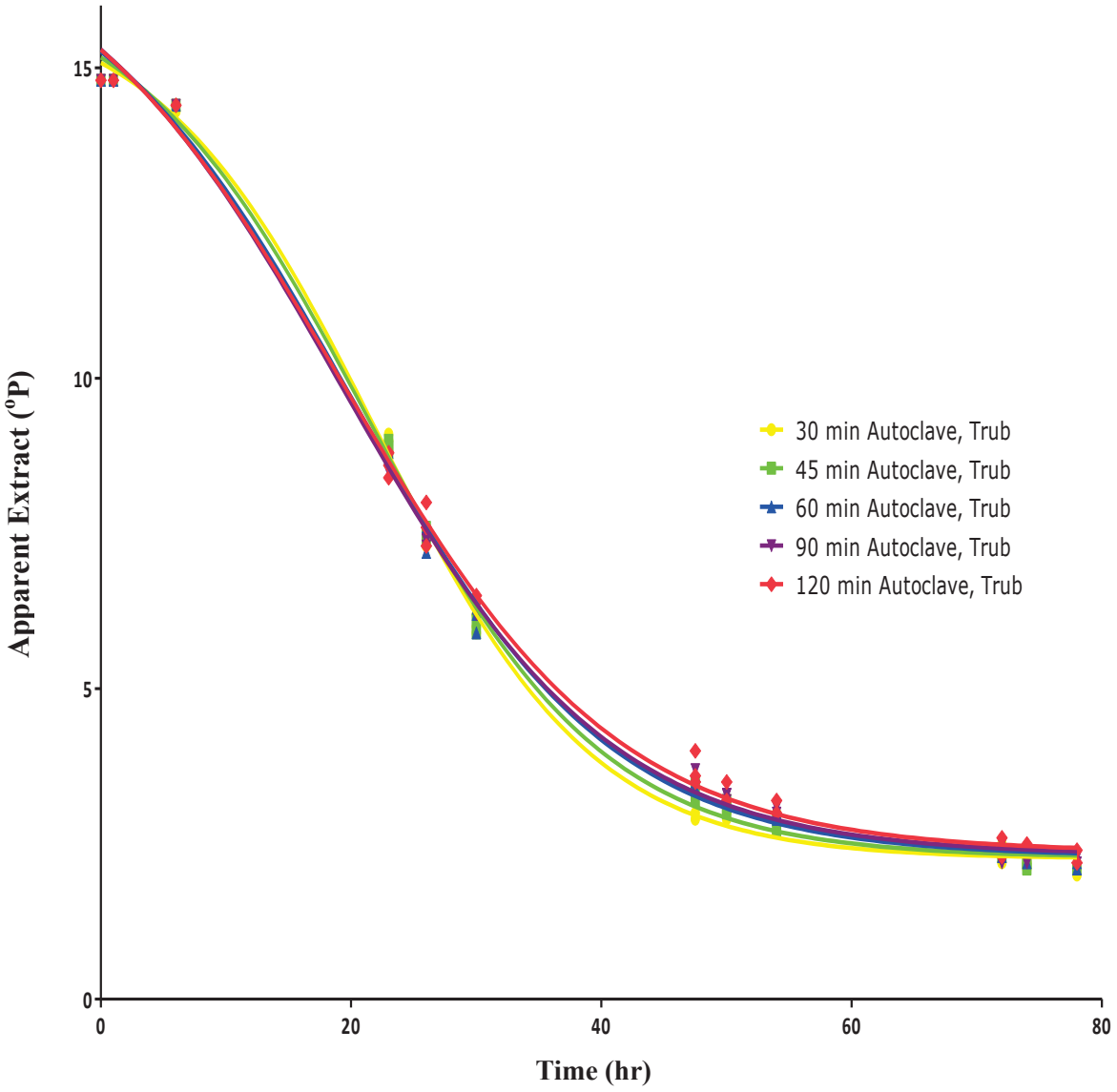

Figure 5.4 Comparison of decline in apparent extract during fermentation of wort prepared by autoclaving (at 100°C) at 1 atm pressure for different time periods and fermented along with trub. The curves represent non-linear logistic fits of the density data collected. Error associated with each curve is reported in Table 5.2 (n=32).

The decline in apparent extract of fermentation runs with wort autoclaved (at 121.1 ^{o}C, 2 atm) at higher temperature for periods of 30, 45, 60, 90 and 120 min is shown in Figure 5.5 (Group V). The trub was centrifuged and removed after heat treatment. Significant differences were obtained between each run when subjected to F-test ($p<0.05$). The best fit values for fermentability parameters along with associated asymptotic standard errors are provided in Table 5.2. From the curve fits, significantly higher final extract values (F-test, $p<0.05$) was obtained with increasing duration, suggesting that wort fermentable sugar fraction was not completely used by the yeast cells yielding to less yeast activity or growth. Similar observations were made when wort was autoclaved for same time and temperature but fermented with trub (Group VI) (Figure 5.6). However, the curve fit values of slope and midpoint do not show consistent trend suggesting an influence of trub on the sugar uptake rate of yeast cells (Table 5.2).

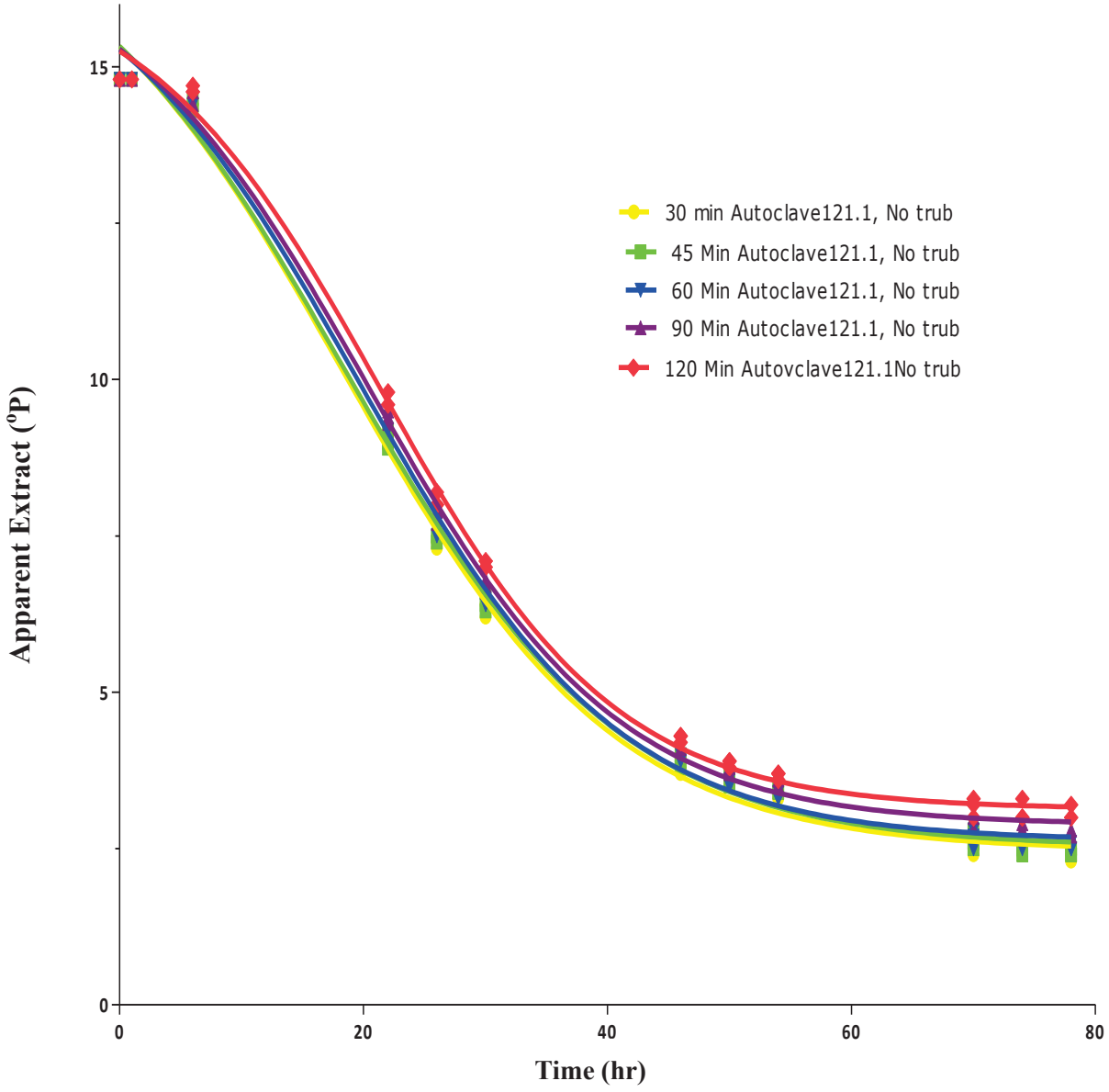

Figure 5.5 Comparison of decline in apparent extract during fermentation of wort prepared by autoclaving (at 121.1°C) at 2 atm pressure for different time periods and fermented without trub. The curves represent non-linear logistic fits of the density data collected. Error associated with each curve is reported in Table 5.2 (n=32).

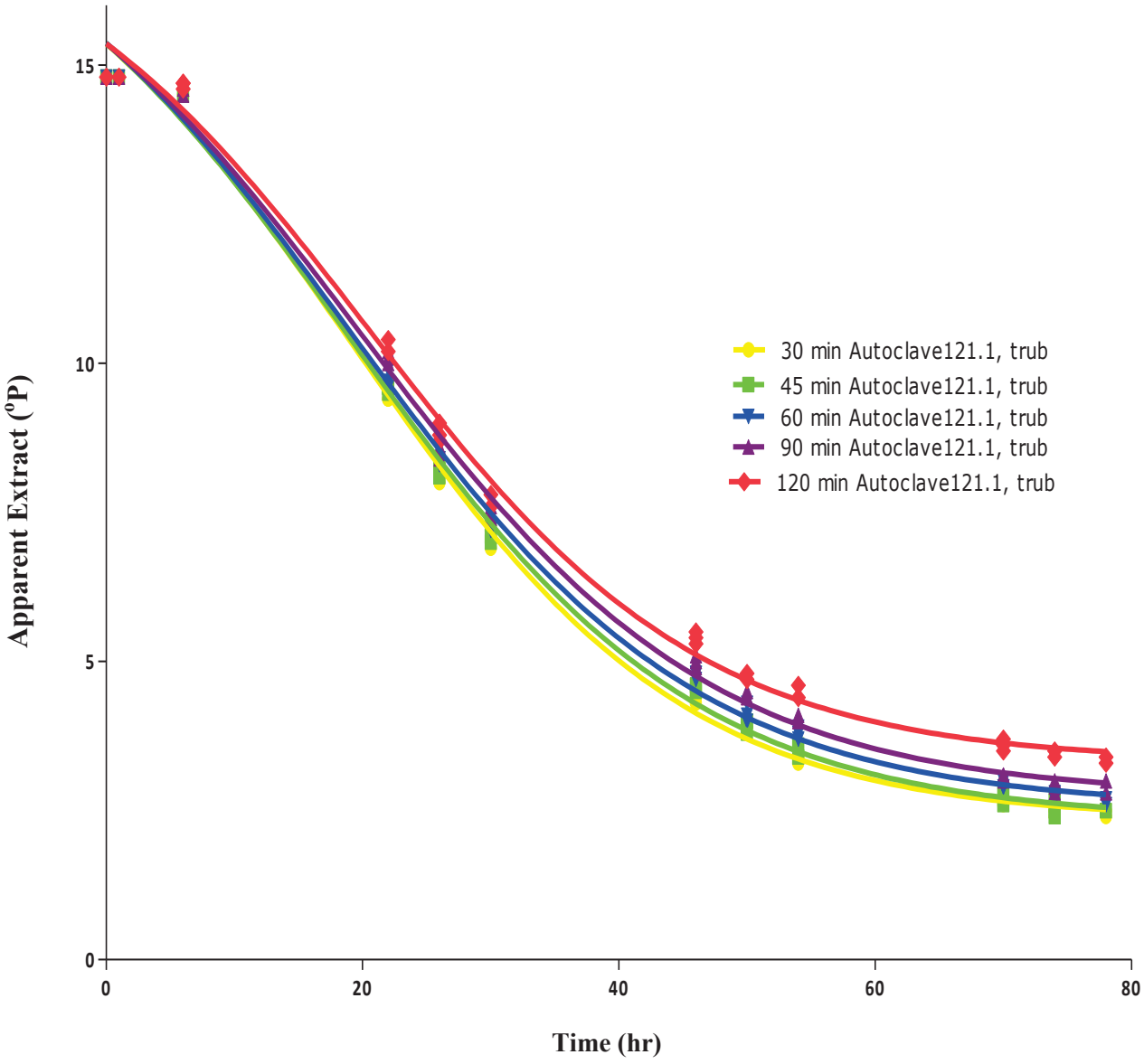

Figure 5.6 Comparison of decline in apparent extract during fermentation of wort prepared by autoclaving (at 121.1°C) at 2 atm pressure for different time periods and fermented along with trub. The curves represent non-linear logistic fits of the density data collected. Error associated with each curve is reported in Table 5.2 (n=32).

Table 5.2 Logistic model parameter values estimated from equation (1) for all fermentations.[a] (Type: 1- Boil (100°C), 2- Autoclave (100°C), 3- Autoclave (121.1°C); Trub: 0- No trub, 1- Trub)

Period	Type	Trub	P_e(°P)	ASE	P_i(°P)	ASE	B(h^{-1})	ASE	M (h)	ASE	r^2
30	1	0	2.174	0.07	17.23	0.463	0.099	0.0054	18.75	0.81	0.998
45	1	0	2.228	0.071	17.16	0.469	0.101	0.0057	18.81	0.824	0.998
60	1	0	2.22	0.075	17.36	0.498	0.097	0.0054	18.7	0.874	0.998
90	1	0	2.252	0.082	17.47	0.545	0.094	0.0057	18.7	0.962	0.998
120	1	0	2.352	0.083	17.43	0.543	0.095	0.0058	18.8	0.966	0.998
F-test			0.016		0.0837		0.0649		0.9147		
30	1	1	2.335	0.065	16.46	0.354	0.114	0.0063	19.94	0.606	0.998
45	1	1	2.443	0.072	16.86	0.43	0.107	0.0062	19.31	0.739	0.998
60	1	1	2.508	0.071	16.83	0.428	0.108	0.0063	19.2	0.738	0.998
90	1	1	2.539	0.068	16.71	0.404	0.111	0.0064	19.34	0.698	0.998
120	1	1	2.61	0.064	16.54	0.369	0.114	0.0063	19.53	0.643	0.998
F-test			0.0167		0.8203		0.6258		0.65		
30	2	0	2.302	0.066	16.67	0.398	0.111	0.0064	19.52	0.705	0.998
45	2	0	2.362	0.066	16.72	0.407	0.111	0.0064	19.4	0.721	0.998
60	2	0	2.416	0.067	16.67	0.407	0.112	0.0067	19.51	0.725	0.998
90	2	0	2.435	0.074	16.86	0.467	0.108	0.0069	19.23	0.83	0.998
120	2	0	2.455	0.078	17.1	0.492	0.101	0.0064	19.19	0.877	0.998
F-test			0.0333		0.0194		0.0377		0.0397		
30	2	1	2.262	0.074	16.11	0.319	0.115	0.007	21.97	0.564	0.998
45	2	1	2.281	0.079	16.51	0.381	0.106	0.0065	21.25	0.675	0.998
60	2	1	2.296	0.093	17.16	0.534	0.096	0.0065	19.93	0.949	0.997
90	2	1	2.318	0.087	17.49	0.546	0.093	0.0058	19.13	0.968	0.998
120	2	1	2.364	0.091	17.54	0.6175	0.091	0.0063	19.24	1.104	0.996
F-test			0.001		0.0311		0.0381		0.0379		
30	3	0	2.466	0.103	17.83	0.692	0.09	0.0063	18.26	1.224	0.997
45	3	0	2.527	0.104	17.85	0.69	0.088	0.0063	18.37	1.229	0.997
60	3	0	2.623	0.092	17.27	0.52	0.094	0.006	19.67	0.941	0.997
90	3	0	2.874	0.086	17.03	0.454	0.097	0.006	20.21	0.836	0.997
120	3	0	3.128	0.073	16.58	0.333	0.103	0.0056	21.39	0.624	0.998
F-test			0.0004		0.0061		0.0057		0.0042		
30	3	1	2.347	0.113	18.33	0.664	0.077	0.0048	19.12	1.217	0.997
45	3	1	2.345	0.131	18.67	0.788	0.073	0.005	18.73	1.455	0.997
60	3	1	2.555	0.133	18.62	0.79	0.073	0.0051	18.86	1.488	0.997
90	3	1	2.737	0.146	18.41	0.797	0.072	0.0054	19.6	1.541	0.996
120	3	1	3.31	0.138	17.92	0.706	0.076	0.0058	20.32	1.42	0.996
F-test			0.0087		0.192		0.9639		0.045		

[a]The total number of points for each fit represents a minimum of 32.

5.1.2 Turbidity

Turbidity measured at 600 nm provides the relative amount of suspended solids present in the fermentation media at a given time. Since wort solids are removed during filtration and centrifugation stages prior to pitching, the relative absorbance gives a measure of yeast cells suspended in wort. However, presence of trub can interfere with the measurement of yeast in suspension as it contains complex proteins and polyphenolic materials, which can contribute to UV absorption at 600 nm.

Each turbidity curve describes the characteristic growth phase of yeast cell in a nutrient media. The start of the curve denotes the suspended cells present in a given volume of wort after pitching. Yeast cells then start consuming sugars and nutrients present in wort and produce alcohol and CO_2. The log phase involves growth and cell division which increases yeast cell numbers causing an increase in turbidity. At the point where nutrient depletion occurs, the cells reach stationary phase where the growth and division stops. When yeast cells stop dividing they begin to flocculate and sediment at the bottom of the fermentation tubes. This marks the decline in turbidity. Relative absorbance was measured at regular intervals by taking the upper 3.5 mL of fermenting media from each test tube that represents yeast in suspension without disturbing the flocculated and sediment cells. No CO_2 bubbles were observed in the cuvette indicating no interference during turbidity measurements.

Figure 5.7 shows the changes in turbidity of Group I fermentations. The decline in turbidity obtained during fermentation of boiled wort samples (at 100^{o}C, room pressure), without trub yields significantly different curves ($p<0.0001$) as compared by global F-test. The best fit values for equation parameters along with associated standard errors are provided in Table 5.3. On increasing wort boil (i.e., to 90 and 120 min), the number of suspended cells in wort all along the fermentation is reduced as denoted by a depressed

turbidity curve. This implies that the growth and cell division of yeast cell is compromised at the start of fermentation itself which results in high final extract value, seen in section 5.2.1. An F-test analysis shows significant decreases in amplitude and slope of the turbidity curve with increase in boiling ($p<0.05$).This implies that with increase in wort boiling, the biochemical changes that take place hinder yeast growth and cause rapid flocculation. Phenomenon of flocculation is governed by several factors such as shear rate, sugar and nitrogen availability, pH, ion concentration etc., and most of these factors are influenced by wort boiling. In the following sections factors that are affected by wort boil are analyzed to establish any correlations between wort boil and yeast flocculation.

Turbidity curves obtained for Group II fermentations where wort was subjected to same heat treatment but fermented with trub are found to be significantly different by F-test ($p<0.0001$, Figure 5.8). At the beginning of all fermentations, the turbidity was found to be parallel suggesting comparable yeast cell concentration. Unlike Group I runs, presence of trub increased yeast growth rate at initial stages even when the wort was boiled for longer durations. However, the turbidity curves show rapid yeast settling in later fermentation stages with increasing wort heat treatment intervals. The best fit values for equation parameters along with associated standard errors are provided in Table 5.3. With increase in boiling a significant decline in mean of the curve was found implying an early flocculation and settling of flocs formed ($p<0.05$). Also, the amplitude of group II runs was found to be less than group I for particular boil duration. This suggests that presence of trub along with longer boiling results in decrease of the maximal yeast cell concentration during fermentation along with an early flocculation.

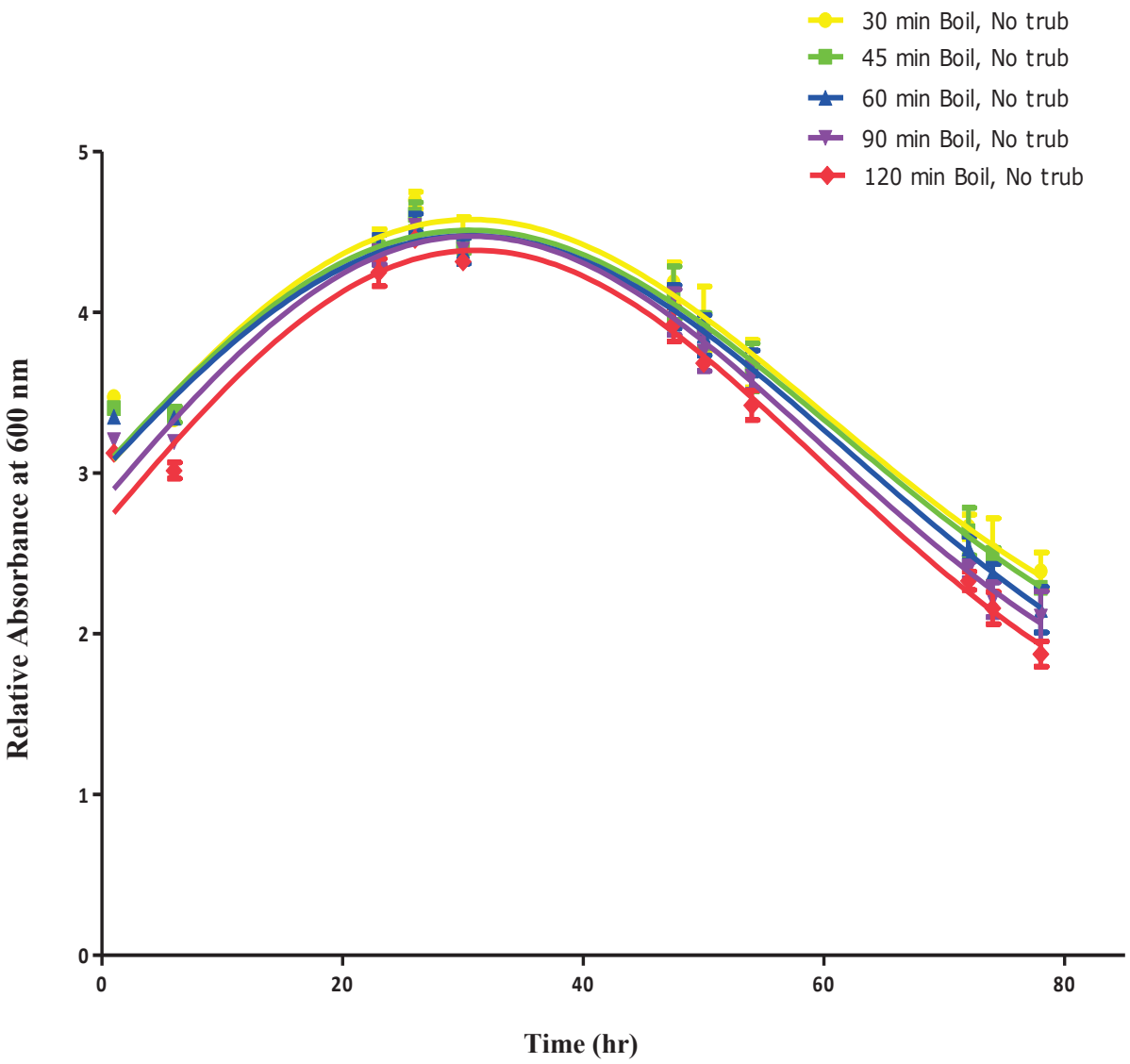

Figure 5.7 Comparison of absorbance measured at 600nm during fermentation of wort prepared by boiling (at 100°C) under room pressure for different time periods and fermented without trub. Curves represents non-linear, tilted Gaussian fit of the turbidity data collected in triplicates along with error bars representing standard deviation (n=32).

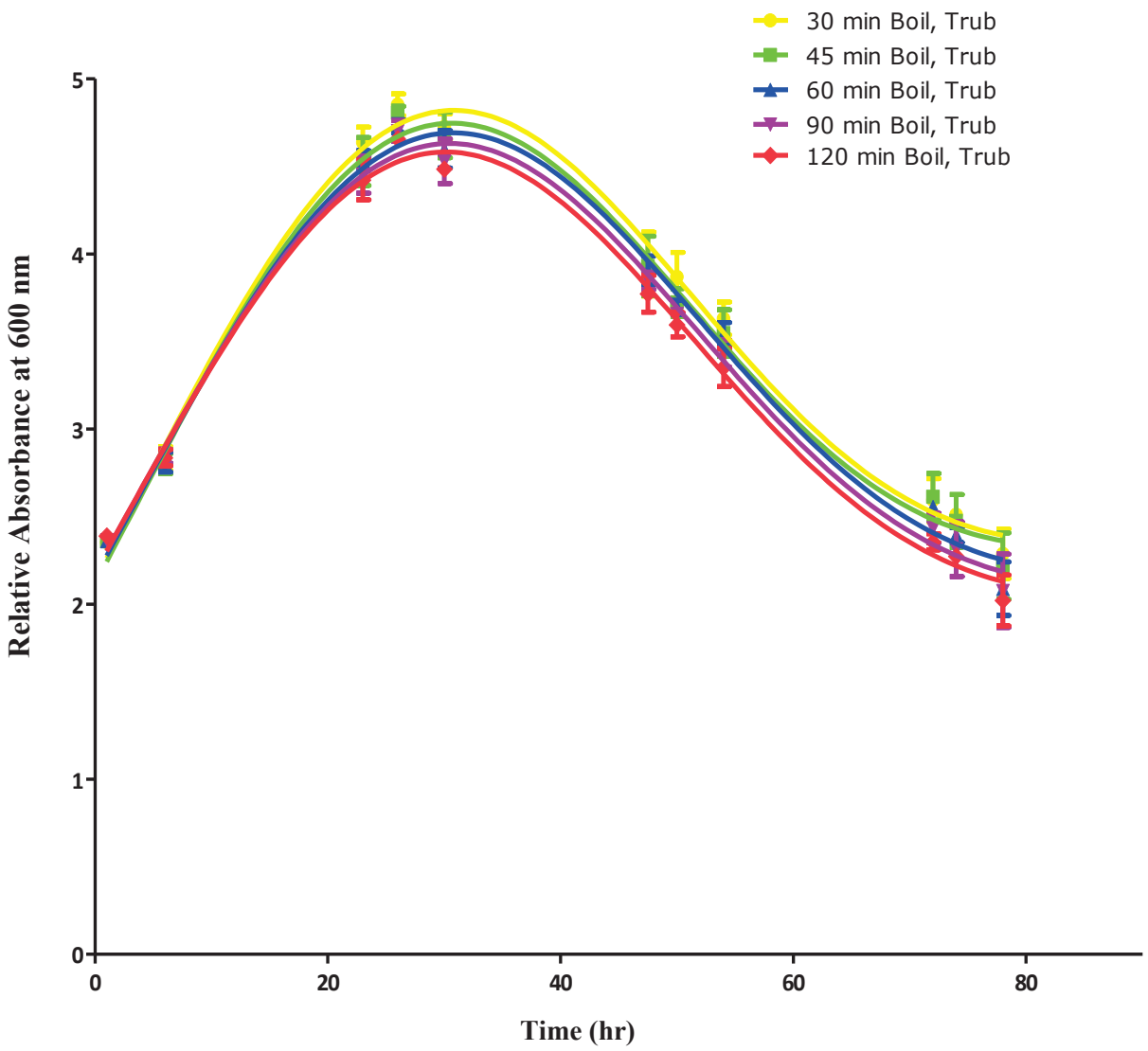

Figure 5.8 Comparison of absorbance measured at 600nm during fermentation of wort prepared by boiling (at 100°C) under room pressure for different time periods and fermented with trub. Curves represents non-linear, tilted Gaussian fit of the turbidity data collected in triplicates along with error bars representing standard deviation (n=32).

Turbidity curves obtained for Group III fermentations where wort was subjected to autoclaving (at 100^{o}C, 1 atm) and fermented without trub were found to be significantly different by F-test ($p<0.0001$, Figure 5.9). After pitching, an initial decline in wort turbidity (t=6 h) was observed for runs with longer wort autoclaving times, suggesting that the yeast settling can be taking place due to insufficient CO_2 induced shear. However, yeast settling was found to be higher in longer autoclaved wort implying that with increase in heat treatment the yeast cells start settling if not sufficient shear is produced. Also, a low cell density was observed all along the run for longer autoclaved wort i.e., 90 and 120 min runs. The best fit values for equation parameters along with associated standard errors are provided in Table 5.3. No significant changes were observed in equation parameters.

Turbidity curves obtained for Group IV fermentations where wort was subjected to autoclaving (at 100^{o}C, 1 atm) and fermented with trub were found to be significantly different by F-test ($p<0.0001$, Figure 5.10). Among the equation parameters, significant decline in slope (R) was obtained ($p<0.05$) (Table 5.3). This suggests that cells in suspension decrease more rapidly with longer autoclaved wort. Presence of trub could aid in precipitating of yeast out of suspension as no such observations were made with runs without trub (Group III).

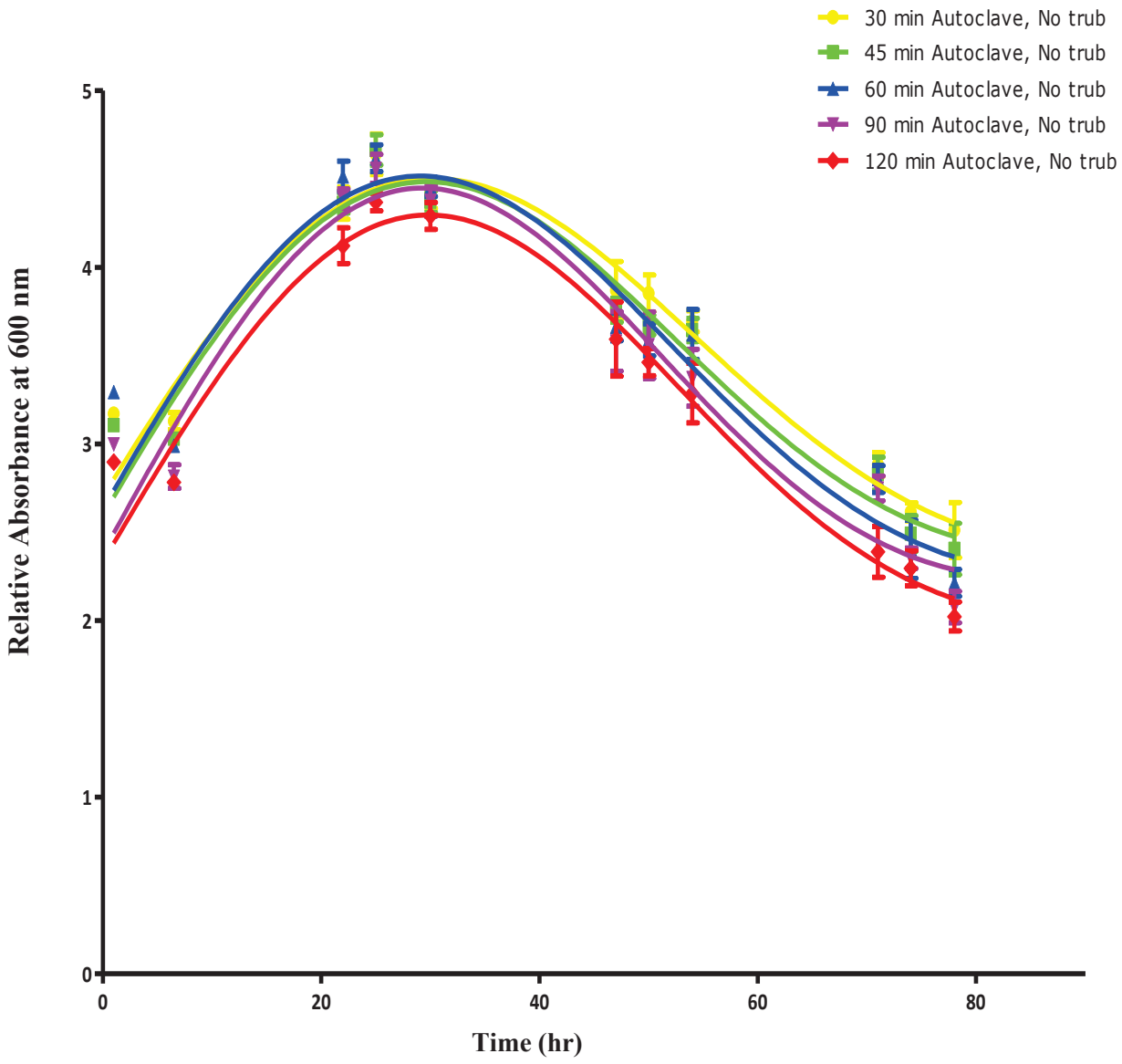

Figure 5.9 Comparison of absorbance measured at 600nm during fermentation of wort prepared by autoclaving (at 100°C) under 1 atm pressure for different time periods and fermented without trub. Curves represents non-linear, tilted Gaussian fit of the turbidity data collected in triplicates along with error bars representing standard deviation (n=32).

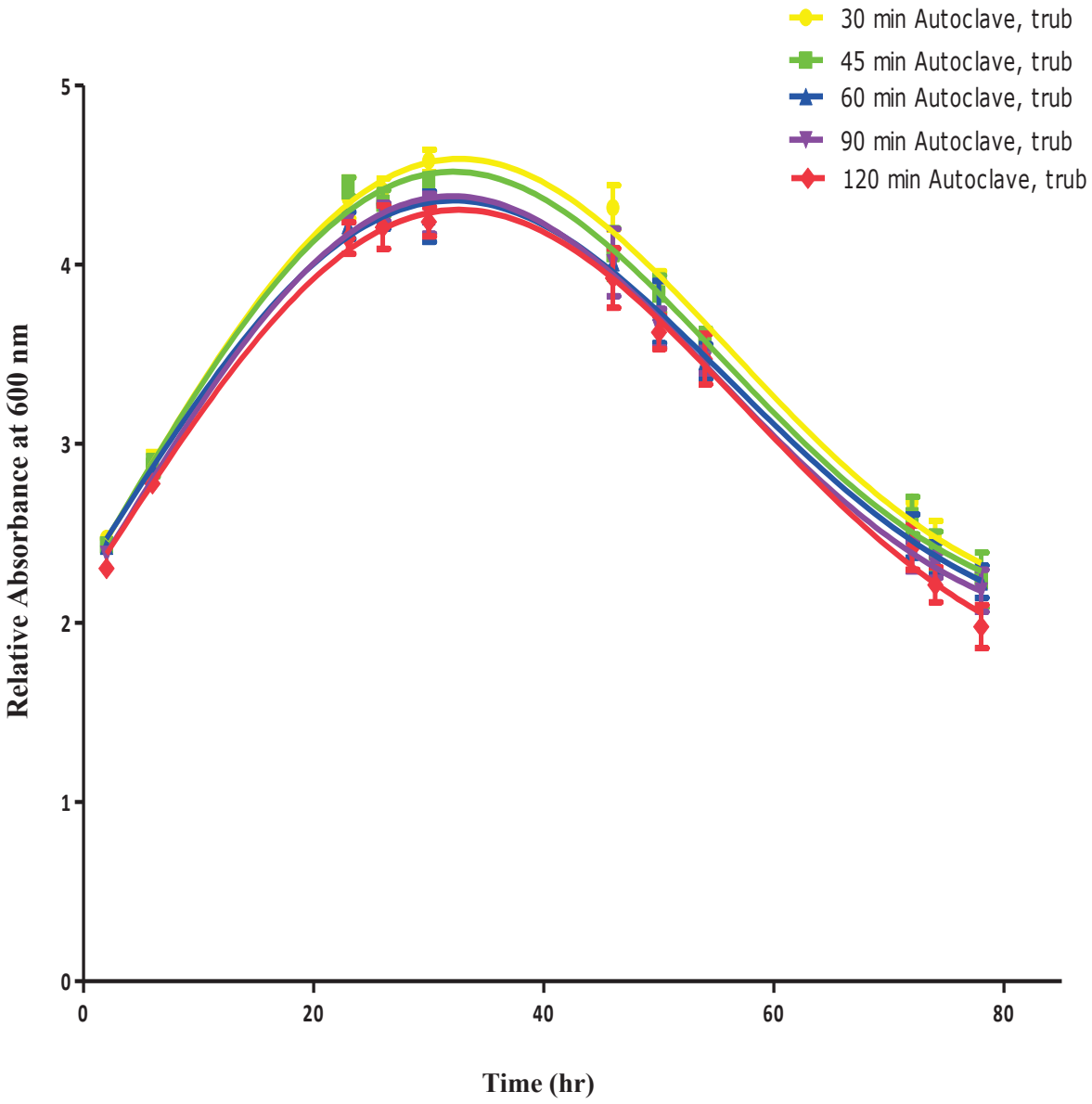

Figure 5.10 Comparison of absorbance measured at 600nm of wort prepared by autoclaving (at 100°C) under 1 atm pressure for different time periods and fermented containing trub. Curves represents non-linear, tilted Gaussian fit of the turbidity data collected in triplicates along with error bars representing standard deviation (n=32).

Turbidity curves obtained for Group V and VI fermentation runs are shown in Figure 5.11 and 5.12 respectively. A global F-test showed turbidity curves to be significantly different ($p<0.0001$). The best fit equation parameters for turbidity curves are listed for each group in Tables 5.3 along with associated errors. An increase in duration resulted in less yeast density and more intense flocculation, as amplitude, curve width and slope showed significant decline in both groups (F-test, $p<0.01$). Moreover, in absence of trub, curve means showed significant decline indicating early onset of flocculation with increasing autoclaving ($p<0.01$). The reason for early flocculation presumably lies with increased biochemical changes taking place in wort with longer heat treatment that interferes with yeast growth and sugar uptake ability. The decreased sugar uptake ability can be associated with high final extract values found in runs with longer wort heat treatment.

The presence of trub was found to negatively affect cell density which can be observed from low amplitude, mean and width of the curves compared to runs without trub (Group V). With an increase in wort treatment temperature and duration, formation of trub correspondingly rose. In presence of sufficient vigor, extreme heat denaturation can take place leading to the formation of much larger trub particle size. Therefore, on fermenting in test tubes there is a high probability of trub particle settling under gravity and being unavailable to suspended cells. A visual examination of wort (t=2 hr) showed a sediment of large trub particles with a comparatively less turbid wort as supernatant. After pitching (t=6 hr) a considerable decline in cell density was observed suggesting that yeast cells along with sedimenting trub particles would have been pulled down from the solution. This results in an overall less cell concentration and incomplete fermentation as seen in Figure 5.6.

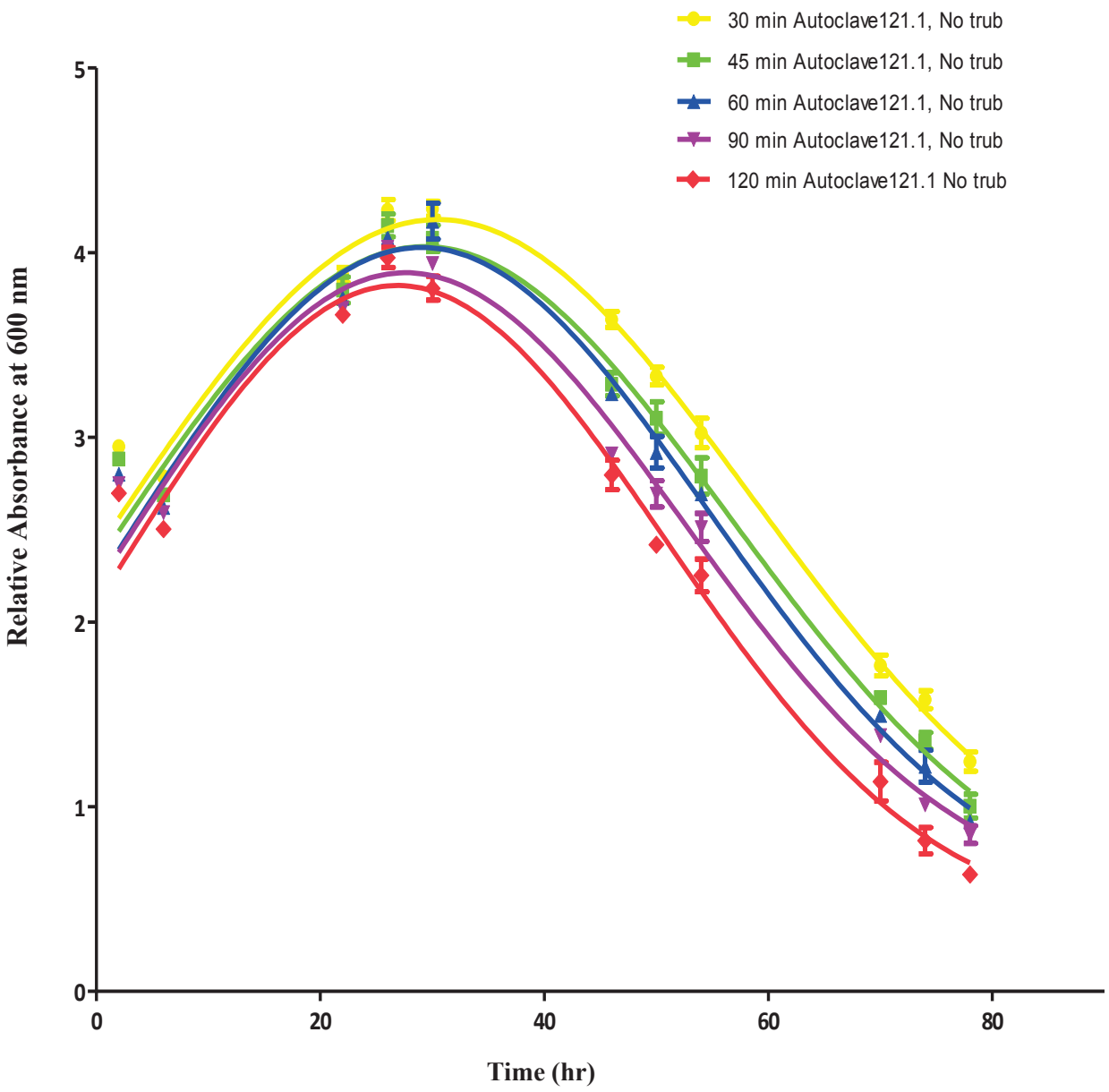

Figure 5.11 Comparison of absorbance measured at 600 nm of wort prepared by autoclaving (at 121.1^{o}C) under 2 atm pressure for different time periods and fermented without trub. Curves represents non-linear, tilted Gaussian fit of the turbidity data collected in triplicates along with error bars representing standard deviation (n=32).

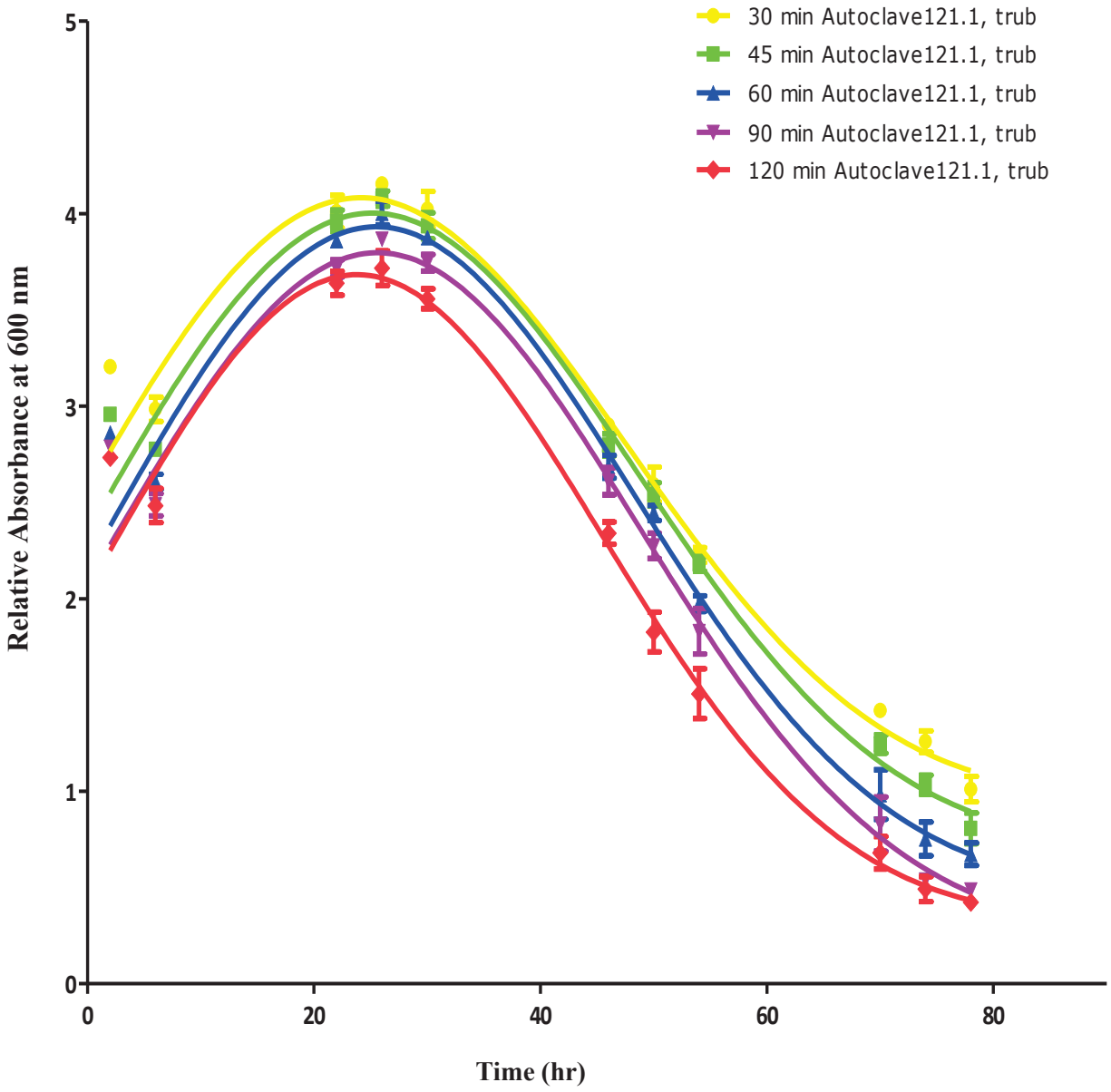

Figure 5.12 Comparison of absorbance measured at 600 nm of wort prepared by autoclaving (at 121.1^{o}C) under 2 atm pressure for different time periods and fermented containing trub. Curve represents non-linear, tilted Gaussian fit of the turbidity data collected in triplicates along with error bars representing standard deviation (n=32).

Table 5.3 Turbidity equation parameter values estimated from equation (2) for all fermentations.[b] (Type: 1- Boil (100°C), 2- Autoclave (100°C), 3- Autoclave (121.1°C); Trub: 0- No trub, 1- Trub)

Period	Type	Trub	A	ASE	μ (h)	ASE	σ	ASE	R	ASE	r^2
30	1	0	4.175	0.044	26.96	0.455	33.05	0.561	0.014	0.001	0.971
45	1	0	4.169	0.051	27.25	0.529	33.98	0.653	0.0119	0.001	0.974
60	1	0	4.206	0.043	28.02	0.464	34.15	0.581	0.0092	0.001	0.985
90	1	0	4.15	0.043	28.04	0.475	31.77	0.597	0.011	0.001	0.981
120	1	0	4.091	0.037	28.75	0.41	31.02	0.515	0.0099	0.001	0.974
F-test			0.022		0.153		0.0854		0.022		
30	1	1	4.09	0.082	27.29	1.374	23.93	1.705	0.0251	0.004	0.939
45	1	1	4.028	0.072	27.11	1.23	23.9	1.474	0.0249	0.003	0.966
60	1	1	4.03	0.072	27.41	1.195	24.5	1.368	0.0228	0.003	0.981
90	1	1	4	0.062	27.12	0.966	24.63	1.165	0.022	0.003	0.977
120	1	1	3.971	0.064	26.86	0.956	24.57	1.131	0.0215	0.003	0.944
F-test			0.102		0.0133		0.088		0.2222		
30	2	0	3.974	0.06	28.96	0.956	27.1	1.368	0.02	0.002	0.909
45	2	0	3.91	0.063	28.35	0.943	26.97	1.337	0.0202	0.002	0.938
60	2	0	3.792	0.078	28.26	1.12	27.87	1.583	0.0188	0.003	0.926
90	2	0	3.806	0.076	28.36	0.994	26.83	1.346	0.0191	0.002	0.925
120	2	0	3.822	0.059	29.44	0.813	27.87	1.11	0.0156	0.002	0.906
F-test			0.1088		0.0899		0.0852		0.1319		
30	2	1	3.871	0.041	25	0.53	29.46	0.663	0.0229	0.001	0.964
45	2	1	3.839	0.04	24.73	0.534	27.92	0.682	0.0238	0.001	0.98
60	2	1	3.913	0.038	24.61	0.547	27.61	0.697	0.0225	0.001	0.984
90	2	1	3.818	0.036	25.19	0.493	25.95	0.629	0.0232	0.001	0.984
120	2	1	3.739	0.048	25.99	0.669	26.77	0.817	0.02	0.002	0.965
F-test			0.1727		0.4531		0.494		0.0456		
30	3	0	4.071	0.054	29.75	0.713	28.75	0.847	0.0036	0.002	0.989
45	3	0	3.932	0.054	28.63	0.733	27.79	0.921	0.0035	0.002	0.986
60	3	0	3.907	0.051	28.26	0.645	26.4	0.824	0.0042	0.002	0.987
90	3	0	3.758	0.049	26.76	0.625	25.74	0.82	0.0049	0.002	0.986
120	3	0	3.722	0.048	26.27	0.574	24.52	0.737	0.0038	0.001	0.987
F-test			0.01		0.0043		0.0051		0.0456		
30	3	1	3.85	0.043	22.52	0.534	24.96	0.707	0.01	0.001	0.918
45	3	1	3.824	0.04	24.05	0.473	24.33	0.61	0.0073	0.001	0.971
60	3	1	3.809	0.044	24.81	0.483	23.41	0.6	0.0049	0.001	0.988
90	3	1	3.739	0.046	25.28	0.512	23.36	0.635	0.0023	0.001	0.974
120	3	1	3.602	0.046	23.36	0.488	21.96	0.566	0.0035	0.000	0.92
F-test			0.0064		0.6581		0.0098		0.0361		

[b]The total number of points for each fit represents a minimum of 32.

5.2 Apparent Degree of Fermentation

Apparent degree of fermentation (ADF) signifies the extent or degree of fermentation by providing relative amount of fermentable solids left in wort at the end of fermentation. ADF is calculated using the equation (3);

ADF= (Original Extract-Apparent Extract)/Original Extract = (OE-AE)/OE = $(P_0-P_{75})/P_0$(3)

Where, OE refers to the amount of wort fermentable solids present at the start of fermentation when time, t = 0; AE is the wort fermentable solids at the end of fermentation (i.e., t = 75 h). OE and AE were calculated as P_0 and P_{75} using the logistic equation for each run and t = 0 and 75 hr, respectively.

The calculated OE and AE values are plotted with respect to heat treatment periods as shown in Figures 5.13 and 5.14, respectively. OE and AE show trends similar to P_i and P_e fermentability parameters. Original extract values of Group I and III runs significantly increased with increase in treatment time periods (F-test, $p<0.001$). Apparent extract values show a significant increase with increase in treatment periods for all groups (F-test, $p<0.05$) signifying incomplete fermentations. Lowest apparent extract values were obtained for runs of boiled wort (group I) carried without trub suggesting higher conversion of fermentable sugars and better alcohol yield. High apparent extract values were obtained for runs with autoclaved wort (at 121.1^{o}C).

ADF calculated using equation (3) is linearly plotted as shown in Figure 5.15. As discussed in ASBC Yeast 14, the error was calculated as shown in Figure 5.15. ADF values showed a significant decline with respect to increasing treatment times of wort of each respective groups (F-test, $p<0.05$). The maximum decline in slope of linear regression line was found for groups V and VI. This suggests that the amount of

fermentable solids left in wort increases with higher autoclaving temperature (at 121.1^{o}C) and treatment duration. The degree or extent of fermentation was found to be highest with Group I fermentation (Boil at 100^{o}C) carried out without trub. Therefore, fermentations that are subjected to less boiling or autoclaving provide better alcohol yields with higher consumption of wort solids. However, presence of trub gave mixed results. When wort was subjected to boiling (at 100^{o}C) trub decreased fermentability while on autoclaving (at 100^{o}C), runs carried with trub fermented better. Moreover, ADF values of wort autoclaved at 121.1^{o}C for 30 min were comparable when fermented with and without trub, but with increase in autoclaving the differences in ADF became more prominent (increase in gap width in Figure 5.15). This suggests negative effect of trub on wort fermentability. A similar observation was made with increasing gap width of AE values with group V and VI runs.

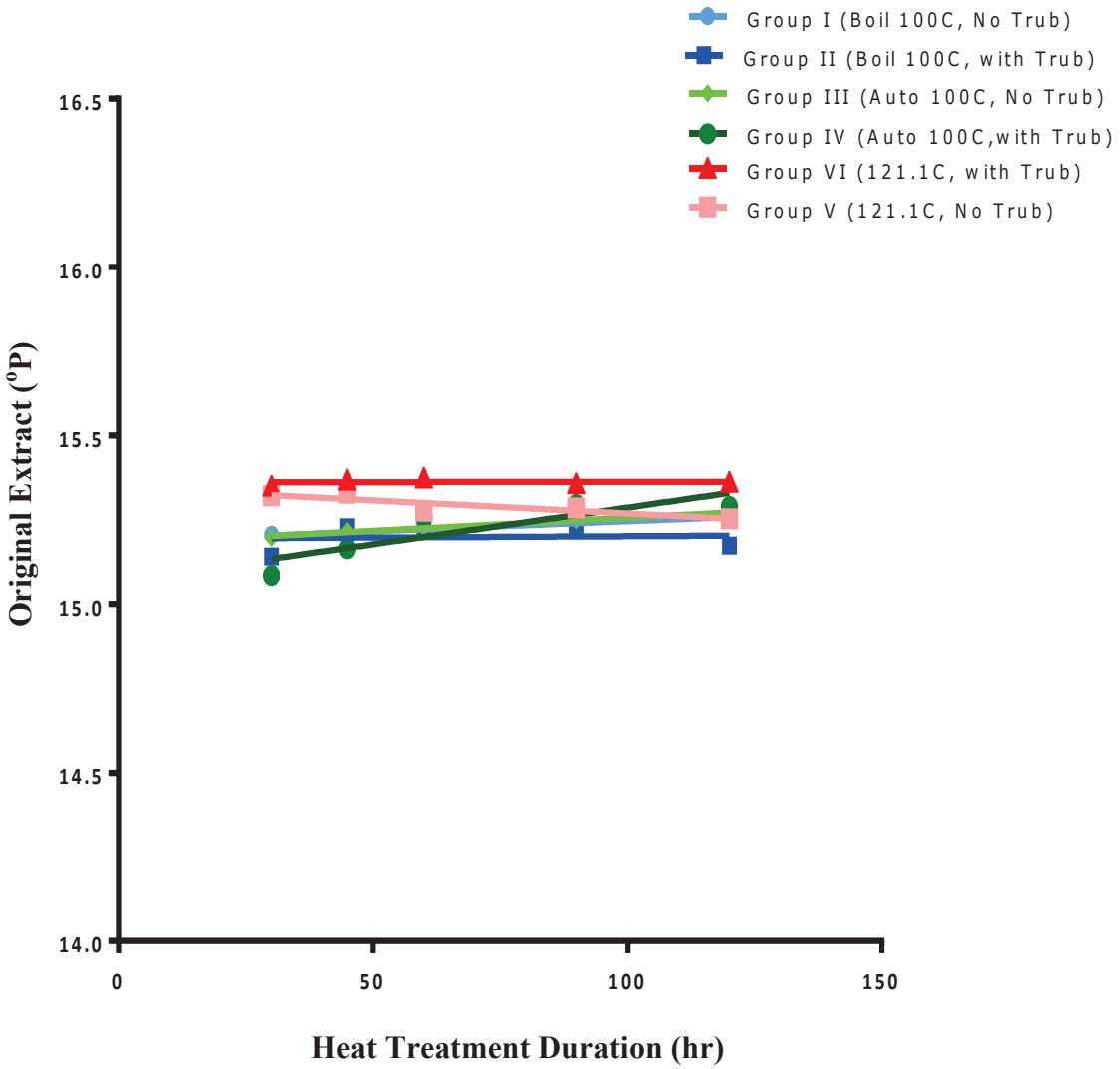

Figure 5.13 Comparison of Original Extract (OE) calculated from best fit value of logistic parameters provided by non-linear regression. Each line represents a linear fit of OE of all fermentations grouped according to table 5.1. Error associated with each point is reported in Table 5.2 and is a maximum of 0.024 (n=32).

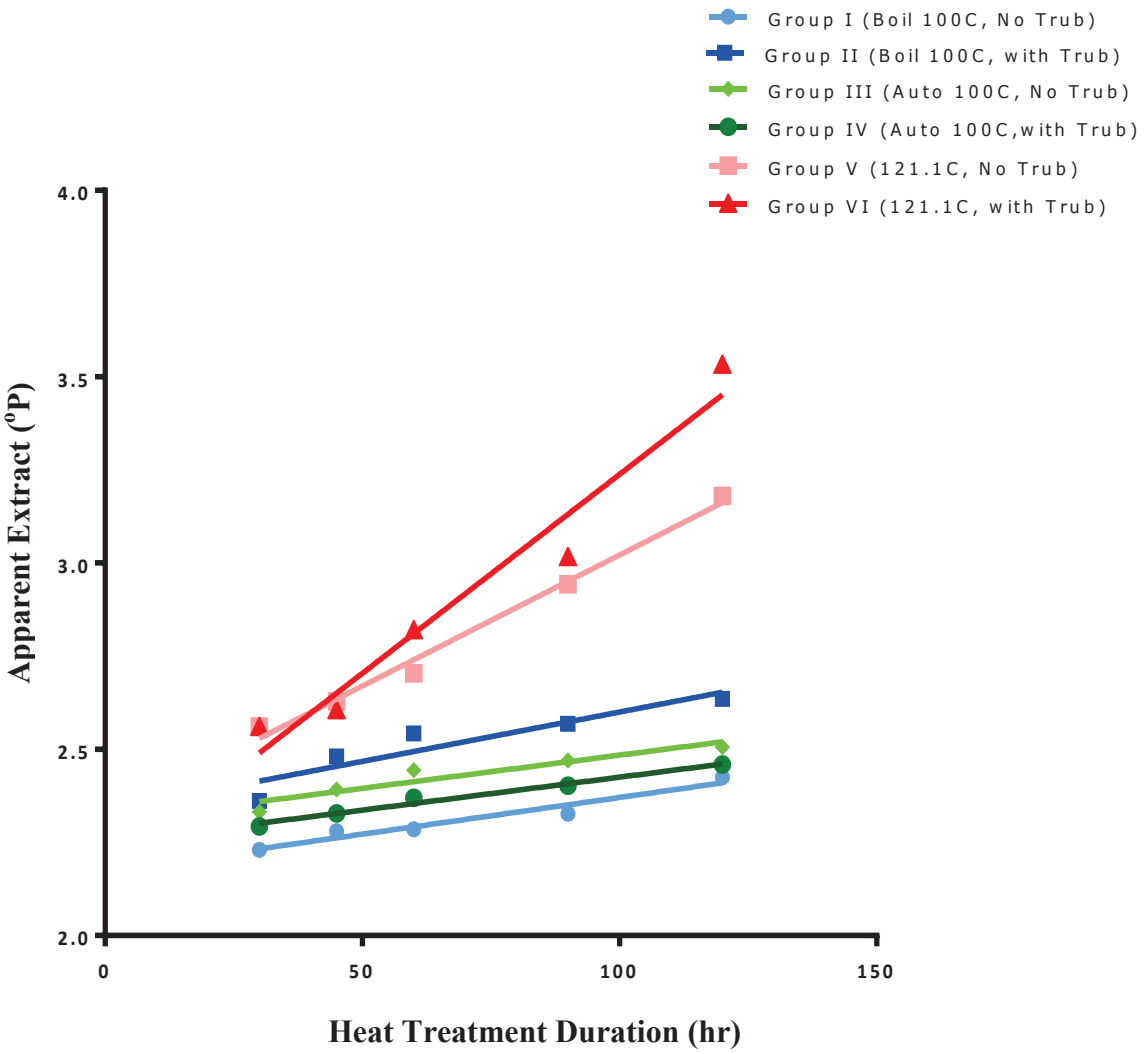

Figure 5.14 Comparison of Apparent extract (AE) calculated from best fit value of logistic parameters provided by non-linear regression. Each line represents a linear fit of AE of all fermentations grouped according to table 5.1. Error associated with each point is reported in Table 5.2 and is a maximum of 0.141 (n=32).

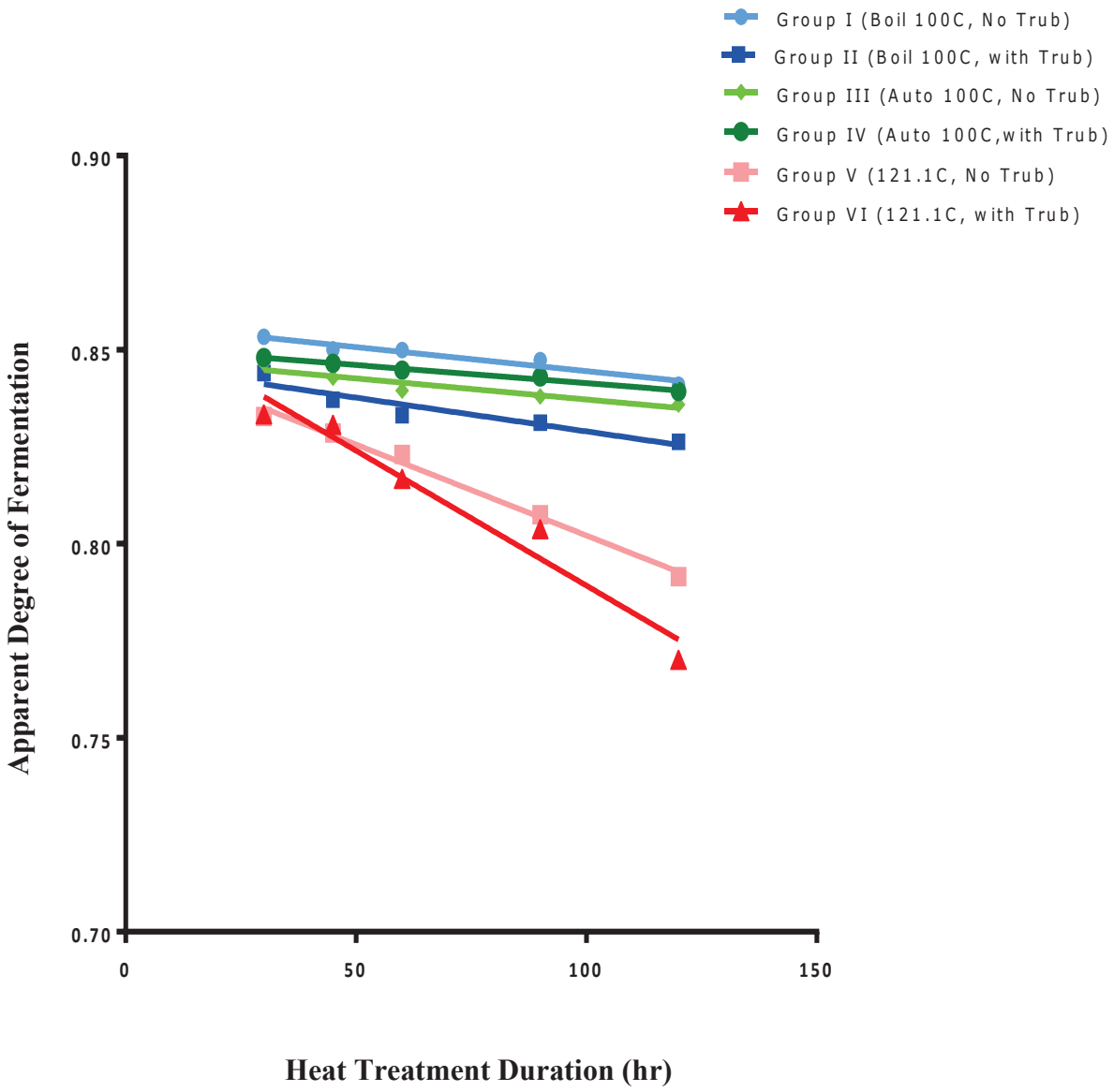

Figure 5.15 Comparison of Apparent degree of fermentation calculated from best fit value of logistic parameters provided by non-linear regression. Curves represent linear fit of ADF of all fermentations grouped according to table 5.1. The mean associated standard error, $ADF_{error}= \sqrt{[(AE/OE^2)^2\times(ASE_{OE})^2+(-1/OE)^2\times(ASE_{AE})^2]}$ was calculated to be 0.0081±0.002 (n=32).

5.3 Rate of Fermentation

A fermentation rate can be deduced from either the slope or the point of inflection of their density curves. However, in certain situations, when two density curves are very close, one fermentation run can have a lower slope while the other can have a low point of inflection value, which makes it hard to determine which of the two runs is slower. In such situations, the area under each curve provides a better estimate of their relative speed of fermentation. A larger density curve area would signify a slower fermentation with respect to time. Similarly, with turbidity curves the area under each curve could provide a relative estimate of total yeast in suspension.

The total area under each curve was integrated for all fermentation runs using Graphpad PRISM ver. 5. Figure 5.16 shows the integrated density curve area for all 6 groups of fermentation with respect to treatment durations. A significant increase in area was observed with increase in both heat treatment temperature and time interval (F-test, $p<0.05$) for all groups except group II. This implies that the rate of fermentation declines with increasing wort heat treatment. Although for group II runs the area under density curve declined from 475.7 to 488.1 on increasing boil from 30 to 120 min, but this was not statistically significant ($p>0.05$). Slowest fermenting wort was found to be the autoclaved runs carried at 121.1^{o}C and fermented containing trub.

Figure 5.17 shows integrated turbidity density curve area of all runs with respect to treatment durations. Area under turbidity curves significantly decreased with increase in the duration and temperature of the heat treatment (F-test, $p<0.05$) for all groups. This implies that the yeast in suspension declined with increasing heat treatment time intervals of the wort, leading to decreased fermentation. Maximum decline was again obtained for runs carried with wort autoclaved at 121.1^{o}C and fermented containing the trub. This suggests an early flocculation of yeast cells took place when wort was treated to high

temperature for longer durations resulting in decreased wort fermentability and sugar uptake.

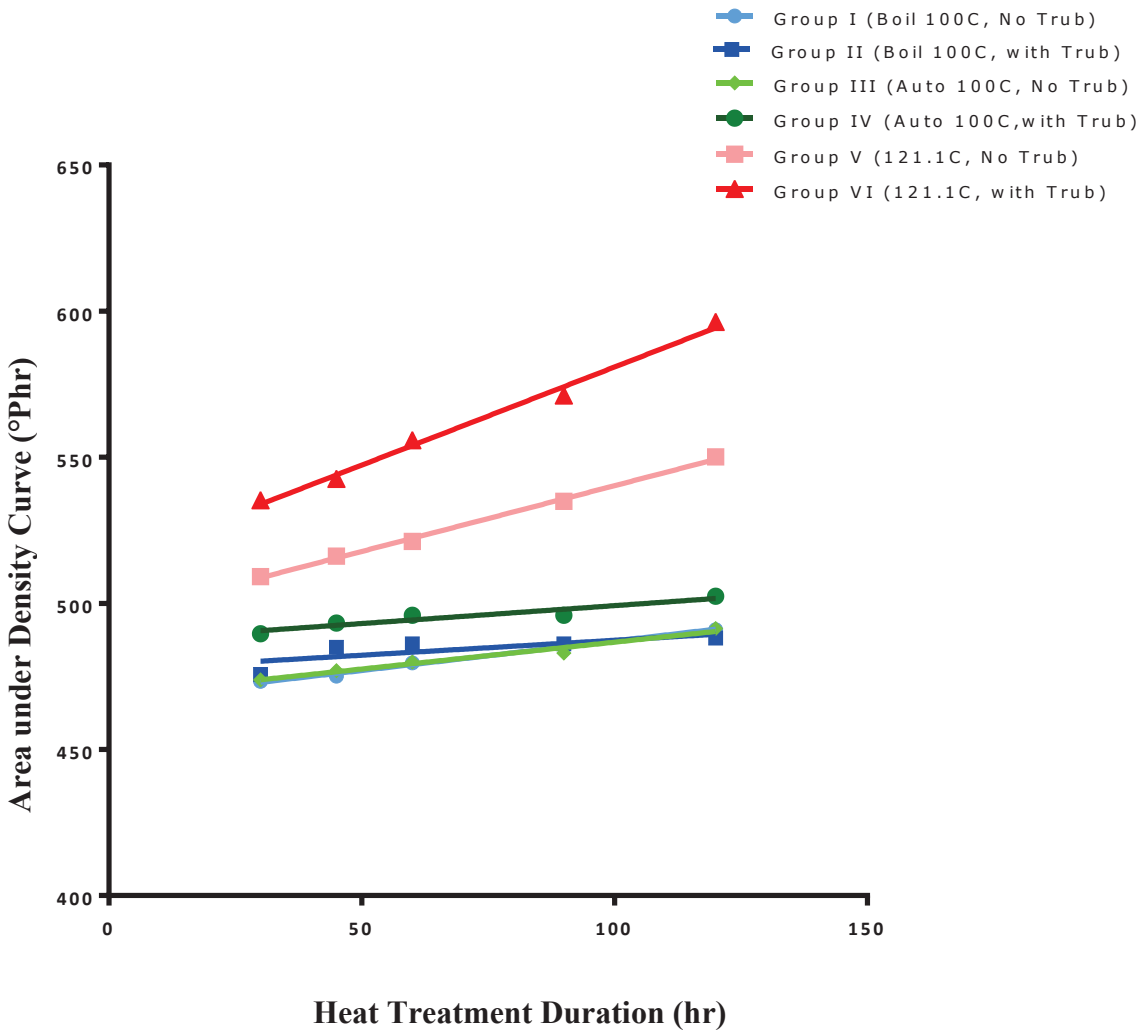

Figure 5.16 Comparison of Area under Density calculated from logistic curve fit by non-linear regression. Each line represents a linear fit of Area of all fermentations grouped according to table 5.1. Associated standard error is provided in Table 5.2 (n=32).

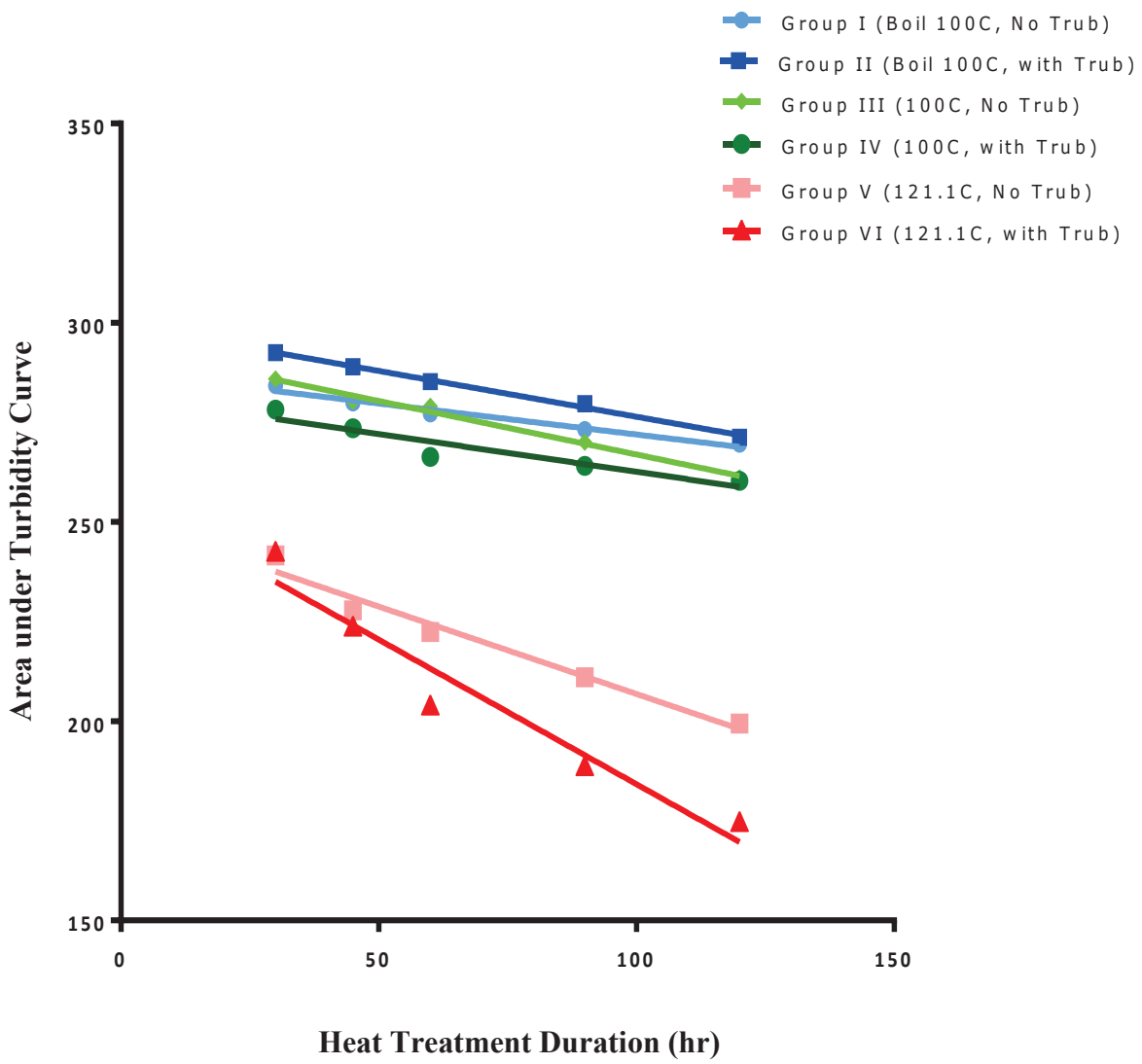

Figure 5.17 Comparison of Area under Turbidity calculated from tilted-Gaussian curve fit by non-linear regression. Curves represent linear fit of Area of all fermentations grouped according to table 5.1. Associated standard error is provided in Table 5.3 (n=32).

5.4 Sugar Profiling

Mashing conditions decide the amount of fermentable sugars present in wort as the amylase activity is regulated by controlling mash bath temperature. The coefficient of variations for laboratory error when using HPLC for individual sugar profiling was stated to be 3.6-7.7% by ASBC (38). Therefore some variations in individual sugar levels can be considered normal. With wort boiling, a loss in sugar concentration is expected as they participate in Maillard reaction. Upon treating wort to high temperature boiling the differences in sugar concentration, if any, would be further amplified. Therefore, wort samples of group V and VI were selected for sugar profiling. Wort samples were autoclaved (at 121.1°C, 2 atm) for varying periods and analyzed using HPLC. Concentration (g/mL) of maltotriose, maltose, glucose and fructose obtained from wort free of trub and containing trub are shown in Figures 5.18 and 5.19. No significant differences in the respective sugar concentrations were obtained with change in treatment time intervals ($p>0.05$). Presence of trub in wort did not show any significant differences in individual sugar concentrations either with change in treatment periods or with wort without trub ($p>0.05$). It has been previously found that wort does contain substantial amount of unfermentable sugars as well such as xylose, arabinose etc. Previous studies have showed that non-fermentable sugars show high reactivity in Maillard browning than glucose and fructose (32, 64). Since HPLC analysis did not show the amount of unfermentable sugar fractions present in wort, therefore, effect of wort boiling on sugar levels cannot be ascertained.

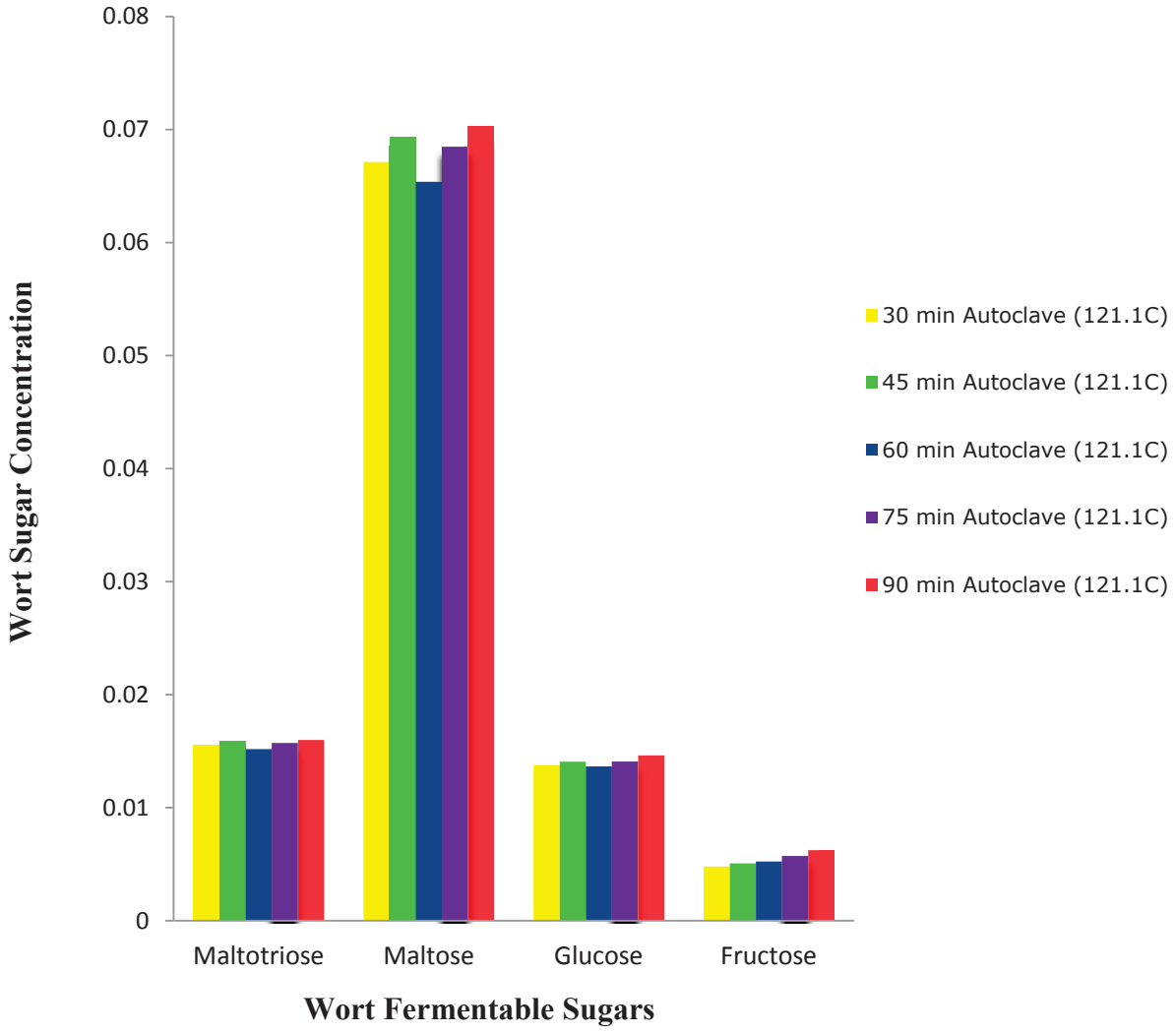

Figure 5.18 Comparison of wort sugar profiles obtained from HPLC analysis. Each column represents concentration of a particular sugar after respective wort treatment at 121.1°C. Wort was filtered prior to sugar analysis. Bars represent average values. Absolute differences were less than 0.015% (n=2).

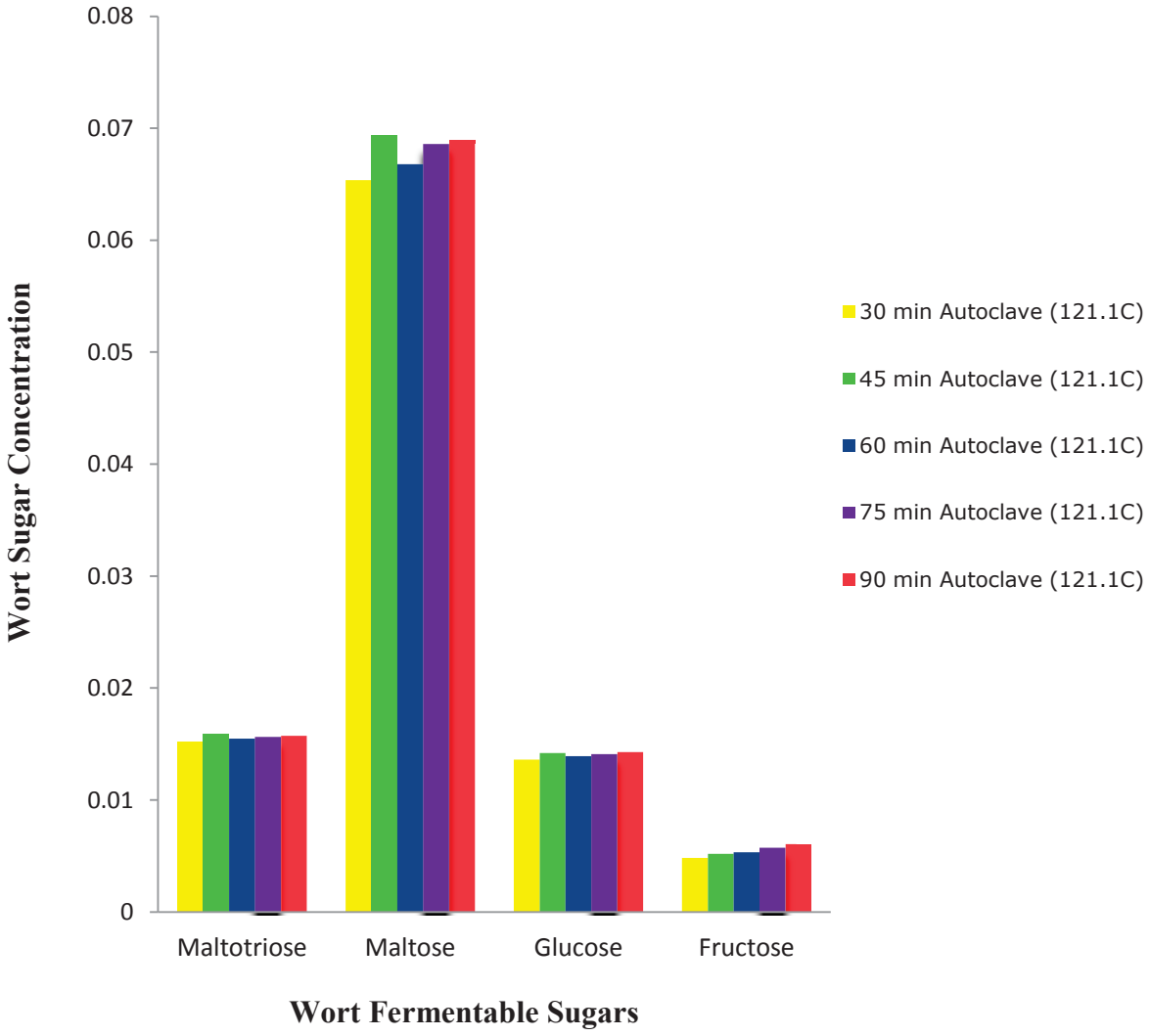

Figure 5.19 Comparison of wort (containing trub) sugar profiles obtained from HPLC analysis. Each column represents concentration of a particular sugar after respective wort treatment at 121.1^{o}C. Wort along with trub was used for sugar analysis Bars represent average values. Absolute differences were less than 0.015% (n=2).

5.5 FAN Content

Free amino nitrogen (nitrogen source) is considered most significant factor affecting fermentation performance as it controls yeast growth (46). During the course of wort boiling and trub formation, a substantial amount of wort nitrogen is lost due to Maillard reaction and precipitation. Therefore, FAN content of wort after each heat treatment was measured in triplicates using the Ninhydrin method (ASBC, wort-12). Figure 5.20 shows FAN levels (mg/L) of all the groups with respect to treatment durations, along with associated standard errors. Regression analysis revealed significant decline in FAN levels with increasing wort treatment duration (F-test, $p<0.0001$). The concentration of FAN decreased considerably when wort was subjected to higher treatment temperature (at 121.1°C) than when boiled or autoclaved at 100°C. This implies that FAN levels decline both with increase in wort treatment temperature as well as with treatment duration. Final FAN levels in wort play a crucial role in deciding the rate of fermentation and yeast density as discussed in section 6.

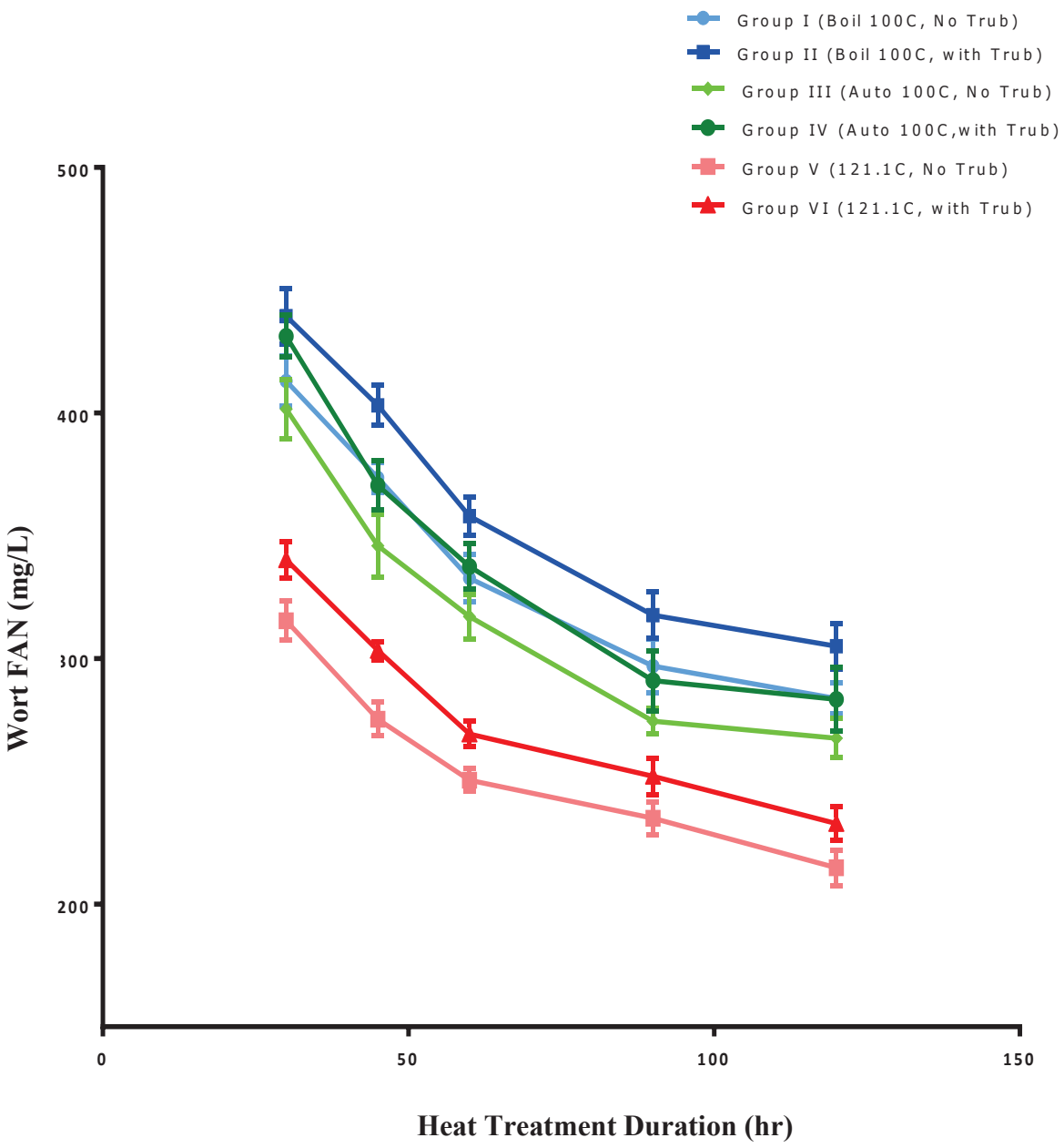

Figure 5.20 Comparison of wort free amino nitrogen levels calculated after respective heat treatment, grouped according to table 5.1. Each FAN level represents mean of triplicate along with error bars representing standard deviation (n=3).

5.6 Wort Color

Wort color was measured by spectrophotometric method (ASBC, Beer-10). Following absorbance at a wavelength from 400-460 nm provides the relative amount of Maillard reaction products or extent of Maillard browning in wort (65). After each heat treatment, trub was removed to avoid turbidity and absorbance measured at 430 nm in duplicates. Figure 5.21 shows the calculated wort color obtained from various heat treatments (group wise) versus treatment duration. With increase in treatment durations from 30-120 min, the wort color also correspondingly increased. Also, when the treatment temperature was high (at 121.1°C), the increase in wort color with longer treatment intervals was comparatively larger. This implies that when wort was subjected to high temperature, the rate of Maillard reactions increases which results in added concentration of melanoidins and other colored intermediates.

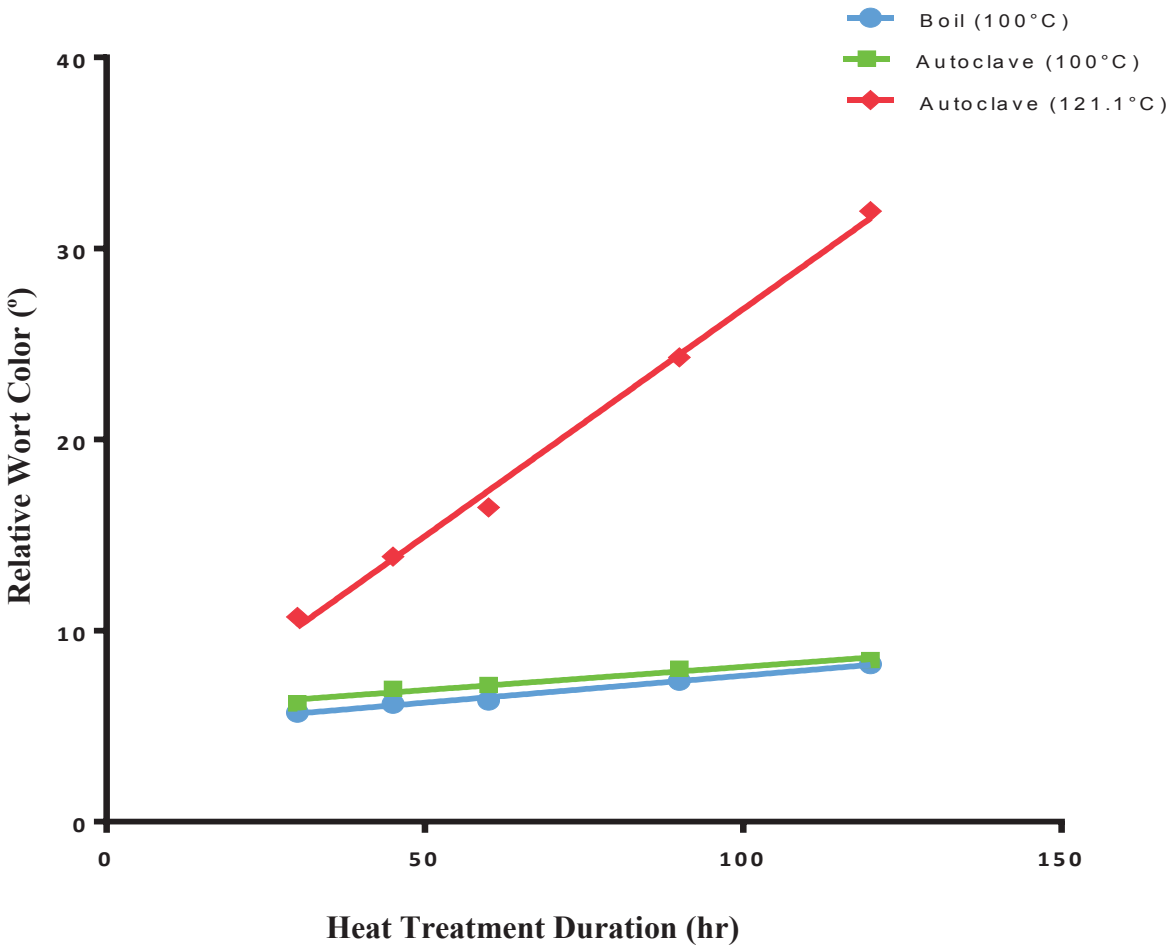

Figure 5.21 Comparison of wort color calculated after respective heat treatment, grouped according to table 5.1. Each line is a linear fit of wort color representing mean of two trials. Absolute differences were less than 0.005% (n=2).

5.7 Wort pH and Trub Content

Wort pH measurement is considered important in order to establish the effect of wort boiling. Boiling causes a decline in pH due to precipitation of ionic components in trub such as alkaline phosphates, formation of acidic Maillard products as well as due to loss of amino acids which act as wort buffering systems. pH changes ranged from 0 to 0.3 units during a wort boil (8). Figure 5.22 shows wort pH measured after each heat treatment (group-wise). Wort pH showed a significant decline with increasing heat treatment duration (F-test, $p<0.01$). A severe decline in pH was observed with increasing wort heat treatment duration at high temperature. Autoclaved runs (at 121.1°C) containing trub showed lowest pH values.

Dried Trub was weighed after each type of heat treatment and duration in triplicates. Figure 5.23 shows the amount of trub (g/167 mL of wort) obtained after each heat treatment. A significant increase in trub levels is obtained with increase in heat treatment temperature and durations (F-test, $p<0.05$). This implies that with increase in wort heating temperature and duration more polypeptides get denatured and coagulate as protein-protein and protein-polyphenol complexes constituting trub.

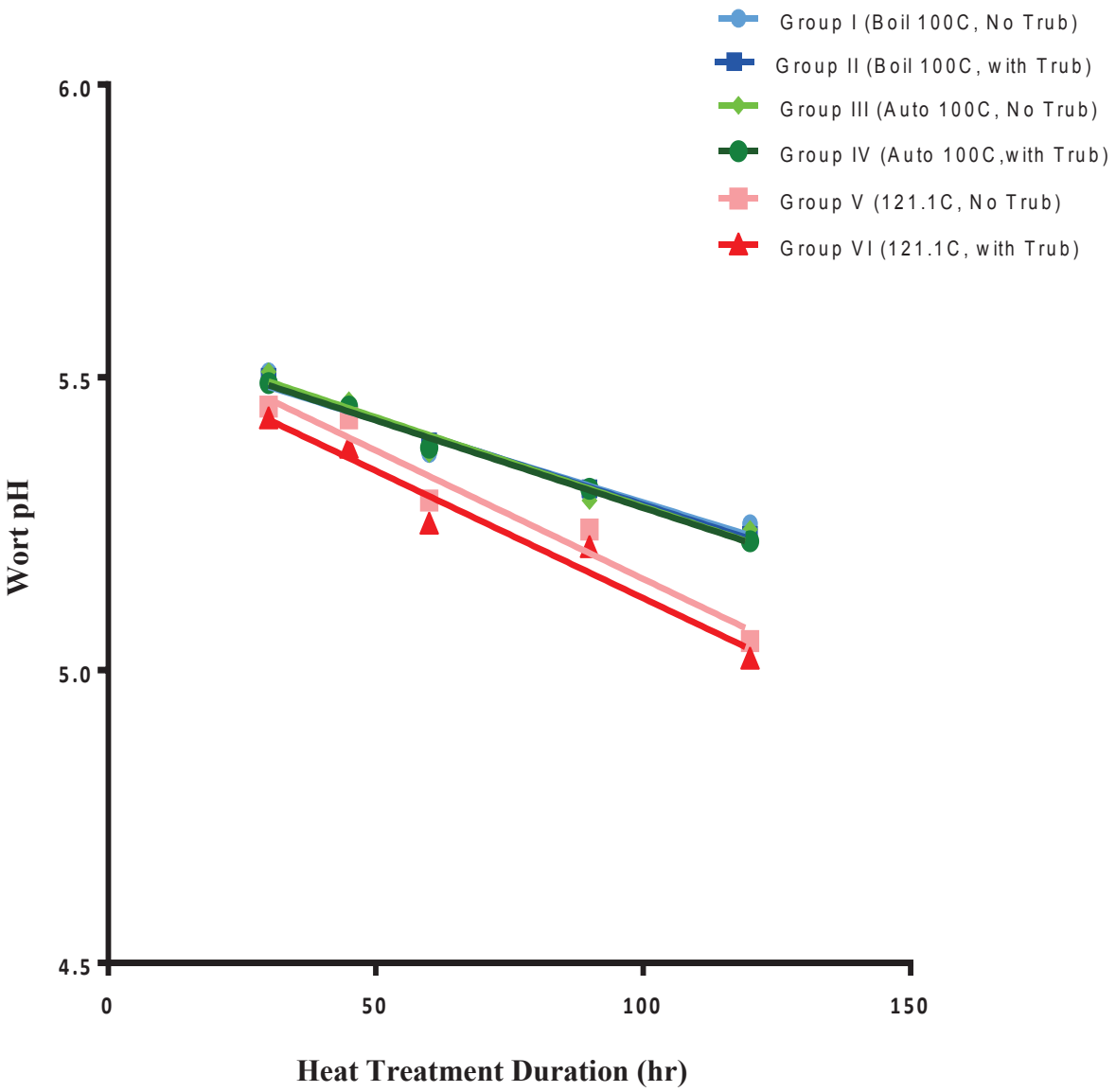

Figure 5.22 Comparison of wort pH after respective heat treatment, grouped according to table 5.1. Line represents linear fit of wort pH. Associated Standard error was found to vary from 0.09 to 0.15.

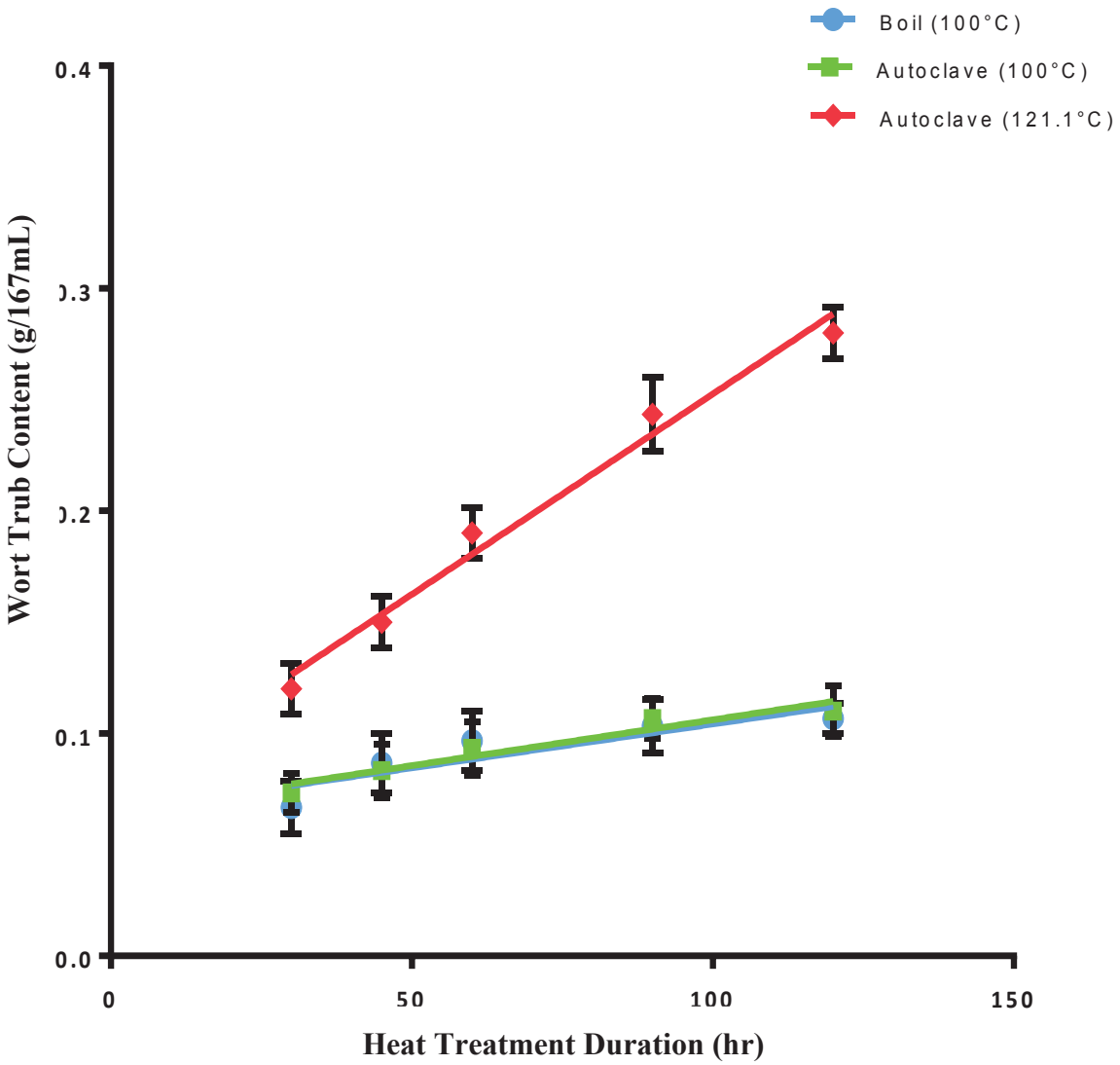

Figure 5.23 Dried trub levels along with associated error, in wort after respective heat treatment. Line represents linear fit of total trub formed after each trial grouped according to table 5.1.

CHAPTER 6 DISCUSSION

For this study, a small scale fermentation method was used to understand the effect of heat treatment on malt fermentability. Trials were completed to evaluate the effect of variation in wort treatment temperature and durations, along with trub levels, on wort fermentations. The results obtained are discussed below.

6.1 Fermentation

A miniature fermentation method was selected as it requires small amounts of grist/malt and gets completed fairly quickly (i.e., within 72 hr). Similarities in the yeast grow up stages and procedures during all the runs, allowed the control of inoculums viability, physiological state of cells, and cell numbers. Wort preparation and treatment were consistent and were easily manipulated with the use of an automated autoclave and rheostat controlled boilers. Any differences in fermentation due to wort or yeast handling procedures were certainly eliminated through this fermentation method.

6.1.1 Rate of Fermentation

The density curves showed significant differences in the rate of sugar consumption with increasing heat treatment of the wort (F-test, $p<0.001$) when the trub was absent. This was further established by measuring the area under the density curves which proved that the sugar uptake rate declined significantly with increase in both temperature and duration of wort heat treatment (F-test, $p<0.05$) (Figure 5.16). An increase in the area under density curve signifies slow and relatively less uptake of sugar, and thus a low rate

of fermentation. A high correlation was found between the area under density curves and the heating temperature (heat type) yielding an r value= 0.785, $p<0.0001$. With an increase in the treatment temperature from 100°C boil to 121.1°C autoclave, maximum increase in area under the curve was found. These observations suggest that during the heat treatment, physical or biochemical changes taking place in the wort increase with increasing time and temperature. This significantly changed the wort composition. Changes in wort composition can affect yeast sugar uptake ability and malt fermentability.

The rate of fermentation of runs without the trub, declined by 7-12% with the wort that was autoclaved at 121.1°C rather than the one boiled at 100°C for 30-120 min. However, the rate of fermentation declined by only 3.7% when wort boiling or autoclaving (at 100°C) was increased from 30 to 120 min. An 8% decline in fermentability was obtained for increasing autoclaving (at 121.1°C) duration from 30-120 min. This signifies that an increase in temperature and duration of wort boiling affects fermentability to a greater extent than boiling at low temperature for less duration.

When heat treated wort was fermented along with the trub formed, the runs gave mixed results. For Group II runs, where boiled wort (at 100°C) was fermented along with the trub, no significant differences were obtained between the rate of fermentation with increasing treatment intervals (F-test, $p>0.05$) (Figure 5.2, 5.16) . This suggests that trub interferes with the sugar uptake ability of yeast cells, thereby affecting the rate of fermentation. However, for autoclaved runs containing the trub, both 100°C and 121.1°C runs showed significant increase in the corresponding areas under the density curves with increasing treatment durations (F-test, $p<0.05$) (Figure 5.16). Fermentability of the wort autoclaved at 100°C was reduced by 2.6%, while it declined by 22% after being autoclaved at 121.1°C for durations ranging from 30 to 120 min. This suggests that increase in heat treatment negatively affects fermentations both with and without trub, although the extents may vary.

Area under the density curves of the runs autoclaved for the same duration at 100°C and 121.1°C but fermented along with the trub showed larger area compared to that without the trub counterparts. A decrease of 5-8% in the rate of fermentation was found on fermenting the wort autoclaved at 121.1°C with trub compared to that without the trub (Figure 5.16). This indicates that the presence of trub lead to a greater decline in the fermentability compared to that done solely by wort heat treatments.

As previously mentioned, several biochemical changes take place during wort boiling and can alter wort fermentability such as amino-carbonyl reactions, trub formation and decline in pH. Each of these changes has several factors that govern their development and characteristics as discussed in section 6.2.

6.1.2 Degree of Fermentation

Final gravity (P_e) of all the fermentation groups increased significantly with increase in wort heat treatment duration (F-test, $p<0.05$) (Figure 5.14). A high final gravity implies that high fermentable sugar concentration is present in the wort at the end of fermentation. Decreased sugar uptake will result in less alcohol production, indicating less wort fermentability. Final gravity estimated from regression fits showed maximum increase of 41% and 26% for autoclaved (121.1°C) runs with and without trub, respectively, on increasing the duration from 30 to 120 min. These results suggest decreasing sugar consumption in wort containing trub, when heat treated for longer durations and at high temperatures.

Apparent Degree of fermentation for all the groups declined significantly with increase in the wort heat treatment durations (F-test, $p<0.05$) (Figure 5.15). Fermentability declined 7.6% when the wort was autoclaved for 120 min instead of 30 min at 121.1°C, and fermented with the trub. The decline in fermentability for similar duration at 121.1°C was found to be 4.97% when fermentations were done without the trub. This suggests that

presence of trub along with wort heat treatment does not allow effective uptake of wort sugars and nutrients, resulting in poor wort fermentability and high final gravity.

6.1.3 Yeast in Suspension

Yeast in suspension is critical for determining malt fermentability. Yeast in suspension refers to total yeast cell count that are actively fermenting the wort components, leading to higher ethanol production at a given time. A decline in yeast in suspension during fermentation is marked by flocculation. It is a complex process depending on several physiological and environmental factors. An early flocculation results in decline of suspended cells and leads to high final gravity and low alcohol content of the wort. These factors can significantly affect the malt fermentability.

The total amount of suspended cells during fermentation can be determined by measuring the area under the turbidity curve. A large turbidity curve area will signify more yeast cells in suspension during fermentation, and therefore, delayed flocculation. Area under the turbidity curve showed a significant increase when the wort was heat treated for less periods, resulting in a higher wort fermentability and low ADF (F-test, $p<0.05$) (Figure 5.16). This suggests that actively fermenting yeast cell density declines with increased heat treatment periods, which negatively affects rate of fermentation. Area under the turbidity curve was found to negatively correlate with the area under density curve ($r= -0.972$, $p<0.0001$), while positively correlate with ADF ($r= 0.903$, $p<0.0001$). Maximum decline of 9.2% and 27.9% in yeast in suspension was observed upon autoclaving the wort (121.1°C) from 30 to 120 min and fermenting without and with the trub respectively. This decline in yeast in suspension explains the poor wort fermentability characteristics of heat treated wort at high temperature and longer durations.

It was found that autoclaved (121.1°C) wort runs showed more intense flocculation, as amplitude, curve width and slope showed significant decline in both groups (F-test,

$p<0.01$) implying the negative effects of high temperature and increased duration (Figure 5.11, 5.12). A higher decline in suspended yeast was observed with increasing heat treatment temperature and periods along with the presence of the trub. Runs carried with the trub showed a greater decline in yeast in suspension when compared to runs without trub for each type and period of heat treatment. Several factors could be responsible for a premature and rapid decline of the yeast in suspension. However, as the trub interferes with cell nutrient uptake, it can also be considered as a factor promoting early flocculation during fermentation. These observations suggest a collective effect of change in the wort nutrient levels, browning compounds, presence of the trub, and pH on yeast growth during fermentation.

Factors such as floc size and density, and medium turbulence, viscosity and density are responsible for the settling of flocs (66, 73). Since the fermentations are quite closely related, the formation of flocs could have started earlier in runs that were subjected to longer heat treatments but were suspended and started to settle only when the shear rate declined below a certain level. It has been previously found that supplementation with 4% glucose and high fermentation temperature produces necessary shear by CO_2 evolution, which facilitates the formation of flocs and holds the suspended flocs (31). It is impossible to determine the exact time of the onset of flocculation solely on the basis of turbidity measurement. The onset of flocculation is represented by the turbidity curve mean which can show variations based on shear produced between different wort runs. This explains why no significant trends in curve means were obtained with boiled (100°C) and autoclaved (100°C) wort runs, fermented without trub. To elucidate the effect of boiling on the onset of yeast flocculation, the mean shear rate all along the fermentation run has to be determined.

6.2 Wort Composition

Figure 6.1 shows various wort associated factors that are known or thought to be influenced by boiling and affect yeast flocculation. A diagrammatic representation will enable to understand and establish specific correlations between the two.

Several factors are responsible for affecting yeast growth during fermentation. Yeast cells use a variety of nutrients from the wort, which enable them to convert sugars into alcohol and release carbon-dioxide. The most essential wort components are fermentable carbohydrates and amino acid moieties. Lack of growth nutrients results in cell division arrest, leading to flocculation (64). Since, heat treatment has been associated with decreasing the carbohydrate and nitrogen content of solutions by Maillard browning and trub formation, longer wort boiling treatments can affect the rate of fermentation. The availability of essential nutrients and sugars can reach growth limiting amounts, leading to reduced yeast growth and early flocculation.

It has been found that all nutritional needs of the yeast are satisfied by amino-nitrogen and small peptides, while proteins and polypeptides do not affect yeast growth or fermentation (47). Therefore, FAN levels play a major role in deciding yeast growth, the rate of fermentation and the onset of flocculation. Studies have confirmed that boiling influences the physicochemical properties and structure of wort proteins resulting in a decrease in protein and amino acid contents (26). Therefore, it is essential to validate the FAN content of the wort after each heat treatment to establish how it affects flocculation.

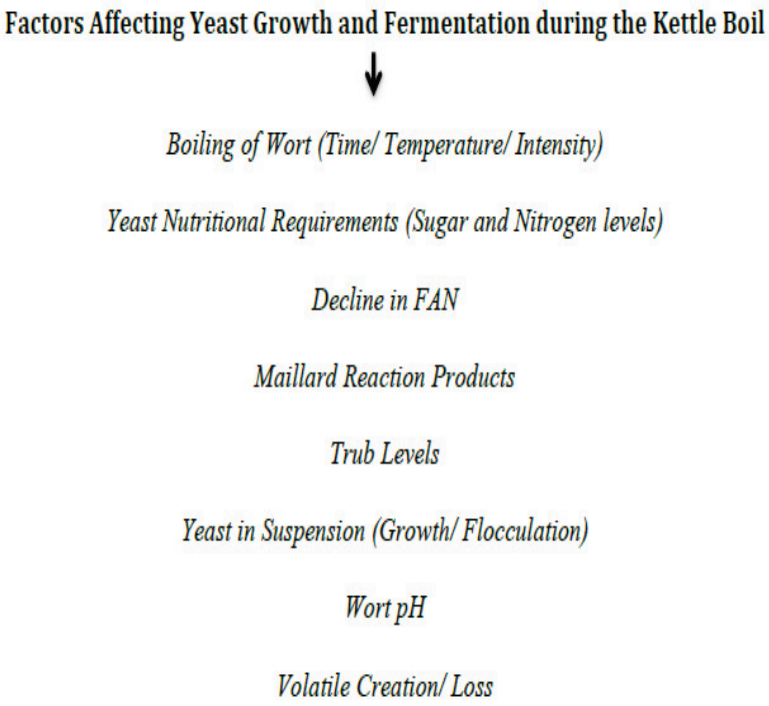

Figure 6.1 Summary of factors affecting cell growth and flocculation.

Growth temperature influences the rate of cellular metabolic activity. Low fermentation temperature is found to result in reduced yeast metabolism and CO_2 evolution, leading to an early flocculation (62, 29). Therefore, an optimum yeast growth temperature of 21°C was selected to carry out a fermentation process that excludes the effect of temperature on flocculation.

Yeast cell growth and flocculation are severely influenced by wort fermentable sugar concentration. Yeast cells prefer taking up glucose, sucrose, and fructose followed by maltose and maltotriose (32). However, since wort sugar analysis (Figures 5.18 and 5.19) did not show any differences in the respective sugar concentrations, early flocculation and reduced uptake due to low sugar concentration is not probable.

Cellular growth and the onset of flocculation are dependent on wort aeration or oxygen concentration. Yeast cells require oxygen to synthesize essential fatty acids and sterols; therefore, the wort was saturated with oxygen before the start of a fermentation run. A number of ions have also been reported to act as flocculation inducers, and Ca^{2+} ions are of significant importance among all ions (62). Since trub formation occurs during boiling which alters sugar, nitrogen, and ionic concentration of the wort, trub levels during fermentation can play a role in decreasing cellular growth and inducing flocculation.

6.2.1 FAN

FAN is essential for yeast growth as it is the principal, easily assimilable nitrogen source. FAN levels in wort vary as they linearly increase with the amount of barley and mashing conditions (7). Boiling has been reported to significantly affect wort nitrogen levels (56). After wort boiling for 60 min, the FAN levels were found to be 358 (±13) and 333 (±16) mg/L for with and without trub samples, respectively (Figure 5.20). These results are in agreement with the previous findings (16).

FAN levels declined significantly in the wort with increase in heat treatment periods at all temperatures (F-test, $p<0.001$). The FAN concentration declined by 33.4% on increasing autoclaving (100 °C) duration from 30 to 120 min, while it decreased by 32%, at 121.1°C (Figure 5.20). FAN showed high negative correlation with wort heat treatment periods ($r= -0.725$; $p<0.0001$). This suggests that wort boiling is responsible for decline in FAN, and the extent and temperature of the boil dictates the loss. On the contrary, some researchers have observed no differences in protein levels before and after the wort boiling and consider wort as free of high molecular weight polypeptides (47).

Biochemical and physical changes happening during wort boiling that affect FAN levels are Maillard reaction and trub formation, respectively. During trub formation amino nitrogen can form complexes with the trub molecules, polyphenols or cations and gets precipitated. Formation of carbonyl-amino compounds involves loss of amino acid moieties resulting in decreased initial wort FAN concentration. The duration and temperature of the boil determines the amount of trub and carbonyl compounds formed, causing FAN precipitation and loss.

Correlation between the initial FAN level and the yeast in suspension yielded an r value of 0.746– significant at the 0.01 probability level. Previous studies have also shown that a low wort FAN concentration coincides with decreased cell numbers and absorbance, even when other nutrients are non-limiting (57, 61). A higher initial wort FAN level due to less heat treatment duration is responsible for an increased total cell density and better fermentability of runs carried with wort heated at low temperature and for less time. With increase in wort heat treatment period, a subsequent decline in wort FAN was obtained that negatively correlated with the area under density curves ($r=-0.658$, $p<0.05$). Therefore, wort having low initial FAN levels will result in decreased rate of fermentation and low cell density. These factors are responsible for poor fermentability of the wort subjected to longer heat treatment. Wort utilizable FAN concentration has been previously found to be positively correlated with the rate of fermentation (48).

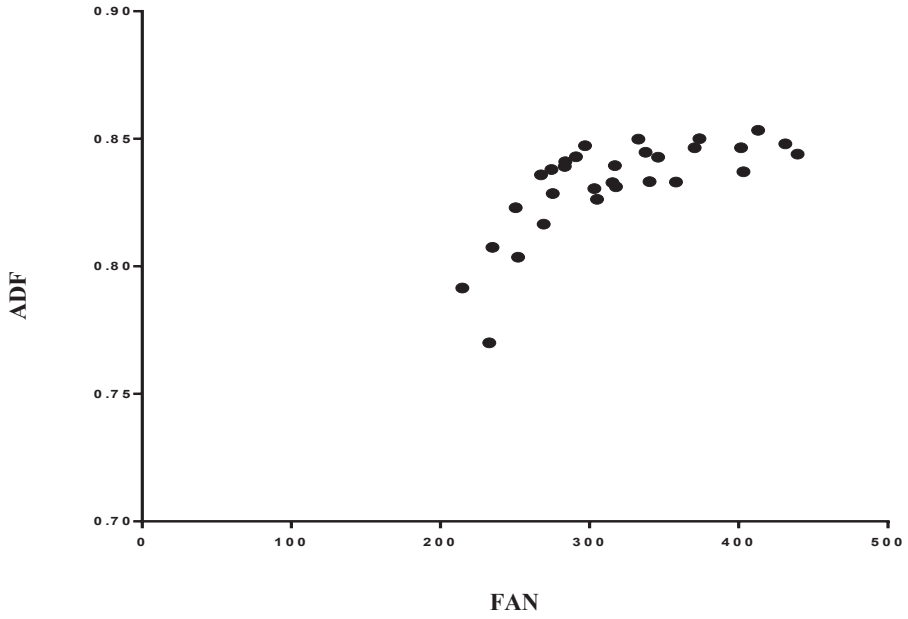

Figure 6.2 Correlation plot of apparent degree of fermentation (ADF) and FAN.

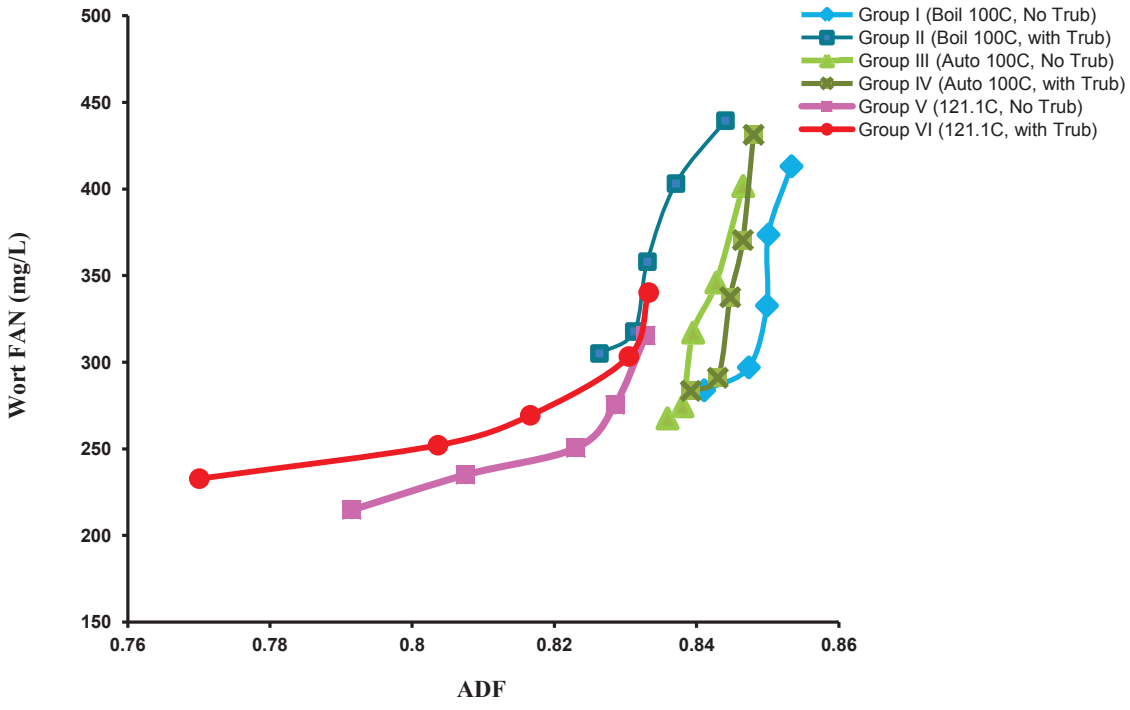

Figure 6.3 Initial wort FAN levels vs ADF for all treatment groups.

A significant and strong positive correlation was observed between apparent degree of fermentation and initial wort FAN levels ($r= 0.700$, $p<0.01$, Figure 6.2). This result indicated that fermentability reduces with decreasing wort FAN levels. The decline in fermentability was higher when initial wort FAN levels reach below 250 mg/l (Figure 6.3). In previous studies, apparent degree of fermentation of the wort containing 317 mg/L FAN was found to be 0.75, when fermented with an initial cell concentration of 6×10^6 cells/mL (48). Similar FAN levels were found in wort subjected to 90 min boiling (group I) but the degree of fermentation was found to be higher (around 0.83). This observation is probably due to the high initial pitching rate used in this study. FAN along with other factors such as initial pitching cell concentration, initial wort gravity, yeast growth rate, and vitality play a vital role in determining the wort fermentability. Fermentation time to 6°P for 90 min Boil wort was found to be 30 hr which was comparably less than previously reported for an all malt wort of 12°P (i.e. 51 hr for wort with initial FAN: 220 mg/L) (48). This suggests that a higher initial wort FAN level reduces fermentation time by correspondingly increasing rate of fermentation.

Wort treatment temperature also plays a crucial role in determining the loss in FAN. Wort autoclaved at 121.1°C and boiled at 100°C for 120 min resulted in initial FAN levels of 215 and 284 mg/L, respectively (Figure 5.20). A loss of 32% in FAN shows that at higher wort boil temperature, more amino nitrogen is lost because of increase in reaction rates. With low initial FAN levels, a decrease in rate of fermentation is obtained. However, the slow rate of fermentation in wort was not only due to inhibitory effects of low FAN levels, but also due to rapid sedimentation of yeast cells which resulted in insufficient cells in suspension to completely ferment wort. This sedimentation and less growth could be attributed to other factors such as low wort pH and high browning compound concentration which increase in wort boiling.

Under extreme wort treatment conditions (i.e., wort autoclaving at 121.1°C for 120 min), the FAN levels were found to be near growth limiting amounts. With initial FAN levels close to 214 mg/L in 14° P wort, yeast cells started to flocculate and settle out early

during fermentation. Interestingly, it was observed that early flocculation was triggered when the level of glucose in the medium is high, but nitrogen sources were low or could even have been depleted. Similar reports have been obtained from other researchers (57, 61, 62). This signifies that exhaustion of fermentable sugars is not necessary to initiate flocculation. These findings are in agreement with previous studies (57).

Initial FAN levels of 120 min boiled (at 100°C) and autoclaved (121.1°C) wort increased by 6.9% and 7.7%, when trub was present than wort without trub. This indicated that presence of trub results in an increase in the initial wort FAN. This increase can be due to the presence of bound trub proteins and amino groups, which are present but are not utilizable by the yeast. Therefore, an increase in trub bound amino nitrogen in the wort with increasing heat treatment durations did not show any increase in the rate of fermentation. Moreover, autoclaved wort (100°C and 121.1°C) containing trub showed higher FAN levels at all instances when compared to the wort autoclaved for the same temperature and duration but without trub.

From the wort FAN analysis, we can state that insufficient FAN levels have negative effect on yeast growth. Although, the rate of fermentation and yeast growth were not severely affected in miniature fermentations, certainly a trend of early flocculation and high final gravity was obtained with decreasing FAN. This suggests that with higher wort FAN concentration, the yeast growth rate certainly increases and leads to a better malt fermentability. Under nitrogen- limiting conditions, even slight increases in FAN levels can show a gradual increase in yeast cell growth and the rate of sugar consumption. Previous studies have shown that the supplementation of slow fermenting worts with FAN was found to considerably increase the speed of fermentation, yeast cell growth rate, and production of volatiles (21, 34, 45, 48).

6.2.2 Wort pH

Wort pH is controlled by numerous factors. Wort FAN and cation concentration are the major pH influencing factors. Wort pH declined as the wort FAN concentration reduced with increase in heat treatment durations and treatment temperature (Figure 5.22). This shows strong correlation ($r= 0.856$, $p<0.0001$) between the two quantities. FAN has been associated with buffering ability because of the presence of amino acids such as glutamate and aspartate. Loss of FAN results in low buffering ability and reduction in wort pH (70). Therefore, as the FAN concentration decreased prominently with increase in treatment duration from 30 to 120 min at 121.1°C, wort pH also declined gradually from 5.44 to 5.03 (±0.02).

Decline in the initial wort pH with sufficient FAN levels (>120 mg/L) will result in increased acidity during fermentation due to H^+ ion excretion by the yeast (organic acids) (4, 70). A pH of 4.5-4.0 is considered optimal for flocculation as it decreases the electrostatic repulsion between cells (29). The pH of wort with initially low FAN will show a quick reduction to flocculation promoting range during fermentation because of less buffering action. However the rate of decline in pH of wort with high initial FAN would be comparable to wort with fewer FAN`s, as high FAN will increase yeast growth. Therefore, the effect of pH on flocculation can be completely elucidated by measuring pH along the fermentation run. A rapid decline in initial wort pH along with low FAN can together contribute to the early onset of flocculation in runs with prolonged wort heat treatment at high temperature.

Wort pH also declines during boiling because of the reaction of Ca^{2+} (cation) with phosphates and polypeptides to form an insoluble complex compound releasing H^+ (56). A fall of 0.27 units and 0.4 units was obtained on increasing duration from 30 to 120 min of wort autoclaving at 100°C and 121.1°C, respectively. These findings are in agreement with previous studies (46). Wort pH was found strongly correlate with heating time

intervals ($r= -0.890$, $p<0.0001$). As wort pH is controlled by numerous factors, a decline suggest a cumulative effect of decreasing amino-acids, increase in acidic Maillard reaction products, loss of cations etc. with increasing heat treatment.

Under acidic conditions, a decline in pH increases the rate of formation of Maillard browning compounds and other volatiles (59). Although, wort pH is not considered as a significant factor, it certainly affects other factors and thus, can cause an early onset of yeast flocculation.

6.2.3 Maillard reaction

During nonenzymatic browning, reducing sugar molecules react with amino acids and form complex structures termed as melanoidins, which cannot be broken down or used up by yeast cells. The Maillard reaction during boiling has been found to involve 0.23-1.56 mM of amino acids and 5.3-7.2 mM of reducing sugars per liter (20). The Maillard browning reaction is complex and includes formation of several intermediate compounds responsible for beer color and flavor. A lowest wort color of 6° was obtained after a 30 min boil at 100°C (Figure 5.21). This result is in agreement with all-malt wort color as provided in previous literature (11).

It was found that wort color increases accordingly with increase in wort boiling temperature and periods (Figure 5.21). At high temperature, the wort color increases substantially with a slight increase in boiling duration due to increased rate of reaction. Maximum increase in wort color (198%) was observed with increase in autoclaving (121.1°C) period from 30 to 120 min. This can be attributed to the increased concentration of melanoidins and reaction intermediate compounds that cause increase in wort color. Oxidation of polyphenols during boiling is also responsible for color development (56). However, some reports have suggested that only about one-third or even less of melanoidins are formed during wort boiling while mostly are produced

during mashing and malting stages (18, 63). The wort FAN content showed a gradual decline with increase in wort color ($r= -0.722$, $p<0.01$) which is in agreement with previous findings (11).

A decrease in the reducing sugar content of wort after prolonged autoclaving at (121.1°C) is expected with increase in wort color and reduced FAN levels. However, the HPLC sugar profiling did not show any significant decline in wort fermentable sugar levels with increase in heat treatment at 121.1°C (Figures 5.18 and 5.19). This suggests that at higher temperature, the Maillard reaction could have also involved not only fermentable sugars but also various non-fermentable sugar fractions such as arabinose and xylans to react with amino-acids. Xylose has been reported previously as highly reactive saccharide in Maillard reaction systems than other fermentable sugar units (32). The concentration of each saccharide in the wort after heat treatment depends on multiple factors such as, the levels of individual saccharides after mashing, rate and extent of non-enzymatic browning, and participation level of individual saccharides in it. Therefore, it is essential to determine all sugar fractions present before and after the heat treatment to correctly establish their reactivity pattern.

Wort color showed high positive correlation with the area under density curve ($r= 0.898$, $p<0.0001$), while high negative correlation with the area under the turbidity curve ($r= -0.931$, $p<0.0001$). This suggests that with increasing wort heat treatment, both rate of fermentation and yeast in suspension decrease. Melanoidins, along with other Browning intermediate compounds are formed all along during wort boiling and are present in high concentrations in longer boils. Melanoidins have been associated with inhibitory effects on yeast growth (5, 11). Some of the examples of browning intermediate compounds are: 5-hydroxymethyl furfural, maltol, and furfural. They have been associated with affecting yeast growth by decreasing CO_2 production and evolution rate in concentrations as low as 1-5 mg/mL (68, 69). Studies showed that furfural in low concentrations inhibited yeast growth from 14-70% (5). Therefore, with longer wort heat treatment, Maillard reaction products might reach to harmful or toxic levels causing inhibition of yeast growth. Yeast

flocculation is positively correlated with mean shear rates in a fermenter (65). Carbon dioxide evolution is the sole source of shear in small scale fermentation, even a slight decline in CO_2 production caused by browning compounds, can be responsible for yeast settling and flocculation. Also, CO_2 evolution has been associated with increased yeast growth and vitality; therefore, browning compounds negatively affect the rate of alcohol production and wort fermentability.

Wort pH also plays a deciding role for the rate of Maillard reaction. Production of furfural increased 137% on decreasing wort pH from 5 to 4.5, signifying that a decline in pH will eventually result in increased inhibition of yeast activity (59). Browning compounds interfere with the metabolism of essential nutrients. Therefore, under nitrogen-limiting conditions (prolonged wort boiling), the inhibitory effect increases further compared to the condition wherein amino acids are present abundantly (5). Previous researchers have found that browning intermediate compounds in a concentration of less than 2mM affects cell glycolytic pathway by causing inhibition of alcohol dehydrogenase (activity <90%), aldehyde dehydrogenase, and pyruvate dehydrogenase (42). These compounds have also been reported to affect non-glycolytic enzymes and cause low cellular growth (42, 68, 69). Therefore, a cumulative effect of these factors together results in reduced cell growth, less sugar uptake, and decreased alcohol production in wort with a high concentration of Maillard compounds.

6.2.4 Trub

Trub comprises precipitated wort nitrogenous material and polyphenol compounds along with lipids and carbohydrates. Since hops were not added during the wort boil, all polyphenolic materials and lipids in the trub are derived from the barley grain. It has been previously suggested that only soluble and small protein fractions are present in the wort (47). However, an increase in the trub content was found with increasing wort boil temperature and durations (Figure 5.23) suggesting that more proteinaceous and

polyphenolic material are precipitating. This incurs a loss in wort peptide and amino acid levels as observed earlier with increasing wort heat treatment.

Although, the presence of trub showed an initial increase in yeast in suspension with increasing treatment durations, with wort boiled (100°C) and autoclaved runs (100°C), more intense cell settling was found at the end (Figures 5.8 and 5.10). Similar observations were made previously, where rate of ethanol production increased in wort containing 0.05% suspended solids during initial fermentation stages ($t<18$ hr), however, the final alcohol concentration at the end of fermentation was higher in worts free from solids (67). Previous studies also show that the presence of trub in the wort stimulates yeast growth and fermentation (30) while others show that high ion binding ability of the trub does not allow the bioavailability of nutrients to yeast cells, thereby decreasing fermentation rate (7, 18). Therefore, the contradictory effect of trub at different fermentation stages can be owing to its complex nature. It has also been reported that at the end of fermentation, trub has a positive zeta potential which would attract negatively charged yeast cells causing decline in electrostatic repulsion between cells (71). This would have promoted cell flocculation in runs containing trub.

Trub formation also causes loss of ionic compounds such as calcium, sodium and potassium from wort. A high Ca^{2+} concentration was found to induce flocculation and high Na^{2+} and K^{+} ions also cause efflux of Ca^{2+}, therefore, promoting flocculation (56). Significant loss of Ca^{2+} ions with trub formation and removal can be a factor contributing to delayed cell flocculation in runs without trub. A 50-60% loss of calcium ions has been reported after mashing and boiling procedures (56).

The presence of the trub during fermentations of autoclaved runs (100°C, 121.1°C) showed an overall decrease in the rate of fermentation and the yeast in suspension, suggesting negative effects on cell growth and sugar uptake (Figures 5.16 and 5.17). Certain trub components such as lipids have been associated with inhibiting nutrient

uptake by coating yeast cells (49). Since trub levels increase with longer boils, copious amount of suspended solids in wort can cause less nutrient uptake by decreasing effective surface absorption area and lipid coating of yeast cells. Also, it has been reported that longer boils encourage formation of polyphenol complexes with larger protein molecules. This suggests settling of denser and larger trub particles in test tubes under the influence of gravity. The settling solids can pull down suspended cells leading to decreased initial cell concentration. This explains initial decline in absorbance at t=6 hr in turbidity curves of autoclaved wort (121.1°C) fermentation runs containing trub, suggesting that trub particles previously interfering with UV absorption have settled down leaving comparably clear supernatant (Figure 5.12). In certain reports it has been found that settling of suspended wort solids can cause decreased yeast activity and can also lead to yeast autolysis (7, 39).

6.3 Volatiles

During pressure boiling of the wort, a number of volatile compounds are formed. Previous reports state that although, pressure boiling reduces the time and energy consumption, the undesirable volatiles formed is not steam volatized sufficiently (18). This results in high levels of volatile compounds which increase with increase in wort boiling. Fermentation of the boiled (100°C) and autoclaved wort (100°C) carried without the trub, showed comparable differences in the rates of fermentation and yeast in suspension when heated for the same period (Figures 5.16 and 5.17). This suggests that heat treating the wort under pressure causes changes in wort composition, which that in turn alters yeast growth. During autoclaving evaporation of volatiles takes place while during boiling a condensing unit was attached to limit evaporation and loss of volatiles. However, there is still a possibility that minimal evaporation can occur during boiling. Volatiles formed during boiling and that did not evaporate can be affecting yeast growth, resulting in differences in fermentation behaviors or even a different set of volatiles could have been formed during steam autoclaving.

A majority of volatiles that are formed during boiling have not been characterized to date. This suggests that a wide range of unknown volatile compounds persist and can affect fermentation performance. Moreover, the amount of volatiles formed during boiling increases significantly with a slight decline in wort pH (59). A variety of browning intermediate compounds formed during boiling are also volatiles (69).

CHAPTER 7 CONCLUSION AND FUTURE WORK

The effect of wort boil on fermentability was investigated by use of miniature fermentation method. Our studies showed that with increase in boil temperature from 100°C to 121.1°C, malt fermentability declined significantly (F-test, $p<0.05$). A decline in malt fermentability was accompanied by increasing unfermented extract (AE) present in the wort at the end of fermentation. These observations suggest that cell growth and sugar uptake ability gets compromised with increased wort boiling. Several biochemical and physical changes take place during boil which can directly affect wort fermentability or can also alter other related processes. The presence of suspended solids in wort also negatively affected yeast growth and fermentability. The increase in temperature and boil periods favor trub formation and cause decline in initial wort FAN and pH levels.

Trub was found to stimulate initial yeast growth in wort autoclaved at low temperature (at 100°C); however, when wort was autoclaved at 121.1°C for increasing time periods, trub negatively affected yeast growth and cell density. It is essential to establish the effect of trub particle size and sedimentation rate in test tube fermentation which can decrease its bio-availability. The measurement of shear rate all along fermentation will help to understand how variations in conditions cause onset of flocculation.

FAN requirements during fermentation depends on the amount of yeast growth required to achieve desired final beer characteristics such as the alcohol concentration, final wort gravity and flavor profiles in a given period of time (53). Therefore, for an efficient fermentation the optimum FAN required will be decided based on multiple factors. The assimilable nitrogen fraction in wort declined 6-7% as the boiling period was increased

from 30 to 120 min. The decline was further increased at high temperature to even growth limiting amounts.

Yeast cell density as well as growth was found to be highly dependent of wort FAN levels. Increasing wort FAN levels showed higher cell density and delayed flocculation. An early and intense flocculation was found with longer heat treated wort proposes a cumulative effect of increasing trub levels, low FAN, low pH and high Maillard browning compounds. On scale up of these fermentations the differences observed in yeast growth and fermentation would be more enhanced.

Previous studies show that flocculation is metabolically regulated in brewing yeasts; therefore, when supplied with necessary growth nutrients, flocculation gets reversed (62). The inhibition effects of nitrogen limitation on yeast growth can be further investigated with reference to their mode of action. By supplementing wort with calculated FAN amounts one can establish the necessary amount of FAN required obtaining optimum yeast activity after heat treating wort for specific periods. One can eliminate the nutrient deficiencies caused by boiling by assessing the fermentation performance. FAN limitation effects glycolytic flux decreasing rate of fermentation (45).

FAN affected fermentations have been previously stated to correspond to the time of depletion of valine amino acid from wort (53). Lysine has also been stated as key amino acid causing high speed fermentations completed in 48 h (35). Therefore, measuring the level of individual amino acids can help to better understand how FAN affects fermentation performance.

Significant differences were obtained between wort boiled at 100°C and autoclaved at 100°C (F-test, $p<0.05$) for all treatment periods. Pressure of 1 atm was applied to the closed system during autoclaving which could have resulted in loss of volatiles while during boiling a condensing unit was attached which did not allow release of volatiles. It

is believed that differences obtained between boiled and autoclaved wort runs carried at same temperature and duration was due to a chemical factor (yet to be determined) such as a volatile or due to differences in temperatures applied.

Increase in boiling period and temperature significantly increased the wort color resulting from high concentration of Maillard browning compounds (F-test, $p<0.05$). An increase in browning compounds was associated with decreased yeast growth and density. It is essential to quantify final browning compounds and intermediates to establish the concentrations that are tolerated by yeast cells to help better optimize wort boiling period.

HPLC analysis did not show any differences in fermentable sugar levels of wort autoclaved at 121.1°C for varying periods. Since boiling at high temperature increases the rate of sugar-amino acid reaction, therefore, sugar fractions were expected to show differences. This makes it essential to determine all sugar fractions present before and after heat treatment to correctly establish their reactivity pattern in Maillard reaction. It has been previously found that pH plays a major role in determining the rate of Maillard reaction (59). Further investigation is much required to determine the relative reactivity of sugars with corresponding amino acids at different wort pH levels.

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APPENDIX A

	45 Boil No Trub	60 Boil No Trub	90 Boil No Trub	120 Boil No Trub	Global (shared)
Comparison of Fits					
Null hypothesis					One curve for all data sets
Alternative hypothesis					Different curve for each data set
P value					0.0211
Conclusion (alpha = 0.05)					Reject null hypothesis
Preferred model					Different curve for each data set
F (DFn, DFd)					1.943 (16,140)
Different curve for each data set					
Best-fit values					
Pe	2.228	2.220	2.252	2.352	
P0	17.16	17.36	17.47	17.43	
B	0.1007	0.09659	0.09427	0.09474	
M	18.81	18.70	18.70	18.80	
Std. Error					
Pe	0.07138	0.07487	0.08241	0.08304	
P0	0.4693	0.4976	0.5453	0.5433	
B	0.005663	0.005439	0.005679	0.005801	
M	0.8238	0.8743	0.9621	0.9658	
95% Confidence Intervals					
Pe	2.082 to 2.374	2.067 to 2.373	2.084 to 2.421	to 2.182 to 2.522	
P0	16.20 to 18.13	16.34 to 18.38	16.36 to 18.59	to 16.31 to 18.54	
B	0.08912 to 0.1123	0.08545 to 0.1077	0.08264 to 0.1059	to 0.08286 to 0.1066	
M	17.12 to 20.50	16.91 to 20.49	16.73 to 20.67	to 16.82 to 20.77	
Goodness of Fit					
Degrees of Freedom	28	28	28	28	
R square	0.9980	0.9980	0.9977	0.9976	
Absolute Sum of Squares	1.161	1.177	1.353	1.384	
Sy.x	0.2036	0.2050	0.2199	0.2223	

Figure A.1 An example of statistical output of nonlinear logistic modeling of decline in apparent extract value collected from fermentation of boiled wort (at 100°C) runs without trub from Prism software package.

	45 min Boil, No trub	60 min Boil, No trub	90 min Boil, No trub	120 min Boil, No trub	Global (shared)
Comparison of Fits					
Null hypothesis					One curve for all data sets
Alternative hypothesis					Different curve for each data set
P value					< 0.0001
Conclusion (alpha = 0.05)					Reject null hypothesis
Preferred model					Different curve for each data set
F (DFn, DFd)					6.581 (16,135)
Different curve for each data set					
Best-fit values					
Amplitude	4.169	4.206	4.150	4.091	
Mean	27.25	28.02	28.04	28.75	
SD	33.98	34.15	31.77	31.02	
r	0.01186	0.009246	0.01103	0.009877	
Std. Error					
Amplitude	0.07219	0.07219	0.06221	0.06376	
Mean	1.230	1.195	0.9658	0.9559	
SD	1.474	1.368	1.165	1.131	
r	0.003069	0.002971	0.002506	0.002475	
95% Confidence Intervals					
Amplitude	4.021 to 4.317	4.058 to 4.355	4.023 to 4.278	3.960 to 4.222	
Mean	24.72 to 29.77	25.57 to 30.47	26.05 to 30.02	26.79 to 30.72	
SD	30.95 to 37.00	31.34 to 36.96	29.38 to 34.16	28.70 to 33.34	
r	0.005566 to 0.01816	0.003150 to 0.01534	0.005887 to 0.01617	0.004798 to 0.01496	
Goodness of Fit					
Degrees of Freedom	27	27	27	27	
R square	0.9776	0.9822	0.9825	0.9822	
Absolute Sum of Squares	0.4502	0.3866	0.4150	0.4376	
Sy.x	0.1291	0.1197	0.1240	0.1273	
Constraints					
Amplitude	Amplitude < 6.000	Amplitude < 6.000	Amplitude < 6.000	Amplitude < 6.000	
Mean	Mean < 60.00	Mean < 60.00	Mean < 60.00	Mean < 60.00	

Figure A.2 Example of statistical output of non-linear modeling of yeast in suspension data collected from fermentation of boiled wort (at 100°C) runs without trub from Prism software package.

APPENDIX B

Table B.1 Correlation Coefficient - Pearson correlation matrix (df=190, P= 0.000)

	BOILTIME	TRUB	HEATTYPE	AMPLITUDE	μ
BOILTIME	1.000				
TRUB	0.000	1.000			
HEATTYPE	0.000	0.000	1.000		
AMPLITUDE	-0.381	-0.221	-0.765	1.000	
μ	-0.018	-0.104	-0.309	0.455	1.000
σ	-0.154	-0.572	-0.511	0.645	0.336
r	-0.184	0.188	-0.629	0.238	0.058
TOTALHEIGHT	-0.348	-0.065	-0.876	0.742	0.299
AREATURBID	-0.331	-0.139	-0.832	0.710	0.325
P_e	0.535	0.057	0.522	-0.630	-0.318
P_i	0.022	0.148	0.524	-0.261	-0.269
B	-0.099	-0.194	-0.617	0.375	0.262
M	0.090	0.273	0.166	-0.343	-0.037
OE	0.225	0.067	0.678	-0.533	-0.338
AE	0.486	0.147	0.628	-0.676	-0.403
ADF	-0.489	-0.147	-0.615	0.670	0.398
AREADENSITY	0.296	0.272	0.785	-0.682	-0.409
FAN	-0.725	0.188	-0.572	0.603	0.151
PH	-0.890	-0.051	-0.325	0.558	0.210
COLOR	0.424	0.000	0.702	-0.649	-0.339

	σ	r	TOTALHEIGHT	AREATURBID	P_e
σ	1.000				
r	0.056	1.000			
TOTALHEIGHT	0.506	0.816	1.000		
AREATURBID	0.610	0.784	0.978	1.000	
P_e	-0.617	-0.486	-0.737	-0.809	1.000
P_i	-0.167	-0.694	-0.629	-0.589	0.121
B	0.220	0.785	0.756	0.714	-0.265
M	-0.288	0.119	-0.123	-0.158	0.349
OE	-0.363	-0.682	-0.786	-0.746	0.407
AE	-0.650	-0.610	-0.848	-0.912	0.968
ADF	0.650	0.596	0.835	0.903	-0.974
AREADENSITY	-0.595	-0.768	-0.948	-0.972	0.761
FAN	0.306	0.636	0.788	0.746	-0.678
PH	0.351	0.480	0.664	0.672	-0.787
COLOR	-0.491	-0.740	-0.899	-0.931	0.910

	P_i	B	M	OE	AE
P_i	1.000				
B	-0.968	1.000			
M	-0.540	0.342	1.000		
OE	0.903	-0.900	-0.444	1.000	
AE	0.347	-0.483	0.259	0.577	1.000
ADF	-0.317	0.456	-0.282	-0.550	-0.999
AREADENSITY	0.645	-0.777	0.178	0.749	0.893
FAN	-0.438	0.511	0.074	-0.684	-0.711
PH	-0.232	0.356	-0.183	-0.456	-0.786
COLOR	0.358	-0.519	0.317	0.560	0.949

	ADF	AREADENSITY	FAN	PH	COLOR
ADF	1.000				
AREADENSITY	-0.882	1.000			

FAN	0.700	-0.658	1.000		
PH	0.786	-0.648	0.856	1.000	
COLOR	-0.948	0.898	-0.722	-0.746	1.000

Table B.2 Matrix of bonferroni Probabilities (Number of observations: 30)

	BOILTIME	TRUB	HEATTYPE	AMPLITUDE	μ
BOILTIME	0.000				
TRUB	1.000	0.000			
HEATTYPE	1.000	1.000	0.000		
AMPLITUDE	1.000	1.000	0.000	0.000	
μ	1.000	1.000	1.000	1.000	0.000
σ	1.000	0.181	0.743	0.022	1.000
r	1.000	1.000	0.038	1.000	1.000
TOTALHEIGHT	1.000	1.000	0.000	0.001	1.000
AREATURBID	1.000	1.000	0.000	0.002	1.000
P_e	0.442	1.000	0.589	0.036	1.000
P_i	1.000	1.000	0.560	1.000	1.000
B	1.000	1.000	0.053	1.000	1.000
M	1.000	1.000	1.000	1.000	1.000
OE	1.000	1.000	0.007	0.460	1.000
AE	1.000	1.000	0.038	0.008	1.000
ADF	1.000	1.000	0.056	0.010	1.000
AREADENSITY	1.000	1.000	0.000	0.006	1.000
FAN	0.001	1.000	0.181	0.080	1.000
pH	0.000	1.000	1.000	0.256	1.000
COLOR	1.000	1.000	0.003	0.020	1.000

	σ	r	TOTALHEIGHT	AREATURBID	P_e
σ	0.000				
r	1.000	0.000			
TOTALHEIGHT	0.823	0.000	0.000		
AREATURBID	0.065	0.000	0.000	0.000	
PE	0.054	1.000	0.001	0.000	0.000
P_i	1.000	0.004	0.037	0.117	1.000
B	1.000	0.000	0.000	0.002	1.000
M	1.000	1.000	1.000	1.000	1.000
OE	1.000	0.006	0.000	0.000	1.000
AE	0.019	0.066	0.000	0.000	0.000
ADF	0.019	0.097	0.000	0.000	0.000
AREADENSITY	0.099	0.000	0.000	0.000	0.000
FAN	1.000	0.030	0.000	0.000	0.007
pH	1.000	1.000	0.012	0.009	0.000
COLOR	1.000	0.001	0.000	0.000	0.000

	P_i	B	M	OE	AE
P_i	0.000				
B	0.000	0.000			
M	0.393	1.000	0.000		
OE	0.000	0.000	1.000	0.000	
AE	1.000	1.000	1.000	0.160	0.000
ADF	1.000	1.000	1.000	0.312	0.000
AREADENSITY	0.022	0.000	1.000	0.000	0.000
FAN	1.000	0.737	1.000	0.006	0.002
pH	1.000	1.000	1.000	1.000	0.000
COLOR	1.000	0.627	1.000	0.245	0.000

	ADF	AREADENSITY	FAN	pH	COLOR
ADF	0.000				
AREADENSITY	0.000	0.000			
FAN	0.003	0.015	0.000		
pH	0.000	0.021	0.000	0.000	
COLOR	0.000	0.000	0.001	0.000	0.000

APPENDIX C

Table C.1 Malt quality analysis of a 'Control malt'.

ID Lab No.	Control ML-11-08	PYF ML-11-09
MALT		
Moisture, %	5.1	6.9
Protein, %	12.2	13.0
Friability, %	75	74.8
WORT		
Fine Grind Extract, %	80.5	81.0
Soluble Protein, %	5.42	6.32
Ratio S/T, %	44.6	48.7
ß-Glucan, mg/L	194	118
Viscosity, cps	1.47	1.43
Colour, °	2.03	2.51
Diastatic Power, °L	129	152
Alpha-Amylase, D.U.	52.4	67.1
FAN, mg/L	170	196

**Results reported on a dry weight basis