DECLARATION

I declare that this submission is my own, unaided work, both in conception and execution, and that apart from the normal guidance of my supervisor, I have received no assistance except for the information obtained from literature sources. All sources of information have been adequately acknowledged and referenced.

Neither the substance nor any part of the thesis has been submitted in the past, or is being, or is to be submitted for a degree in the University or any other University. I hereby grant the University free license to reproduce the above thesis in whole or in part, for the purpose of research.

I now present the thesis for examination for the degree of Masters of Science.

Name: Gail Lorato Kgari

February 2008
Cape Town

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ABSTRACT

In the brewery, yeast cropped on completion of the fermentation is used to pitch subsequent fermentations. Proper handling of yeast within the brewery yeast circuit is thus crucial for production of quality beer. During handling, yeast is exposed to physiological and mechanical stresses, including cold stress. In this study, the effect of cold stress at varying ethanol concentrations was investigated while exposing yeast to shear. The main objective of this study was to understand the effect of cooling rates on brewer's yeast quality. The following hypotheses were investigated:

(1) Yeast cooled at a low cooling rate will yield better fermentation performance than yeast cooled at rates closer to the critical cooling rate, previously proposed as $2^{o}C.s^{-1}$ (Nkosi, 2001).

(2) Lower cell viability, cell growth and fermentation performance are expected on yeast cooled in the presence of beer, compared to that cooled in a suspension where the beer is diluted.

(3) Flexibility of the cell envelope increases with an increase in cooling or heating rate.

(4) Yeast structural strength is indirectly proportional to the cooling rate beyond a critical rate.

(5) Haze generation is aggravated by higher cooling rates.

From the findings, it can be concluded that cooling rate is directly proportional to the flow rate. Moreover, there was no significant difference between cooling rates obtained with the type of diluent used. When both the linear velocity and the yeast consistency were varied, the cooling rate decreased with increasing yeast consistency due to a decrease in fluid velocity and an increase in fluid viscosity.

A base case small-scale fermentation was demonstrated using the control yeast with 58 % consistency. At the end of the small-scale fermentation, the final cell concentration was 4 times the starting cell concentration. The method used to quantify carbon dioxide evolved due to maintenance was not sufficiently sensitive.

The cooling rates obtained ranged between 0.1 and $2.15^{o}C.s^{-1}$. The extent of growth in small-scale fermentation following cooling was less than that of the control, but consistency did not have a dominant effect except when the yeast consistency was 30 %. The final cell concentration obtained at 40 % yeast consistency was approximately equal to that at 60 % consistency.

At all yeast consistencies, the final cell concentrations of control yeast suspensions were greater than the final cell concentration of cold stressed yeast. Cell vitality was not sensitive to varying yeast consistency.

The ethanol concentrations across all samples ranged between 2.0 % (v/v) and 8.0 % (v/v). Irrespective of the level of ethanol concentration, the final cell concentration in the small-scale fermentation following inoculation with control and cooled yeast were approximately the same. For all diluents used, there was no significant difference in the amount of carbon dioxide evolved. Further, cell vitality was not sensitive to varying cooling rate and yeast consistency. It can be concluded that when yeast is suspended in alcohol pre-inoculation), the yeast performance is not compromised at low alcohol concentrations (below 10 % v/v).

Different degrees of cell flexibility were observed upon injecting yeast slurries into media of different osmotic potential (hypotonic and hypertonic solutions). The temperature of the Malvern diluent was shown to play a major role in the estimation of the cell envelope flexibility. On examining the effect of the difference in the temperatures of the yeast sample and the Malvern diluent on the measured flexibility of yeast, it was shown that it is crucial not to introduce a temperature shock during the assay when determining the relation between the yeast sample and cell envelope flexibility. However, for cooled yeast sample, the diluent temperature could not meaningfully be maintained at the same temperature as the sample because condensation on the lens reduced the accuracy of data.

For both heated and cooled yeast, rate and extent of change in size increased as heating or cooling rate to which yeast was exposed increased. The nature of the yeast suspension on cell envelope flexibility was observed when using PBS.

Fragility test results showed that yeast cooled at higher cooling rates was most susceptible to breakage using the French Press. The increased fragility of the cells when cooled at higher cooling rates compared to slower cooling rates may be attributed to either a higher degree of shear stress or rate of temperature drop during yeast cooling.

The average particle size of haze particles was also found to be in a range of 0.05–2 µm. More fine haze material was generated on rapid cooling. An increase in consistency of the yeast suspension cooled increased haze generation although the cooling rate decreased.

GLOSSARY

Consistency	A measure of yeast biomass concentration as percent wet weight
Cropping	The transfer of yeast from the fermentation vessel to the yeast collection vessel for re-inoculation into subsequent fermentation
Fermentation	The anaerobic process by which yeast cells convert sugars to carbon dioxide and ethanol
Generation number	Indicator of the number of times a batch of yeast has been cropped and re-used for fermentation of wort
Knock-out strain	yeast with specific gene e.g. HSP12 gene deactivated
Structural integrity	The intactness of the cell envelope
Physiological state	Yeast quality in terms of functional integrity
Pitching	Inoculation of fermentation
Propagation	The step-wise aerobic generation of biomass from laboratory to plant scale to obtain sufficient biomass to catalyse the production fermentation
Replicative deactivation	Loss of the ability of yeast to reproduce
Viability	A term used to describe the ability of cells to grow and reproduce; losses of viability may occur via loss of structural integrity, cell death or replicative deactivation
Vitality	A term used to describe the metabolic activity of yeast; in some cases, overall fermentation capacity may be implied
Wort	A cereal-based extract containing a range of carbohydrates, which are converted to ethanol, carbon dioxide and other organoleptic compounds during beer production
Wild type strain	Yeast in which the natural genetic complement has not been altered
Yeast quality	The physiological condition of the yeast in terms of its fermentative capacity and the quality of the beer produced

NOMENCLATURE

GREEK SYMBOLS

φ	Chemical potential
π	Osmotic pressure (kPa)
μ	Specific growth rate (h^{-1})

GENERAL SYMBOLS

a_w	Free water activity
con	Control yeast
C	Consistency (%)
f_m	Particle size distribution by mass (%)
h_s	Hypertonic salt solution ($mg.ml^{-1}$)
k_3	Scaling factor used to normalise the area under the graph to 100 %
L_p	Hydraulic permeability
P	Pitching rate ($g.100ml^{-1}$)
m_s	Medium strength salt solution ($mg.ml^{-1}$)
N	Cell number ($cells.ml^{-1}$)
X	Cell concentration ($cells.ml^{-1}$)
T_m	Phospholipid transition temperature (^{o}C)
v	Viability of yeast (%)
w_1	Mass of the Eppendorf tube (g)
w_2	Combined mass of the Eppendorf tube and the centrifuged yeast suspension (g)
w_3	Combined mass of the Eppendorf tube and the wet yeast pellet (g)
x	Particle diameter (μm)

SUBSCRIPTS

f	Final
i	Initial
max	Maximum
s	Salt solution
280 nm	Wavelength

ABBREVIATIONS

A	Absorbance
ADP	Adenosine Diphosphate
ATP	Adenoside Triphosphate
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
C	Consistency
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicyclic Acid
FV	Fermentation Vessel
GSH	Glutathione gene
HSP	Heat Shock Protein
OH	Hydroxide molecule
M	Mass particle distribution
PBS	Phosphate Buffered Saline solution
P	Pitching rate
PAGE	Polyacrylamide Gel Electrophoresis
ROS	Reactive oxygen species
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
SAB	South African Breweries
SOD	Superoxidase dismutase
T	Temperature
U	Uncooled sample, maintained at 14^{o}C
UCT	University of Cape Town
V	Viability
v/v	Volume of sample measured per total volume

W	Water
w/v	Weight of sample measured per total volume
YCV	Yeast Collection Vessel
YPD	Yeast, Peptone and Dextrose media
YPV	Yeast Pitching Vessel

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Yeast finds application in many bioprocesses including the production of pharmaceuticals, food and beverages (Madigan *et al*., 2000). The yeast *Saccharomyces cerevisiae* is commonly used in one of the key reaction stages of brewing known as fermentation. During fermentation, yeast carries out the anaerobic conversion of sugar to ethanol, carbon dioxide and several key compounds required for final beer flavour (Trelea *et al*., 2004; Basson, 1996; Shuler and Kargi, 1992).

Subsequent to the fermentation process on depletion of maltose, the yeast flocculates and settles at the base of the cylindroconical vessel. This yeast may be transferred from the fermentation vessel (FV) to the yeast collection vessel (YCV) for further repitching into subsequent fermentations. This collection of yeast is known as cropping. Typically brewers' yeast is re-used through 6 to 8 successive fermentations or "generations", hence, handling yeast within the brewery yeast circuit forms one of the critical aspects of brewing. Improperly handled yeast with compromised yeast quality may lead to hanging fermentations, poor attenuation and an altered beer profile (McCaig and Bendiak, 1985). One such component of yeast handling includes its thermal treatment through both cooling and heating phases, the subject of this study.

1.2 THE BREWING PROCESS

Yeast cultured in the laboratory is scaled to provide the inoculum for plant production through aerobic propagation. Thereafter, the yeast biomass is added to the cooled, sterile wort, saturated with oxygen, before entering the fermentation vessel (FV) where fermentation commences. Upon depletion of maltose in the wort towards the end of the lager fermentation process, the yeast flocculates and settles at the bottom of the cylindroconical fermentation vessel. The early flocculated yeast from the bottom of the FV is discarded (Hofmann, 2004) and, depending on the quality of yeast and the demand for more yeast, the remainder is collected in the YCV for repitching, as shown in Figure 1.1. The ability of yeast to flocculate is closely related to its cell wall and cell membrane structures as well as maltose availability (Stewart *et al*., 1973; Robinson, 2001). Subsequent to cropping yeast, the remaining beer is pumped from the FV to the storage vessels (SV) prior to the filtration process. The filtration process removes trace yeast from the beer.

It is common practice to cool the cropped yeast from the FV temperature of about 14^{o}C to approximately 2^{o}C to reduce its metabolic activity during storage in the YCV. Yeast is typically cooled using a heat exchanger (such as the plate and frame heat exchanger) or by agitation in a chilled jacketed vessel. It is temporarily stored in the yeast collection vessel (YCV). The storage phase allows the brewer time to detect, eliminate and control microbial contamination of yeast. Should there be contamination, the yeast may be acid washed with phosphoric acid (H_3PO_4) to eliminate bacteria prior to re-pitching. In addition, the storage phase also eases the scheduling time between cropping and wort availability and gives the brewer an opportunity to determine the quantity of yeast suspension to be pitched into the subsequent fermentation to achieve a pre-determined combination of concentration and viability.

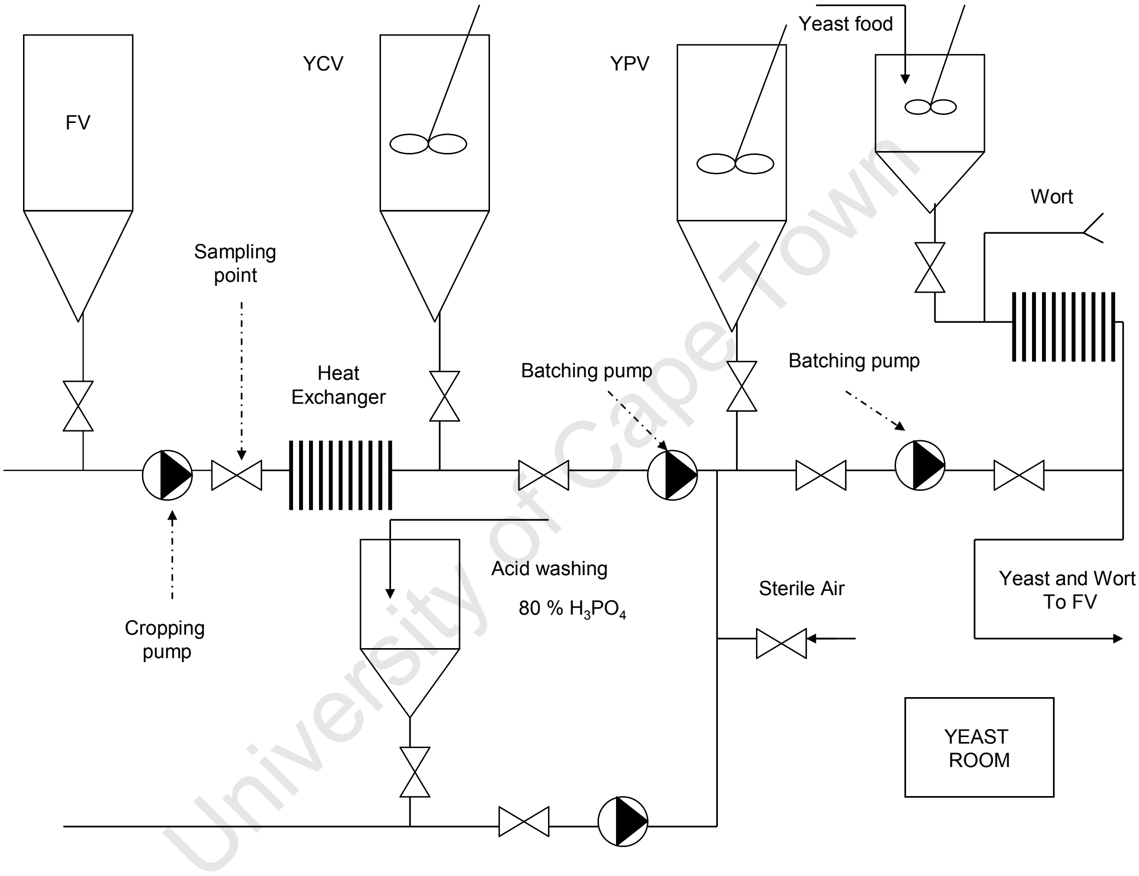


Figure 1-1: Schematic diagram of the yeast handling circuit as obtained from Basson (1996)

1.3 YEAST STRESS RESPONSE

Stress responses in brewer’s yeast have been observed during yeast handling. The understanding of these stresses is essential to ensure a yeast inoculum of appropriate quality while minimizing negative impacts on the beer during beer recovery. These stresses are classified as physiological or mechanical stress. Physiological stresses comprise osmotic stress, oxidative stress, nutrient limitation stress, ethanol stress and thermal stress. Brewers yeast can experience mechanical stress either in the form of hydrodynamic shear caused by agitation and flow or as hydrodynamic cavitation caused on flow restrictions such as valves, bends, pipe and fittings (Basson, 1996).

Following fermentation, yeast may remain as trace yeast in beer or be removed as cropped yeast to the yeast collection vessel for re-use. Damage of either the trace yeast or the cropped yeast can affect the quality of the final beer. On handling the cropped yeast, pumping it between the FV and the YCV renders yeast susceptible to mechanical damage (Figure 1-1) due to shear created in pumping through pipe lines or agitation inside the YCV. Additionally, yeast might also undergo physiological stress due to cold stress in the heat exchanger, nutrient starvation and ethanol stress inside the YCV. If sufficient quality of the cropped yeast is not maintained, it is discarded. Pumping the beer from the FV to the SV for lagering places trace yeast under hydrodynamic stress due to pumping of yeast through heat exchangers, exposure to cavitation (Balasundaram and Harrison, 2006) and trace yeast removal by high speed centrifugation (Robinson *et al*, 2001). Damaged yeast cell walls may result in the release of intracellular compounds into beer. This may lead to the production of off-flavours (McCaig and Bendiak, 1985) and haze formation (O'Connor-Cox, 1994), resulting in reduced beer quality and shortened shelf life.

This study aims to investigate the effect of cold stress on brewer's yeast quality. The study investigates cooling rates typical of the heat exchanger between the FV and the YCV and determines the cooling rate that has the least detrimental effect on the yeast physiology, such that subsequent fermentations are not adversely affected. Further, it investigates the combined effect of cold shock, hydrodynamic stress and ethanol stress.

1.4 FACTORS AFFECTING THE RESPONSE OF YEAST TOWARDS COLD STRESS

In order to understand the response of yeast to cold stress within the heat exchanger, it is vital to understand the numerous factors contributing to yeast quality at low temperatures. Many studies have attributed the enhanced survival of yeast cells when subjected to low temperature to cryoprotectants present inside the cell (Kandror *et al*., 2004; Nkosi, 2001; Komatsu *et al*., 1990). Examples of cryoprotectants that have the potential to be induced by cold shock in yeast are trehalose and heat shock proteins (HSP).

In this study, the effect of HSP12 on yeast recovery subsequent to cold shock is explored further. The heat shock response was the first stress response observed and was found to be triggered when the cells were exposed to stress resulting from increased temperatures (Sales *et al*, 2000; Kandror *et al*, 2004). Heat shock proteins (HSPs) are largely molecular chaperones (Mager and Varela, 1993; Sales *et al*., 2000) for protein molecules and are named according to their molecular weights. Previously, the heat shock response has been shown to be induced by a variety of stress inducers, including high ethanol concentration (Birch and Walker, 2000), low temperature (Praekelt and Meacock, 1990; Nkosi, 2001; Kandror *et al.,* 2004; Palhano *et al.,* 2004) and high pressure (Fernades *et al*., 2004).

Research has shown that HSP12 can protect the membrane against desiccation and ethanol-induced stress (Sales *et al*., 2000), enhance cell viability during subsequent rapid cooling (Komatsu *et al*., 1990) and protect yeast against low temperature and freezing (Kandror *et al*., 2004).

1.5 STRUCTURE OF THE THESIS

In this thesis, Chapter 2 introduces the theoretical framework of yeast cell morphology, including the cell wall, the periplasm and the cell membrane in Section 2.1. The primary carbohydrates found in yeast such as glucan, mannan, glycogen and trehalose are discussed. The description of the yeast cell is given in Section 2.2. The storage carbohydrates of yeast are outlined in Section 2.3. Possible stresses experienced by Brewers' yeast throughout the Brewer's yeast circuit are reviewed (Section 2.4). Special emphasis is placed on cold stress and the mechanisms of yeast protection from such stress are discussed. Loss of yeast quality is classified and mechanisms of protection discussed. Lastly, in Sections 2.6, the research problem, objectives and hypotheses to be tested are given.

In Chapter 3, various physiological states that affect yeast response towards the stress are presented. The analytical techniques used to assess these states and experimental approach and plan used in this study to identify and characterize loss of yeast quality are provided and their selection motivated.

In Chapter 4, the cooling rate of yeast is related to its fermentation performance. The effects of thermal stress (heating and cooling) were investigated in terms of yeast quality and subsequent performance in small-scale fermentation. Furthermore, the effect of biomass consistency on the cooling rate was illustrated. Biomass concentration was studied at 30 %, 40 % and 60 % consistency (wet weight). The effect of biomass consistency of the cold stressed yeast on fermentation performance was also investigated across the range of yeast consistency from 30 % to 60 % and ethanol (beer supernatant) concentrations of 8 % (v/v) to 2 % (v/v).

In Chapter 5, cell wall flexibility was related to the rate at which yeast was heated and cooled. Moreover, the effect of the temperature gradient between the injected yeast sample and the Malvern diluent was investigated. Cell wall flexibility was observed both in terms of the rate of change of cell size when placed in water and the extent to which the yeast cell can stretch or contract when injected into different osmotic media.

In Chapter 6, the investigation of the effects of cooling rate on the structural strength of brewers yeast was observed through both the quantity of haze formed in the beer and the fragility of the cell envelope on exposure to sublytic pressures. Furthermore, the amount of protein released on fragility testing of cooled yeast was investigated.

In Chapter 7, an overall discussion of the results reported in Chapters 4, 5 and 6 is integrated with the literature reviewed to enable critical analysis. In Chapter 8, the conclusions are drawn based on the findings of this study and recommendations made.

CHAPTER 2: THEORETICAL FRAMEWORK

2.1 INTRODUCTION

In this chapter, the yeast cell structure is described. The four major carbohydrates found in yeast, including glycogen and trehalose are also described. An illustration of the key roles played by each carbohydrate is given. Thereafter, the various types of stress to which yeast is exposed in Brewers' yeast circuit are briefly discussed. The stresses are divided into physiological and mechanical stress. The chapter particularly emphasizes the effect of cold stress on the quality of Brewers' yeast. The response of yeast to cold stress is discussed in terms of the fermentation performance and the molecular level. A brief summary of how trehalose, HSP and ethanol protect yeast against cold stress is given. Lastly, the findings from the reviewed literature are used to derive the key questions and the hypotheses of the thesis are presented.

2.2 THE YEAST CELL

The yeast *S. cerevisiae* belongs to a class of unicellular microorganisms known as eukaryotes. The cell contents are protected from the external environment by the cell envelope comprised of a rigid cell wall, cell membrane and periplasm. Mature *S. cerevisiae* cells are generally ellipsoidal in shape, with a diameter of 5 to 10 µm. During cell multiplication, the yeast cell looses its ellipsoidal shape to accommodate the bud attached to the mother cell. The cell wall and membrane act as a first barrier against shear (Prokop and Bajpaj, 1992). The outer and the inner layers of the yeast cell wall are covered with mannoproteins and ß-glucans respectively (Klis, 1994) as illustrated in Figure 2-1. The periplasmic space mainly contains secreted proteins that are unable to penetrate the cell wall, but play a vital role in hydrolyzing substrates that cannot permeate through the plasma membrane into the cell. Periplasmic proteins include hydrolytic enzymes, binding proteins and chemo-receptors involved in cell signalling (Madigan et al., 2000). Whenever a receptor protein receives the signal from the external environment, the signal is transferred into a new intracellular signal (Alberts *et al*., 1998). However, not all signals can penetrate the plasma membrane, especially large hydrophilic signals.

The cytoplasmic membrane is internal to the cell wall of eukaryotic cells. It serves as a selective barrier and surrounds the cell completely. It contains highly selective channels and protein pumps to control in and outflow of specific substances to the cytoplasm (Alberts *et al*., 1998). Since the plasma membrane is the site of the primary contact of the cell with its surrounding environment as well as the primary biological boundary, its flexibility and adaptation capacity determine the survival of the cell. The membrane acts as a principal interface between the extracellular environment and the cytosol (Steponkus and Lynch, 1989 as cited in Sales *et al*., 2000) and is susceptible to dehydration damage (Steponkus and Webb, 1992 as cited in Sales *et al*., 2000). The cytoplasmic membrane is generally made up of lipid bilayer with embedded proteins (Williams, 1990; Alberts et al., 1998). The major lipid components, phospholipids, consist of a hydrophilic head and hydrophobic tail (Alberts et al., 1998). The phospholipid bilayer of biological membranes is maintained in a fluid lamella, liquid–crystalline phase at physiological temperatures and levels of hydration (Figure 2-2). The lipid bilayer serves as a permeability barrier, separating the cell contents and the surrounding medium (Alberts et al., 1998). The cytoplasmic membrane is not a rigid structure since the lipids and proteins are in constant motion, known as lateral diffusion. The three types of movement possible for phospholipid molecules in a lipid bi-layer are rotation, lateral diffusion and flip-flop (Alberts *et al.,* 1998). The phospholipid membrane may show phase transition (e.g. liquid to gel) on change in temperature. The phospholipid transition temperature (T_m) varies with membrane composition and with osmotic pressure of the surrounding medium. Membrane phase transitions were shown to occur at higher temperatures for dehydrated cells compared to hydrated cells. For example, the transition temperatures of the *E. coli* membrane were 50°C and 10°C for freeze-dried and rehydrated cells respectively (Leslie *et al*., 1995).

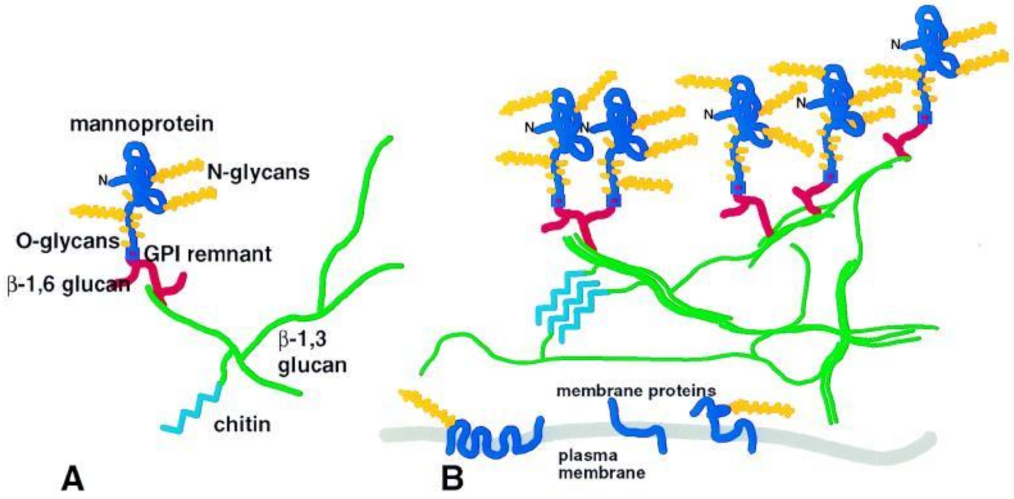


Figure 2-1: Relationships among components of *S. cerevisiae* cell walls. (A) Prototypical module with components individually labelled and coloured. The mannoprotein polypeptide is blue, and oligosaccharides are shown in yellow, labelled as N or O linked. Only a few of the of the glucans are shown. Chitin can also be linked to the β-1, 6 glucans. (B) Association of modules to form a wall lattice. Colours are as in panel A. The β-1, 3 glucans chains are intertwined to designate triple helices, and chitin is shown as a crystalline micro-domain. Cross-linking of mannoproteins through disulfide and other bonds is not depicted Lipke and Ovalle, 1998; Smits et al., 1998).

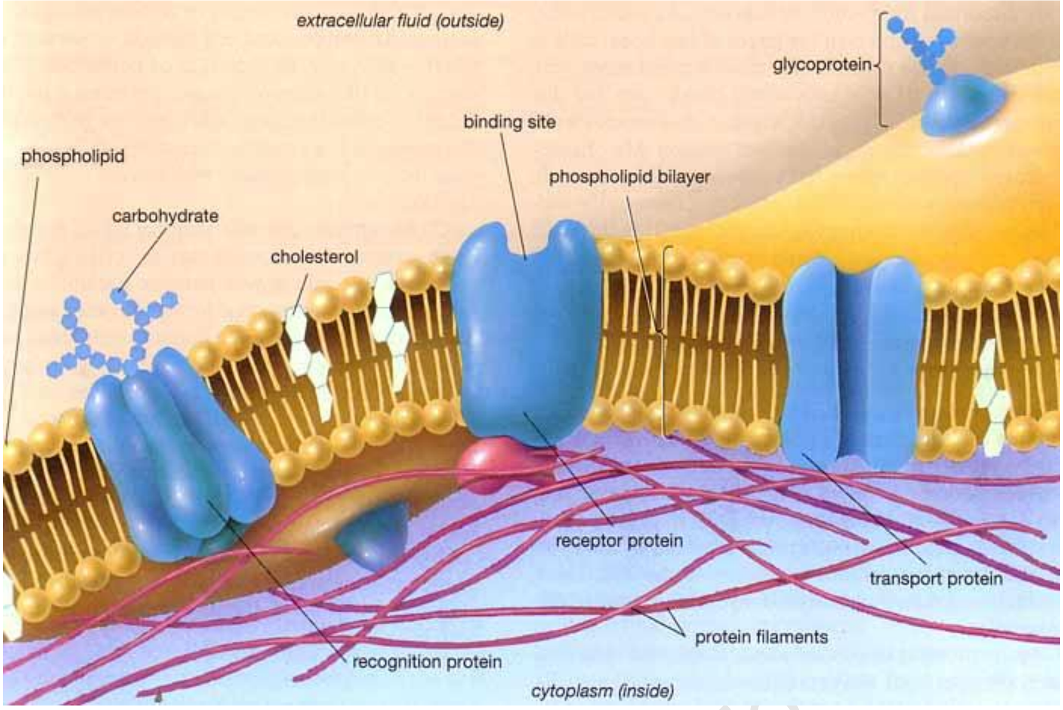


Figure 2-2: The cytoplasmic membrane of yeast showing a phospholipid bilayer of a eukaryotic cell membrane (Audesirk and Audesirk, 1999). Integral membrane proteins embedded in the cytoplasmic membrane cross both ends of the membrane.

2.3 STORAGE CARBOHYDRATES OF YEAST

The storage (energy reserves) carbohydrates found in yeast are glycogen and trehalose (Klis, 1994; Sall *et al.*, 1998). Glycogen is a polysaccharide made entirely of glucose units and it is known as a source of carbon-based intermediates (Sall *et al*., 1998). Trehalose has been identified as source of carbon and energy during the various cycles of the yeast growth phase, especially during starvation. It is also cited to be synthesised in response to stress (Kandror *et al*., 2004; Wiemken, 1990).

2.3.1 Glycogen

Glycogen accumulation was shown to be triggered by limitation of nitrogen (Lillie and Pringle, 1980; Patel and Ingledew, 1973), phosphorus and sulfur (Lillie and Pringle, 1980) whereas glycogen consumption during fermentation is associated with the provision of metabolic energy (Quain, 1988) and lipid synthesis (Murray *et al*., 1984). The glycogen content of yeast during wort fermentation was classified into three phases (Quain *et al.*, 1981). On inoculation into fresh medium, the glycogen content decreased from ca. 40 % to below 10 % of the total yeast dry weight while substrate utilization was negligible, illustrating that glycogen was used as the sole source of metabolic energy. In the second stage there was a steady accumulation of glycogen with concomitant consumption of extracellular glucose and maltose. The final stage commenced at the end of growth with an increase of glycogen. Quain (1988) reported a decline in the content of glycogen toward the end of fermentation and attributed that to the provision of maintenance energy under conditions of carbon exhaustion.

Murray and co-workers (1984) demonstrated glycogen utilization during the early aerobic stages of yeast growth and attributed it to lipid synthesis based on studies that during the early stages of yeast growth there is an intense demand for ATP to support the synthesis of sterols (lipids) and fatty acids (Quain and Tubb, 1982). Murray and co-workers (1984) demonstrated that the relationship between lipid formed and glycogen assimilated was stoichiometric: for every 1 gram of glycogen assimilated, 69 milligrams of sterol were produced. Lipids play a major role in regulating the rate and extent of fermentation and also influence the cell's ability to reproduce (Quain and Haslam, 1979).

The availability of glycogen during the initial stages of fermentation is critical. When the glycogen content of pitched yeast was low, fermentation was slower than for yeast having high glycogen content. The minimal yeast glycogen content required to permit fermentation upon pitching was 200 $mg.l^{-1}$ (Quain and Tubb, 1982).

2.3.2 Trehalose

Aranda *et al*. (2004), D'Amore *et al*. (1991) and Lillie and Pringle (1980) showed that an increase in the trehalose concentration in yeast occurred on depletion of nitrogen in the growth medium. When cells were depleted of nitrogen, the amount of accumulated trehalose increased 4-fold (Aranda *et al*., 2004). Trehalose was also shown to accumulate during heat shock and at the inception of the stationary phase. The highest concentration of trehalose during heat shock was found to be 500 nM (Hottiger *et al*., 1987*a* as cited in Benaroudj *et al*., 2001). Trehalose is widely recognized as a stress response indicator.

2.4 PROCESS STRESSES EXPERIENCED BY BREWERS YEAST

The yeast is exposed to various types of stress during processing in the brewery. These stresses are classified as physiological and mechanical stresses. Proper handling of yeast in the brewery is required to prevent the deterioration of yeast quality as a result of exposure to excessive stress. For survival and adaptation, it is necessary for cells to sense and respond to the changing environment. The receptors found on the plasma membrane help the cell to transmit the signal received from the external environment. This typically results in the production and activation of molecules that have protective functions (Welch, 1993; Thieringer *et al*., 1998). In brewing, yeast is exposed to various types of stress: nutrient limitation, heat shock, osmotic shock, oxidative stress, ethanol stress (toxic), pH fluctuation, oxygen limitation and exposure to metals and other ions.

Handling of yeast between fermentations includes its separation from beer, transfer between vessels and agitation of yeast slurries prior to the inoculation of subsequent fermentations (Robinson, 2001). Basson (1996) reported that the typical yeast handling process entails:

- Transfer of yeast from propagation vessels into the aerated wort for fermentation
- Transfer of yeast from the storage vessel into aerated wort for further fermentation
- Cropping settled yeast from bottom of cylindroconical fermentation vessel at the end of fermentation
- Flow of yeast through pipes (of specified diameter and length), bends and valves
- Cooling by passage through heat exchanger
- Agitation in the storage tank
- Possible re-circulation through heat exchanger to maintain temperature

2.4.1 Mechanical stress experienced by Brewers' yeast in the yeast circuit

2.4.1.1 Hydrodynamic Shear

Shear is defined as the force applied parallel to the interface between the cell and its environment (Thomas, 1993). Hydrodynamic shear may arise during flow of the yeast suspension through pipework, fittings, constrictions and expansions as well as other units of process equipment such as pumps, heat exchangers, centrifuges and agitated vessels. Potential effects of hydrodynamic shear on Brewers' yeast are loss of molecules (phosphomannan, proteins and lipids) from the cell wall surface (Basson, 1996; Prokop and Bajpai, 1992), loss of cell wall structure, loss of membrane integrity (Basson, 1996; Robinson, 2001; Stewart *et al*., 2006) and loss of cell wall surface properties (Robinson, 2001). This may lead to loss of cell viability and vitality as well as an alteration of the metabolic energy requirement for maintenance. The degree of damage depends on the physiological state of yeast, cell growth phase and magnitude of the force applied (Basson, 1996; Robinson, 2001).

In breweries, yeast is transferred from one point to another using a pump. At the end of yeast fermentation in the FV, the flocculated yeast is pumped to storage (Yeast Collection Vessels) via the chiller. The type of pump used has been considered critical to the quality of beer and yeast. Basson (1996) investigated the effect of pump type on the quality of cropped yeast. A pilot study was carried using different pumps such as peristaltic, lobe, sine, gear, diaphragm and centrifugal pumps. At the brewery production scale, the small-scale fermentation showed that the yeast quality was not affected by transfer of the yeast from the cone of the FV to the chiller outlet. Cropping using the pumps investigated did not have any significant effect on the quality of cropped yeast.

In breweries, the yeast suspension is continually agitated to prevent compaction in the storage vessels, to maintain homogeneity and prevent the development of hot spots inside the vessel (Lewis and Poerwantaro, 1991). The effect of hydrodynamic shear on cell viabilities was shown to be dependent on both the generation number of yeast and the intensity of the shear (Stafford *et al*, 2001; Robinson *et al*., 2001). Shear was introduced to yeast through agitating the yeast slurry (Stafford *et al*, 2001) and by centrifuging the yeast at rotational speed between 4000 and 10000 rpm (Robinson *et al.*, 2001). The cell viability test indicated that the feed flow rate did not have a specific effect on loss of cell viability in the centrifuge and the first generation yeast was more resilient to the loss of viability compared to 5th and the 7th generation yeast slurries (Robinson, 2001).

Stafford *et al.* (2001) investigated the response of Brewers' yeast to a defined shear field in a 2 litre flat-bottomed cylindrical vessel for differing exposure times. Lager and ale yeast slurries were used and maintained at a temperature of 4°C to 4.5°C for about 30 hours. Mixing speeds chosen were 500 rpm (1.44 $m.s^{-1}$ tip speed) and 900 (2.36 $m.s^{-1}$ tip speed) rpm. Generation numbers of ale yeast were 2 and 9 whilst for lager yeast the generation numbers were 2 and 5. The consistencies of both lager and ale were maintained between 47 % and 52 % wet weight. It was shown that on agitation at 500 rpm, cell viability of all the yeast slurries decreased by between 0.8 % and 1.8 %. At 900 rpm, the decrease in viability of generation 9 ale yeast was 3.5 %. The amount of protease released was directly proportional to the agitation rate or centrifuging rate. All strains except the G5 (lager) yeast displayed an increase in the amount of protease released with an increase in shear stress. Robinson (2001) investigated the influence of centrifugation speed on the amount of protease released by the cells. An increase in protease released in the concentrate stream was observed when centrifuging at a speed above the threshold rotational speed of between 4000 and 6875 rpm.

Centrifuges are used at the brewery to remove partial trace yeast at the end of the primary fermentation (in the FV) prior to the secondary fermentation (lagering stage inside the storage vessels). Furthermore, the centrifuge helps during beer clarification. Centrifugation often follows the removal of flocculated brewers yeast. Usually, at the end of fermentation the flocculated yeast is used for repitching whilst the centrifuged yeast is discarded. The loss of flocculation is influenced by the absence of "lectin like" surface properties and mannan receptor sites (Miki *et al.*, 1982a, b). In addition, the loss of flocculation increases the demand on the centrifuge since it results in less yeast settling at the end of the primary fermentation in the FV (Robinson, 2001). Should yeast flocculate before the end of the fermentation period, the attenuation stage will cease and a large amount of residual sugar will remain in the wort. Damage to yeast which has been centrifuged can manifest itself in terms of loss of cell integrity and alteration of the cell surface.

The effect of hydrodynamic shear on cell wall surface properties can affect yeast flocculation potential and fermentation performance, hence beer quality, through rearrangement and loss of surface molecules (Prokop and Bajpai, 1992). The hydrophobicity test was used to investigate the effect of shear on different yeast generations in a disc stack centrifuge (Robinson et al., 2001). A greater hydrophobicity for older generations was reported (Table 2-1). (Stewart et al. (2006) used the same test to investigate the activity of proteinase A from centrifuged yeast and found that proteinase A was released from damaged yeast cells and this enzyme diminished the hydrophobicity of foam-positive polypeptides in beer thus making the beer foam unstable. Proteinase A is known to hydrolyse and inactivate the foam-inducing properties of the hydrophobic polypeptides (Stewart et al., 2006; Robinson, 2001 and Ormrod et al., 1991). Stewart et al. (2006) investigated the influence of centrifuged yeast on beer foam stability using a 2 hl pilot brewery with a 5 hl.l^{-1} Westfalia disk stack centrifuge (Westfalia, Oelde, Germany) (Figure 2-3). The centrifuge was operated at 10000rpm with a gravitational force of 14000 x g. The centrifuged yeast was re-pitched, re-fermented and recycled through a total of six cycles. The beer obtained from each cycle showed a decrease in foam stability with an increase in number of cycles. The reduced foam stability was accredited to reduced foam-positive polypeptides due to increased proteinase A secretion during centrifugation. The increase in proteinase A secretion during centrifugation was also supported by work of Stoupis *et al.* (2003). Stewart *et al*. (2006) concluded that centrifugation following fermentation leads to stressed yeast and the secretion of proteinase A, that later leads to lower hydrophobic polypeptides and thus reduced foam stability.

Table 2-1: The influence of the generation number on the hydrophobicity index and flocculation of the centrifuged yeast (Robinson, 2001).

Generations	Hydrophobicity index for feed stream (%)	Hydrophobicity index for concentrate stream (%)	Loss of flocculation (%)
1st	1.8	2	6
4th	7	1.5	45
7th	11	4	26

Figure 2-3: The pilot brewery 5 hl.l^{-1} disk stack centrifuge (Stewart *et al*. 2006).

2.4.1.2 Hydrodynamic cavitation

When a fluid flowing through a pipe experiences a reduction in fluid pressure such that the localized pressure approaches the vapour pressure, cavitation takes place (Coulson and Richardson, 1990). During this process, the vapour cavities oscillate through collapse and rebound cycles until they are destroyed by pressure recovery. In brewing, hydrodynamic cavitation may result from local increases in linear velocity and dynamic pressure fluctuations as a result of constricted flow through the valves and pumps. Previous research done by Harrison and Pandit (1992) showed that by increasing the pressure drop across the constriction and the number of passes through the constriction, the extent of yeast disruption increased. Higher biomass concentrations ranging from 19 to 140 kg.m^{-3} (dry weight) were found to reduce the extent of cell disruption. This was attributed to the dampening of pressure oscillations as a result of increased viscosity. This phenomenon has been rigorously studied using an orifice plate constriction, cavitation number between 0.9 and 0.13, and the yeast *S. cerevisiae* (Balasundaram and Harrison, 2006).

Hydrodynamic cavitation varies with temperature. At low temperature, the severity of the cavitation may worsen due to a more violent collapse of the vapour cavity as the vapour pressure is reduced. An increase in temperature may lead to a reduction in cavitation intensity but more ready onset of cavitation, owing to the increase in vapour pressure of the water. Pumping of the fluid for a longer duration may increase the temperature of the fluid due to dissipation of energy as heat.

2.4.2 Physiological stress on brewers yeast during yeast handling

2.4.2.1 Nutrient limitation

Yeast, like any other micro-organism, needs nutrients to grow. The yeast nutrients comprise components such as a carbon source, proteins, micro elements, growth factors and vitamins. Each nutrient has a specific function in yeast such as growth, cell repair and energy supply. This section provides an understanding of the survival mechanism used by yeast undergoing nutrient stress.

The yeast enters the stationary phase of its life cycle when it experiences nutrient limitation. On limitations, owing to components other than carbon, large amounts of energy reserves such as trehalose and glycogen (Fraenkel, 1982) accumulate intracellularly for the yeast to survive long starvation periods during storage (Granot and Snyder, 1991; Granot and Snyder, 1993). Sall and co-workers (1988) showed that there was little overall change in the trehalose level during storage using industrial yeast. In contrast, Lillie and Pringle (1980) argued that the level of trehalose in yeast was important in allowing stored yeast to survive prolonged starvation. Further information on the induction of trehalose on stress and the role of glycogen through fermentation can be found in Section 2.3.

2.4.2.2 Osmotic stress

Osmotic stress is brought about by changes in solute concentration in a particular environment. This affects the free water activity (a_w) or water potential (φ) of the medium (Mager and Varela, 1993). The osmotic medium is classified into three categories: hypertonic (higher solute concentration in solution than in cytoplasm), hypotonic solution (lower concentration of solute in solution relative to cytoplasm) and isotonic solutions where concentrations of solute in solution and cytoplasm are similar. During osmosis, the movement of water is from a region of high concentration to a region of low concentration. Hence, placing a cell into a hypertonic solution causes water loss from the cell with cell shrinkage whereas placing a cell into a hypotonic solution causes the cell to swell and possibly burst. Isotonic solutions contain approximately equal concentrations of solute; hence no net flow of water into and out of the cell is expected. When the cell was exposed to a hypertonic solution, the osmotic flow through the cell membrane was proportional to the change in osmotic pressure ($\Delta\pi$) thus leading to a reduced cell size (Wood, 1999).

Yeast experiences changes in osmotic potential throughout the brewing circuit. A decrease in the osmotic potential of yeast is caused by an increase in ethanol production (fermentation) or pitching of yeast into the wort. The yeast moves from high to lower osmotic potential by diluting yeast with water. Modern breweries in Europe and America have set a trend of cooling brewers yeast (Wittl and Maier, 1980). The cropped yeast is cooled by diluting it with 12°C deaerated, non-carbonated water in the yeast collection vessel (YCV) to lower the ethanol stress and reduce suspension viscosity during storage of the cropped yeast. The yeast-water mixture is agitated to maintain homogeneity and provide heat transfer for further cooling to 2-4°C using the cooling jackets (2°C.h^{-1}). In this section, the effect of the osmotic stress on cell growth, cell flexibility and viability are given.

Numerous studies have shown that cell viabilities of the microorganism are influenced by both temperature (Walton and Pringle, 1980; Hottiger *et al*., 1987a; Suutari *et al.*, 1990) and osmotic pressure (Scott, 1957; Esener *et al*., 1981). Moreover, further studies have investigated the coupling effect of temperature and osmotic pressure on cell viability (Mille *et al*., 2002; Beney *et al*., 2001). The response of bacteria and yeast when under osmotic pressure was studied using *Escherichia coli K12 (*Mille and co-workers (2002) and *Saccharomyces cerevisiae (*Beney *et al.,* 2001*)*. Furthermore, studies were carried out using glycerol as a water depressant. For *E.coli,* the osmotic pressure conditions were 26, 30, 35, 40, 82 and 133 MPa whilst the temperature conditions were 4, 10, 30 and 37°C. The control bacteria were thermally equilibrated but were not allowed to undergo osmotic challenge. A slight increase in osmotic pressure led to an active biological response through an active osmoregulatory system of the cell. The cell synthesized solutes in response to the increased osmotic pressure and rapidly modified the membrane permeability in order to allow influx of water from the external environment into the cell. For osmotic pressure levels below 26 MPa, cell viability retained was greater than 80 %. The effect of temperature was negligible within that osmotic shift. When the osmotic shift was between 30 and 35 MPa, loss in cell viability increased with increasing temperature. A more marked decrease in viability of some 40 % was observed when osmotic pressure was approximately 40 MPa. A similar magnitude effect was observed irrespective of varying temperatures. The decrease in viability was more prominent at 37°C. When osmotic pressure was increased to 133 MPa, at low temperatures the viabilities were maintained above 5 % whilst at 30°C and 37°C the viabilities fell to less than 5 %. Overall, the viability decreased with an increase in temperature and osmotic pressure (Table 2-2). Moreover, viability was greatly influenced by the osmotic pressure.

Beney and co-workers (2001) thermally equilibrated the yeast samples and the glycerol solution for 2 minutes in a thermostatically controlled waterbath prior to injecting the yeast into the glycerol. During the exposure of yeast to osmotic stress, osmotic pressures of 14.5, 49.2 and 99 MPa were used. The temperature conditions were 5, 10, 15 and 25°C. Subsequent to yeast rehydration, the yeast was thermally equilibrated to 25°C. At the osmotic pressure below 50MPa and temperatures below 10°C, the cell viability was preserved (Table 2-3). At the osmotic pressure of 99 MPa and temperatures below 10°C, the yeast cells were largely resistant to the osmotic pressure with some 80 to 85 % viability maintained. However, when temperature was increased to between 15 and 25°C, the cell viabilities reduced to 45 and 25 % respectively. The optimal protection of yeast cells against hyperosmotic pressure could be increased by lowering the temperature to below 10^{o}C. Comparing the response of yeast and bacteria to the coupling effect of temperature and osmotic pressure, it can be seen that the viability of *E. coli* was more severely affected by an increase in the osmotic pressure as compared to yeast. At temperatures below 30^{o}C, the viability of *E. coli* was maintained below 30 % at the osmotic pressure of 40 MPa. However, at 49 MPa and temperature below 25^{o}C, the cell viability of yeast was above 80 %. It is thus concluded that yeast is more resistant to the osmotic pressure compared to the bacteria.

The effect of osmotic pressure on cell flexibility was also investigated using water to demonstrate the importance of rehydration kinetics in predicting cell preservation (Gervais *et al*., 1992 and Poirier *et al*., 1999). Increase in rate of rehydration of cells resulted in greater cell damage. Slow rehydration allowed the recovery of the initial surface area to volume ratio. Further, viability was dependent on the magnitude of osmotic pressure fluctuation and temperature. Cell wall flexibility was also tested using a hypertonic glycerol solution (Niedermeyer *et al*., 1977 as cited in Dumont *et al.,* 2003). Initially the cell shrunk rapidly, then it slowly increased in volume as the external solute molecules entered the cell accompanied by water molecules. The final cell volume was proportional to the rate of water exit flow, the initial glycerol concentration and the glycerol entry flow which were all dependent on the osmotic pressure gradient. The experimental data were not presented.

Table 2-2: The average residual viabilities of E. coli K12 strain obtained when the osmotic shift was between 26 and 133 MPa (Mille et al., 2002). Cultures were grown at 37^{o}C for 24 hours. To vary osmotic pressure, a freely permeant polyol, glycerol was used as a water depressor.

Temperature	26 MPa	30-35 MPa	40 MPa	133 MPa
37^{o}C	92 %	54.8 %	22 %	0.8 %
30^{o}C				0.7 %
10^{o}C		69.2 %		6.5 %
4^{o}C	85 %	74.7 %	28.1 %	9.1 %

Table 2-3: Viability of *S. cerevisiae* (CBS 1171) after hyperosmotic shocks achieved as a function of osmotic pressure levels and temperature (Beney *et al.,* 2001)

Temperature (°C)	5°C	10°C	15°C	25°C
Osmotic pressure (MPa)				
14.5	98 ± 2	98 ± 2	98 ± 2	97 ± 2
49.2	92 ± 3	94 ± 3	80 ± 7	94 ± 5
99	81 ± +5	85 ± 6	45 ± 9	25 ± 4

Motshwene *et al*. (2004) reported that the increase in cell volume for the wild type and the knock-out strain (the haploid segregants from the diploid strain 842 (*a*/*α*, *ade2-1/ade2-1*, *trp1-1/trp1-1*, *leu2-3/leu2-112*, *his3-11/his3-15*, *ura3/ura3*, *canr1-100/CAN*) were 8.8 % and 4.5 % respectively over a 7.5 minutes period. The difference in cell volume seen was accredited to HSP12 affecting the flow of water across the cell membrane or the altered flexibility of the cell wall brought about by HSP12. The presence of HSP12 was correlated with cell wall flexibility (Motshwene *et al*., 2004), measured by observing the response of yeast size to rapid changes in the osmotic strength of the environment. In measuring cell wall flexibility, the average yeast volume was measured by laser light scattering using Malvern Mastersizer (Malvern Instruments, Malvern, Worcs, U.K.). A rapid decrease in cell volume of the wild type yeast was observed upon addition to the high osmolality buffer (approximately 1320 mosmol.l^{-1}). A 13 % decrease in volume was seen after 1 minute followed by a gradual decrease in volume until steady state was reached. The approximate decrease in yeast volume was 17 % after 10 minutes. For the knock-out strain, the decrease in cell volume after 1 and 10 minutes were 5 % and 8 % respectively. Due to unavailability of data generated by Niedermeyer *et al*. (1977); it is difficult to compare the work done by Motshwene *et al* (2004) and Niedermeyer *et al*. (1977). In both cases, the yeast was injected into a high osmolality buffer; however, the strength of the buffer used by Niedermeyer was not stated.

2.4.2.3 Oxidative stress

Oxygen forms part of the essential nutrients for aerobic organisms. However, oxygen can also pose as a toxic agent damaging cells. During aerobic growth, cells are exposed to reactive oxygen species (ROS) such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH), which are synthesized as by-products of respiration

(Kwong *et al*., 2003; Stadtman and Berlett, 1997 as cited in Benaroudj *et al*., 2001). Santoro and Thiele (1997) (cited in Belo *et al*., 2005) defined oxidative stress to be incurred whenever the ROS concentration exceeded the antioxidant capacity. In order for a cell to protect itself against oxidative stress (defence mechanism), enzymes including catalase and superoxide dismutase (SOD) as well as the non-enzymatic protective molecules glutathione and thioredoxin (Ames *et al.,* 1993; Moradas-Ferreira *et al.,* 1996 as cited in Benaroudj *et al*., 2001) are induced. The conversion of O_2 to H_2O_2 is catalysed by superoxide dismutases (SODs) (McCord and Fridovich, 1969). Oxidative stress in cells results from the chain reactions that are established between the ROS and the biomolecules in cells, such as proteins, DNA and lipids. ROS damage of yeast is caused by modifying the amino acid side chains to form cross links between proteins and fragmentation of the polypeptide backbone (Berlett and Stadtman, 1997 as cited in Benaroudj *et al*., 2001). Oxidative damage to the cellular components is postulated to cause cell aging (Stadtman and Berlett, 1997; Ames *et al.,* 1993 cited in Benaroudj *et al*., 2001).

The effect of increased aeration and oxygen pressure on the oxidative response of *Saccharomyces cerevisiae* was studied by Belo *et al*. (2005). The fed-batch experiments were carried out in a 600 ml stainless steel bioreactor (Parr 4563) at a temperature of 30°C, stirring rate of 400 rpm and a gas flow rate of 1 $l.min^{-1}$ (at 0°C and 0.1 MPa). The operating pressure was set by the compressed gas pressure (inlet gas) and the regulatory valve position in the exit gas line. The oxidative response of yeast cells was determined by analyzing cell viability, metabolic activity of cell, antioxidant activity of intracellular enzyme, the genealogical profile of the cells and the cell size distribution. The methylene blue staining technique was used to determine cell viability. The genealogical profile of the cell was measured by the number of bud scars per cell. When the initial glucose concentration flux was 0.36 $g.g^{-1}.h^{-1}$ at an aeration pressure of 0.1 MPa, oxygen limitation was experienced. The switch to the fermentative pathway was seen, resulting in low biomass formation and a significant amount of ethanol production. When the aeration pressure was increased to 0.6 MPa (air) or 0.13 MPa (pure oxygen), the biomass formation increased by 1.5 fold whereas the ethanol produced decreased by 1.8 fold. However, a further increase in aeration pressure to 1 MPa did not bring about any significant improvement in cell biomass formation suggesting that oxygen limitation had been overcome at a partial pressure of 0.13 MPa O_2. Due to high number of viable cells obtained, it was concluded that the hyperbaric pressure of 1MPa did not have any negative effect on the physiological state of the cells. A fed-batch reactor was also run at a hyperbaric pressure of air (1.5 MPa, corresponding to an O_2 partial pressure of 0.33 MPa) and using pure oxygen (0.32 MPa). A decrease in cell growth was seen. In addition, cell viabilities decreased with an increase in exposure time of aeration. Belo *et al.* (2004) reported that due to the experimental errors associated to the cell viability determination method, no significant differences (at a 95 %

confidence level) were found on the percentage of viable cells during the first 7 hours of aeration (at different pressures). However, after 24 hours, there was a significant difference between cell viabilities obtained for the culture using 0.32 MPa of pure oxygen and that at an oxygen partial pressure of 0.13 MPa. The cell viabilities obtained when using pure oxygen were lower than those with 1.5 MPa air. This is supported by indicating that the growth inhibition was caused by the toxicity of oxygen exceeding 0.21 MPa. A decrease in cell viability and the lower biomass yield coefficient $Y_{x/s}$ were attributed to the toxicity of oxygen exceeding 0.21 MPa (Table 2-4) rather than the increased air flow. Very long error bars were obtained in all the cell viabilities obtained.

Besides SODs and other antioxidants, trehalose was also found to play a role in protecting the cell and proteins from damage by oxygen radicals (Benaroudj *et al.,* 2001). The wild type S*. cerevisiae* was exposed to a mild heat shock temperature of 38^oC and thereafter split into three batches. The first batch of cells was incubated with an oxygen radical-generating system comprising H_2O_2 and $FeCl_3$ at 38^oC, then cells were maintained at 38^oC. The second batch of cells was incubated with an oxygen radical-generating system comprising H_2O_2 and $FeCl_3$ at 28^oC, then the cells were maintained at 28^oC. The third batch of cells was incubated with an oxygen radical-generating system comprising H_2O_2 and $FeCl_3$ at 38^oC, then the cells were maintained at 28^oC. In the first batch of cells, the trehalose content increased dramatically and so was the cells' resistance to H_2O_2. In the second batch of cells, trehalose was not detectable and the cell resistance to oxidants did not change significantly. In the third batch of cells, the level of trehalose decreased within 20 minutes and the resistance of cells to H_2O_2 decreased concomitantly. It was concluded that low trehalose levels make the cellular proteins more susceptible to covalent damage by free radicals. The accumulation of trehalose enhances cellular resistance to oxygen radicals. Furthermore, the defence of trehalose against oxygen radicals is important when cells are exposed to elevated temperatures.

Table 2-4: Pressure effects on the global cell mass yield, Y x/s, ethanol mass yield, Y e/s, specific cell growth rate, μ, and the maximal cell concentration, X_{max} in the fed-batch cultivation of *Saccharomyces cerevisiae* with air and pure oxygen. The experimental values of μ were determined from the beginning to the end of the fed-batch experiment (Belo *et al.,* 2005)

Pressuring gas (MPa)	Partial pressure O_2 (MPa)	$Y_{x/s}$ (%)	$Y_{e/s}$ (%)	μ (h^{-1})	X_{max} ($g.l^{-1}$)
0.1 air	0.021	24.3	25.4	0.04-0.08	9.8
0.6 air	0.126	35.6	13.6	0.09-0.13	15.4
1.0 air	0.21	37.1	13.2	0.08-0.12	13.7
1.5 air	0.33	8.3	14.0	0.06-0.0	1.7
0.13 O_2	0.13	35.9	14.4	0.08-0.11	14.4
0.32 O_2	0.32	1.8	8.30	0.05-0.0	4.6

2.4.2.4 Ethanol toxicity

In brewing, the fermentation process is used to maximize the production of ethanol. The ethanol produced results from the degradation of the carbon source (e.g. glucose) under anaerobic conditions. The ethanol production stops when the carbon supply is depleted at the beginning of the stationary phase. Maximum ethanol production yield is desired although ethanol concentration can be toxic to the yeast cells. In this section, the influence of ethanol concentration on yeast quality is reviewed. Furthermore, the survival mechanism used by yeast subjected to high ethanol concentration is discussed.

The typical ethanol concentration of yeast suspension from cropped fermentation vessels (FV) ranges between 1.1 M (52 $g.l^{-1}$) and 1.4 M (67 $g.l^{-1}$) (Lodolo, 1995). Ethanol is known to exhibit several inhibitory effects on yeast cells. Previous studies have shown that ethanol toxicity can inhibit yeast cell growth (Mager and Varela, 1993, Walker, 1998; D'Amore *et al*., 1990) by causing membrane leakage (Sajdidor and Grego, 1992) and loss of viability (Sales *et al*., 2000; Pina *et al.,* 2004). Adaptation of yeast to ethanol stress was assumed to be possible by altering membrane fluidity, increasing the catabolism of ethanol, synthesizing stress proteins and increasing membrane unsaturated fatty acids (Walker, 1998). When yeast was exposed to a high ethanol concentration, yeast cells exhibited the collapse of ion gradients across the plasma membrane, dehydration and decreased cell viabilities (Mager and Varela, 1993).

The response of yeast to ethanol toxicity was shown to be dependent on the type of yeast (Pina *et al*., 2004) and the presence of trehalose (Mansure *et al*., 1994), HSP12 (Sales *et al*., 2000) and lipids (Alexander *et al*. 1994). The cryoprotectants were found to reduce cell wall damage due to the toxicity of ethanol. Trehalose was shown to inhibit ethanol–induced leakage of the cell membrane and liposomes (Mansure *et al*., 1994). This suggested the protection role of this sugar when yeast is under osmotic stress. Mansure and coworkers (1997) showed that the concentration of trehalose increases during the fermentation process. Since ethanol production also increases during the fermentation process, this suggests that the survival of yeast at the end of the fermentation may be dependent on trehalose. HSP12 was shown to protect yeast against ethanol-induced stress (Sales *et al.,* 2000). However the mechanism of protection was not understood. A study carried by Alexander *et al*. (1994, cited in Sales *et al*., 2000) also showed lipid playing a key role in ethanol tolerance. The fluidity of the plasma membrane was shown to increase when yeast was supplemented with ethanol. Sales and coworkers (2000) investigated the difference in viability of the wild type yeast and the HSP12 knock-out mutant when grown in YPD media supplemented with ethanol to a concentration up to 15 % (v/v). No difference in growth rate of the two strains was detected after 24 hours when the ethanol concentration was 5 % (v/v). The growth of the knock out strain was affected by ethanol concentration up to 10 % (v/v). The growth rate of the knock-out strain was reduced by about 75 % compared to the growth of this strain without ethanol. When ethanol concentration was increased to 12 % (v/v), a total growth inhibition of the growth of the knock-out strain was observed whereas growth of the wild type strain was only reduced to 75 % (compared to the control wild type strain grown in YPD media with no alcohol). The wild type strain was unable to grow in YPD media supplemented with 15 % ethanol. The above results suggested the role of HSP12 in yeast tolerance against ethanol (Figure 2-4).

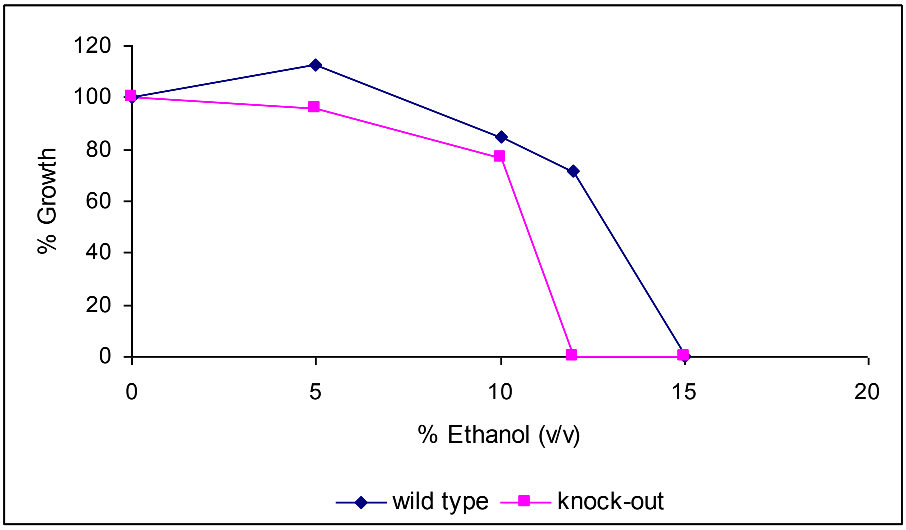


Figure 2-4: The effect of ethanol on the survival of the wild-type and knock-out yeast. Yeasts were incubated in YPD medium containing various concentrations of ethanol for 24 h after which the growth (determined from the 600-nm absorption) was compared with growth in the absence of ethanol (Sales *et al*., 2000).

Pina and co-workers (2004) studied the tolerance of *Saccharomyces* and non-*Saccharomyces* yeasts to ethanol by progressive inactivation. Five non-*Saccharomyces* strains (*Candida stellata PYCC 3044*, *Debaryomyces hansenii* PYCC 2968T, *Hanseniaspora uvarum* PYC 4193T, *Torulaspora delbrueckii* PYCC 2477T and *Hanseniaspora guilliermondii* NCYC 2380) and one *Saccharomyces* strain (*S. cerevisiae* PYCC 3507) were used. Yeast was grown at 25°C then harvested by centrifugation (Pina *et al*., 2004). No ethanol was added to the growth medium during cell growth. The wet pellet was then resuspended in Yeast Malt (YM) broth with ethanol added at 20, 22.5 and 25 % (v/v) for 1, 2 and 4 minutes. The chosen alcohol concentrations are believed to represent an ethanol stress, which generates kinetics of cell inactivation measurable over a short period of time (less than 5 minutes) (Pina *et al*., 2004). Cell viability was measured by plate count. For *S. cerevisiae*, there was no effect of ethanol inactivation at 20 % (v/v) ethanol (Figure 2.5a). Significant decrease in cell viability was observed at 22.5 % ethanol ((Figure 2.5b). When yeast was exposed to 25 % ethanol, greater reduction in cell viability was observed. For non-*Saccharomyces* cells, when the ethanol concentration was 20 % (v/v), greater cell inactivation was only seen in *D. hansenii* PYCC 2968T *and T. delbrueckii* PYCC 2477T*. (*Figure 2.5a*).* These two cells suffered a reduction of approximately 1 and 2 log cycles respectively*.* At 22.5 % (v/v) ethanol, a more significant decrease in cell viability was visible in all cases except for *C. stellata.* The highest reduction in cell viability of non-*Saccharomyces* cells were observed at 25 % (v/v) ethanol. Furthermore, the resistance of *H. guilliermondii* NCYC 2380 was found to be similar to that of *S. cerevisiae* at 25 % (v/v) ethanol. *Debaryomyces hansenii* PYCC 2968T, *Hanseniaspora uvarum* PYC 4193T and *Torulaspora delbrueckii* PYCC 2477T were the most sensitive yeast to ethanol concentration of 25 % (v/v) (Figure 2.5(c)).

Another experiment was carried out to investigate the effect of different ethanol concentrations on yeast growth (Pina et al., 2004). For yeast grown with ethanol supplementation, the alcohol concentration was kept at 4, 6, 8 and 10 % (v/v). Control yeast was grown in growth media not supplemented with ethanol. The lag phase increased and the final biomass decreased with an increase in ethanol concentration (Table 2-6). No growth was detected for most of the non-*Saccharomyces* yeasts at ethanol concentration above 6 % (v/v). *S. cerevisiae* was found to be the most ethanol resistant strain. Comparing the findings of Sales et al. (2000) and Pina et al. (2004), it can be seen that yeast (*S. cerevisiae*) grown on ethanol supplementation showed response at different stages. Sales et al. (2000) reported that the viability of wild type yeast was not affected by ethanol up to 10 % (v/v) whereas Pina and co-workers (2004) reported a prolonged lag phase on cell growth with increasing ethanol concentration. The differences in the experimental conditions for yeast preparation used by the above mentioned authors are shown in Table 2-5.

Ethanol is known to cause membrane leakage (Sajdidor and Grego, 1992). Sales and coworkers (2000) investigated the ability of HSP12 to protect the plasma membrane against ethanol stress. Yeast was grown in YPD media supplemented with various ethanol concentrations. The liposomal membrane incorporating stearylamine and also encapsulating calcein was immersed in various ethanol concentrations (3 % up to 15 % v/v) having the specific concentration of 15 µg HSP12 per µg phospholipid. Calcein leakage was used as the sign for membrane damage. It was shown that the maintenance of membrane integrity by HSP12 decreased with an increase in ethanol concentration (Figure 2-6). When the ethanol concentration was 6 %, approximately 88 % of liposomal membrane integrity in the presence of HSP12 compared to 55 % in its absence was observed. Approximately 78 % of the liposomal membrane integrity was maintained when the ethanol concentration was 9 % for the wild type yeast compared to total calcein leakage in the HSP12 free yeast (Figure 2-6). HSP12 was also shown to interact electrostatically with the charged groups on the membrane surface. Results showed the positively charged liposomal membrane (stearylamine) was protected by HSP12 against dessication. It was thought that the negatively charged amino acids, glutamate and aspartate, interacted with the stearylamine amino group. Similarly the positively charged amino acids, arginine and lysine, were assumed to interact with the negatively charged phospholipid head groups (Sales et al., 2000). The two positively charged amino acids were believed to mimic the action of water by shielding the charge repulsion of the free phosphate group. The mechanism used by HSP12 in protecting yeast against membrane desiccation involves hydrogen bonding with the membrane proteins and the glycolipids. This results in a hydrophilic net preventing membrane fusion during the desiccation process. Moreover, the presence of HSP12 was assumed to lead to increased water content during dessication as a result of some water molecules being tightly bound to the charged groups of the HSP12 hence leading to a stabilized membrane configuration (Sales et al., 2000). The hydrophilic characteristic of HSP12 is also supported by Praekelt and Meacock (1990) cited by Mtwisha et al. (1998). From the above findings, it is clear that an increase in ethanol concentration will lead to a decrease in water content of yeast (dessication). However, incorporating HSP12 into the liposomal membrane will protect the yeast against membrane dessication.

Table 2-5: Differences in inocula preparation on studying the effect of ethanol on cell growth (Pina et al., 2004; Sales et al., 2000). Agitation rates were kept at 100rpm in both cases.

Parameter	Sales *et al.*, 2000	Pina *et al*, 2004
Growth temperature	30°C	25°C
Duration for cell growth	Late stationary phase	Mid-growth phase
Growth media	1 % Yeast, 2 % Peptone and 2 % dextrose supplemented with 100 μg.ml^{-1}penicillin	Yeast Malt

Table 2-6: The growth parameters of five non-Saccharomyces yeasts and one S. cerevisiae strain at increased initial ethanol concentrations (% v/v). Sc: *S. cerevisiae;* Hg: *H. guilliermondi*; Hu: *H. uvarum*; Cst: *C. stellata*; Td: *T. delbrueckii* and Dh: *D. hansenii*. The abbreviation "ngd" means no growth detected. The units of measure for the lag phase and biomass were hours and mg ml^{-1} respectively. (Pina *et al*., 2004)

Final ethanol conc (% v/v)	Parameter	*Hg*	*Hu*	*Cst*	*Td*	*Dh*	*Sc*
0	Lag phase	7	7	22	9	9	6
	Biomass	1.51	1.23	1.28	2.79	2.29	2.67
4	Lag phase	8	10	32	25	10	8
	Biomass	1.11	0.7	1.2	0.86	1.39	2.4
6	Lag phase	10	ngd	48	ngd	ngd	9
	Biomass	0.94	-	0.2	-	-	1.96
8	Lag phase	ngd	ngd	160	ngd	ngd	18
	Biomass	-	-	0.13	-	-	1.51
10	Lag phase	ngd	ngd	ngd	ngd	ngd	55
	Biomass	-	-	-	-	-	0.26

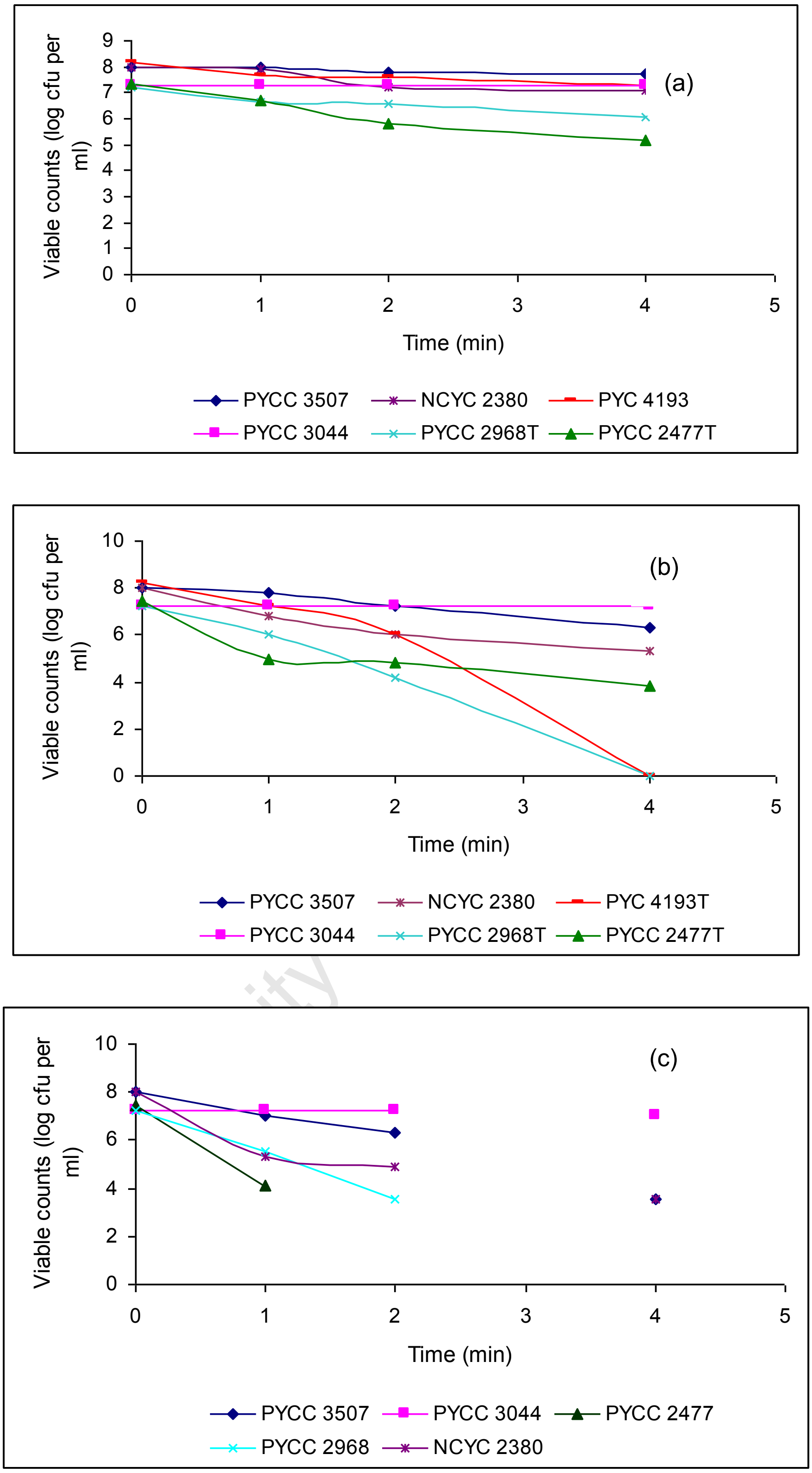


Figure 2-5: The effect of ethanol stress at (a) 20 %, (b) 22.5 % and (c) 25 % (v/v) on the survival of (♦) *S. cerevisiae* PYCC 3507, (#) *H. guilliermondii* NCYC 2380,(__) *H. uvarum* PYC 4193T, (■) *C. stellata* PYCC 3044, (x*)* *D. hansenii* PYCC 2968T and (▲) *T. delbrueckii* PYCC 2477T. Viable counts not detected at (b) 4 min for D. hansenii and H. uvarum; (c) 1 min for *H. uvarum*, 2 min for *T. delbrueckii* and 4 min for *D. hansenii* (Pina *et al*., 2004).

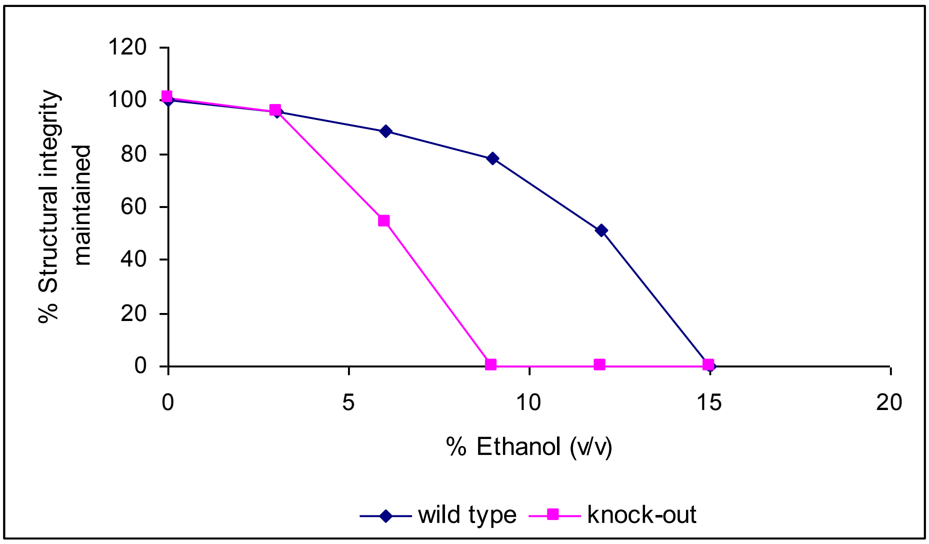


Figure 2-6: The effect of HSP12 on the relationship between ethanol concentration and structural integrity of liposomes. Liposomes were incubated in PBS containing various concentrations of ethanol either alone or together with HSP and the structural integrity assessed by Calcein leakage (Sales *et al.,* 2000).

2.4.2.5 Thermal stress

Yeast experiences temperature changes at different positions within the brewery yeast circuit. At the end of the fermentation period, the temperature of yeast is between 14 and 16°C. This yeast is pumped via the heat exchanger to lower the temperature to about 2°C prior to storage in the YCV in order to minimise metabolic activity during storage (Robinson, 2001). During pitching, the temperature of yeast and wort are maintained around 14-16°C. In this section the response of yeast to heating, freezing and cooling is reviewed. Moreover, the effects of cold stress at molecular and performance levels are discussed in detail. The study entails the molecular response of *E.coli* and yeast to thermal stress.

When *Saccharomyces cerevisiae* was exposed to a sudden temperature change, an alteration in the pattern of protein biosynthesis was displayed (Miller *et al*., 1979 cited in a review by Mager and Moradas Ferreira, 1993). Heat Shock Proteins (HSPs) are molecular chaperones and help in regulating cellular homeostasis and promoting yeast survival (Kopecek *et al*., 2001). Molecular chaperones are known to play a key role in protein folding and translocation and also in the selective degradation of certain proteins with highly abnormal conformation (Welch, 1991 cited in Kandror *et al*., 2002). The exact mechanism used to degrade these proteins with abnormal conformation was not understood.

The growth of yeast was transiently arrested when yeast was shifted to a higher temperature (Johnston and Singer, 1980 cited in a review by Mager and Moradas Ferreira, 1993). McAlister *et al*. (1979 cited in a review by Mager and Moradas Ferreira, 1993) reported that when yeast temperature was increased from 23 to 30°C, minor alteration in the protein synthesis was observed. However, when the temperature was increased from

30 to 36^{o}C, a major alteration in the protein synthesis was observed. Since heat shock proteins were induced when yeast was undergoing stress, it was believed that these proteins are pivotal for yeast survival (emergency response).

Mtwisha and co-workers (1998) investigated the effect of temperature on HSP12 expression by growing yeast at 30 and 37^{o}C respectively. At 30^{o}C, the HSP12 expression increased from the log phase towards the early stationary phase. No effect on the concentration of HSP12 was seen during the stationary phase. Immunologically, the level of HSP12 was lower in yeast grown at 37^{o}C than yeast grown at 30^{o}C. However, no apparent difference was observed between the protein components of the soluble fractions of yeast grown at 30 and 37^{o}C hence it was assumed that the decrease in HSP12 concentration at 37^{o}C was due to reduced biosynthesis at higher temperature.

Nkosi (2001) investigated the effect of cooling rate employed in a heat exchanger on yeast quality of S. cerevisiae harvested from the brewery upon cooling at different cooling rates. Unwashed yeast was resuspended in de-aerated, deionised water at 14^{o}C to a consistency of 30 %. Cooling rates employed using the laboratory cooling coil were between 1.28 to 4.03^{o}C.s^{-1}. Yeast quality was measured based on cell membrane integrity, cell wall strength and cell growth. Haze assay and fragility tests were used to measure cell membrane integrity and cell wall strength. The cell membrane integrity was determined by measuring the amount of protease release. The cell wall damage was determined by measuring the amount of protein release on exposure to sublytic pressures. Rapid cooling of yeast (2 to 4^{o}C.s^{-1}) through the heat exchanger resulted in greater stress than that experienced at slower cooling rates (1^{o}C.s^{-1}), with simultaneous damage to the cell envelope and loss of yeast vitality. Over the range of cooling rates tested using cooling coils in the laboratory and using a plate and frame heat exchanger online in the brewery, the amount of intracellular protease released and haze generated increased with the increase in cooling rate. Cooling rates above 2^{o}C.s^{-1} aggravated damage to the cell envelope and showed the most detrimental effect on the subsequent small-scale fermentation (Table 2-7).

Furthermore, Nkosi (2001) investigated the effect of slow and fast cooling with dilution on the level of HSP12 expressed. Two batches of yeast (both 30% consistency) were cooled at different cooling rates. The first batch was centrifuged and resuspended in water at 4^{o}C. The second batch was centrifuged, resuspended in water at 12^{o}C, then cooled gradually to 4^{o}C. Both the first and the second batch of yeast were stored at 4^{o}C for four hours before assays were taken to facilitate protein expression. The cooling rates for the first and the second batch of yeast were about 3.09^{o}C.s^{-1} and 2^{o}C.h^{-1} respectively. Control yeast was maintained at 14^{o}C for 4 hours. The level of HSP12 was enhanced by 28-37 % for fast cooled yeast compared to slow cooled yeast. The difference in the amount of HSP12 induced was attributed to the variation in rate of exposure of yeast to cold stress.

Table 2-7: The effect of cooling rate on yeast cell membrane integrity, cell wall, yeast growth and yeast metabolism. Yeast was cooled in the laboratory, coolant set at -1°C (Nkosi, 2001).

Cooling rate (°C.s^{-1})	Protease release (as absorbance) an inverse measure of cell membrane integrity	Protein release (as absorbance) an inverse measure of cell envelope damage	Yeast metabolism (carbon dioxide yield in $g.g^{-1}$ glucose	Specific growth rate (x10 cells *ml^{-1}. hr^{-1})*
1.28	0.03	0.03	0.65	0.25
2.31	0.025	0.04	0.64	0.25
3.03	0.07	0.04	0.5	0.23
4.03	0.17	0.20	0.52	0.22

2.5 LOSS OF YEAST QUALITY DURING COOLING

2.5.1 Sublethal injury

From the above literature review, cold shock protection in yeast was shown to be provided by HSP and trehalose. A brief summary of how HSP, trehalose and ethanol affect yeast survival during cooling is given below.

2.5.1.1 Cold shock protection provided by heat shock proteins

The protection of yeast by heat shock proteins has been studied extensively (Kandror *et al*., 2004; Nkosi, 2001, Komatsu *et al*., 1990) and has been demonstrated through comparison of the wild-type and knock-out strains (Section 2.4.2.2 and Section 2.4.2.4). Earlier work done by Walker (1998) showed that the cell size of yeast decreased as a result of cold stress and it was postulated that the decreased cell size may temporarily inhibit budding on returning the yeast to its normal growth temperature. In addition, Komatsu and co-workers (1990) demonstrated that cells plunged into liquid nitrogen (severe stress) without prior exposure to mild stress lost almost 100 % viability. Nevertheless, the viability of cells exposed to mild stress prior to lethal stress improved significantly to 12 % viability. The results were attributed to higher initial HSP content of heat shocked cells (HSP70 and HSP90). The two HSPs were assumed to strengthen the bonds within macromolecules thus reducing denaturation. The bond within the macromolecules was in the form of a hydrophilic net formed by the bonding of the HSPs to OH group from water. This net keeps the cell hydrated at all times. Obuchi and coworkers (1991) also exposed yeast to the liquid nitrogen temperature and arrived at the same conclusions as Komatsu *et al*. (1990). The viability of the cells was reduced by 99 % when they were rapidly cooled in liquid nitrogen (Obuchi *et al*., 1991). However, it was demonstrated that when yeast cells were exposed to a mild heat shock of 43^{o}C for 2 hours prior to being rapidly immersed in the nitrogen, the cell survival was enhanced 20-30 fold. The protection offered was attributed to the induction of HSP90, HSP85 and HSP70.

2.5.1.2 Cold shock protection provided by ethanol

The effect of ethanol on S. cerevisiae was studied under different freezing conditions by Lewis and co-workers (1994). The study was observed under slow cooling (3^{o}C.min^{-1}) and fast cooling (25^{o}C.min^{-1} and 200^{o}C.min^{-1}), using the ethanol concentration range of 0.1 (4.7 g.l^{-1}) to 2 M (92 g.l^{-1}). Control yeast was cooled and immersed in the growth media. Ethanol acted as a cryosensitiser and a cryoprotectant at different stages (Table 2-8). A cryoprotectant refers to the ability of the compound to protect the cell against damage due to cold (cryogenic conditions). A cryosensitizer is a substance that makes the cell susceptible to low temperatures. Lewis et al. (1994) assumed that an increase in cryoprotection with an increase in ethanol concentration was related to ethanol increasing permeability of the plasma membrane enabling the rapid efflux of water during freezing, thus preventing formation of intracellular ice crystals. The cooling rates for most brewery heat exchangers exceed 25^{o}C.min^{-1} (0.4^{o}C.s^{-1}). The average cooling rate for the heat exchanger used for cooling yeast from the FV at SAB Miller (Newlands, Cape Town) was 138^{o}C min^{-1} (2.3^{o}C.s^{-1}) (Nkosi, 2001; Hofmann, 2004). Based on results shown in Table 2-8, it is postulated that, at faster cooling rates, ethanol acts at as a cryoprotectant.

Table 2-8: Effects of ethanol concentration in protecting yeast during cooling (Lewis *et al*., 1994).

Ethanol concentration ($g.l^{-1}$)	**Cooling rate ($^{o}C.min^{-1}$)**	**Ethanol Effect**	**Viability**
4.7 to 92	3	cryosensitizer	Viability of yeast decreased as compared to cooled control yeast grown in growth media
4.7 to 92	25	cryoprotectant	No results shown
92	200	cryoprotectant	61 % viability compared to 1 % of cooled control yeast in growth media

2.5.1.3 Cold shock protection by trehalose

Trehalose accumulated in yeast subjected to temperatures below 10°C (Kandror *et al*., 2004; Kandror *et al*., 2002; Nkosi, 2001). Moreover, trehalose played a key role in protecting components of the cytosol against adverse conditions such as osmotic shock, heat shock and nutrient limitations (Wiemken, 1990; Attfield *et al*., 1994). During cooling, the plasma membrane changes from liquid to gel state. Kandror and co-workers (2004) exposed a mid log phase sub-culture of *S. cerevisiae (*wild-type and trehalose deficient mutant) to mild lower temperature (0 to 4°C) for 48 hours prior to severe freezing at -20°C for 5 days. Trehalose and other heat shock proteins (including HSP12) were induced on pre-stress and protected yeast against low temperature and freezing. After 5 days of freezing, the viability of wild-type and trehalose deficient mutants exposed to 4°C were 70 % and 25 % respectively (Figure 2.7).

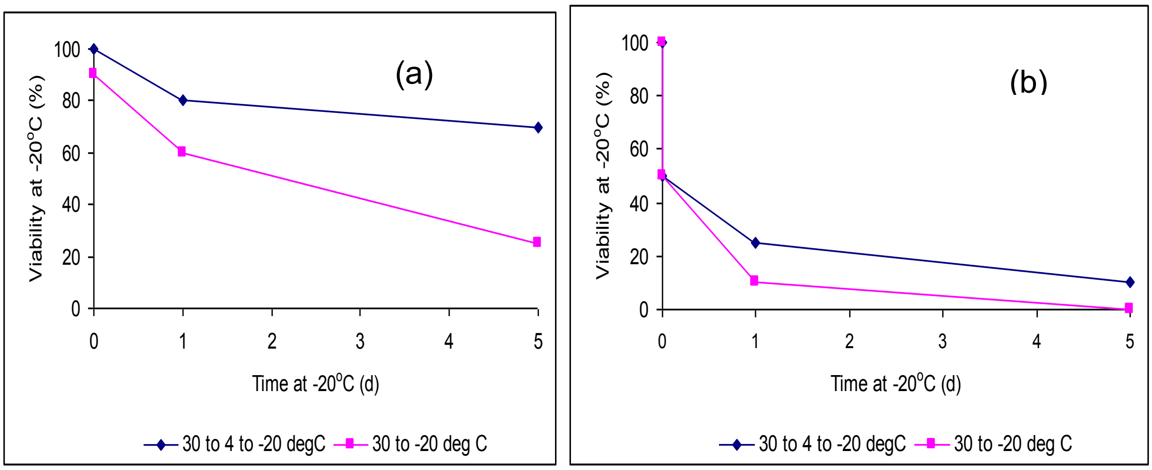


Figure 2-7: Wild-type (a) and trehalose deficient mutant (TPS1, 2) (b) cells were grown until mid-log phase at 30°C, and each culture divided into two. One portion was shifted to 4°C, and after 48 hours, cells were frozen at -20°C. The percent of viable cells was determined after different times at -20°C. The other portion was transferred from 30°C to -20°C and the percent of viable cells was also determined (Kandror *et al*., 2004).

Sales *et al*. (2000) reported that trehalose maintained the membrane integrity of cells upon changes in lipid bi-layer fluidity. Trehalose was assumed to decrease the transition temperature (T_m) for the phospholipid by interacting with the phospholipid head groups thus stabilizing the phospholipid bi-layers. The effect of trehalose in maintaining liposomal membrane integrity was investigated using liposomes incorporating stearylamine and encapsulating calcein (Sales *et al*., 2000) then diluted into PBS containing various concentrations of trehalose. The liposomal membrane was subjected to desiccation prior to rehydration. Desiccation was introduced by drying 10 nmoles of lipid using a speedy-vac at 30°C. The sample temperature was maintained between 28°C and 31°C throughout the dessication process. The liposomes were then diluted into the phosphate buffered saline (PBS) containing trehalose. The liposome incorporating trehalose that was subjected to dessication maintained 70 % of the membrane integrity compared to the lower membrane integrity of the control liposome without trehalose incorporated (the % membrane integrity was not mentioned). Trehalose concentrations up to 30 µg trehalose per µg phospholipid were prepared. A linear relationship between trehalose content during dessication and the structural integrity after dehydration was obtained up to a concentration of 12 *µ*g trehalose per *u*g phospholipid. Further investigation was carried out to see whether HSP12 could replace the role of trehalose after dessication and rehydration. About 77 % of the membrane integrity was maintained upon rehydration in the presence of HSP12 and absence of trehalose compared to the control yeast without HSP12 or trehalose incorporated. The linear relationship between the structural integrity maintained after rehydration and the HSP12 content during dessication was found up to a concentration of 15 *µ*g HSP per *µ*g phospholipid. Further work showed that the liposomal membrane integrity was better maintained when either HSP12 or trehalose was encapsulated in the liposome both internally and externally. However, work comparing trehalose and HSP12 showed the ability of trehalose to replace the role of HSP12 completely when trehalose was encapsulated on the external surface of the membrane. Trehalose and HSP were assumed to prevent fusion between adjacent membrane phospholipids attributed to osmotic and cold stress-induced membrane desiccation, resulting in improved membrane fluidity.

Kandror et al. (2004) compared the concentration of trehalose in yeast cooled at 4°C and 0°C. Since trehalose increases at near freezing temperatures, the effect of cell adaptation at near freezing temperature on the cell recovery after freezing was also investigated. In the experiment, the wild type and the trehalose deficient mutant grown at 30°C till mid log phase were shifted to 0°C to 4°C for 48 hours prior to being frozen at –20°C for 5 days. The viability of the cells that were shifted to 0 to 4°C prior to freezing was about 70 % compared to the control yeast (not shifted to 0°C to 4°C for 48 hours prior to severe freezing) for which 25 % viability resulted (Table 2.9). Pre-adaptation at 4°C showed better protective results than at 0°C (data were not shown). The mechanism used by trehalose to protect yeast at low temperature was still not understood since some of the cell proteins were damaged at near-freezing temperature.

Table 2.9: Cell viability of yeast that was frozen at -20°C for 5 days. Prior to freezing, some of the cells were shifted to 0 to 4°C. Control cells were frozen without being shifted to 0 to 4°C (Kandror *et al.,* 2004).

Type of cells	Viability (shifted to 0- 4°C)	Viability (control cells)
Wild-type	70 %	25 %
Trehalose deficient	dead	10 %

2.5.2 Lethal injury

Under normal conditions the yeast cell membrane remains in a liquid crystalline phase. Phospholipids with unsaturated fatty acids have greater flexibility compared to saturated fatty acids. At low temperature the cell alters the level of fatty acid saturation, reduces the synthesis of sterols and produces molecular chaperones so as to preserve its fluidity (Russel, 1990). Walker (1998) showed that cells exposed to low temperature exhibited reduced sterol synthesis, weakening the cell membrane and compromising cell integrity.

2.6 RESEARCH PROBLEMS AND KEY QUESTIONS

Following the reviewed literature, a number of conclusions may be made. The cell wall is the site of the primary contact of the cell with its surrounding environment; hence its flexibility and adaptation capacity determines the survival of the cell. The yeast carbohydrates play a major role in yeast survival in terms of providing maintenance energy and providing yeast protection during adverse conditions. The survival of yeast during adverse conditions is also influenced by the level of cryoprotectants expressed (e.g. HSP and trehalose). Fast cooled yeast is more sensitive to loss of membrane integrity compared to slow cooled yeast. Excessive hydrodynamic shear on yeast decreases cell growth; compromises cell membrane integrity and reduces yeast flocculation during fermentation. Damage of yeast due to hydrodynamic shear depends on the physiological state of yeast, cell growth phase and magnitude of the force applied. The toxicity of ethanol in the yeast cell can manifest itself by inhibiting cell growth and causing membrane leakage. However, *S. cerevisiae* was more tolerant to ethanol stress compared to other non-*Saccharomyces* species. Survival of yeast in a concentrated ethanol environment was dependent on the concentration of trehalose and HSP12. The maintenance of membrane integrity by HSP12 decreased with an increase in ethanol concentration. Moreover, the presence of HSP12 was assumed to lead to increased water content during dessication as a result of some water molecules being tightly bound to the charged groups of the HSP12 hence leading to a stabilized membrane configuration (Sales *et al.,* 2000).

In this study, the focus is on the maintenance of yeast quality during cooling. The aim of this study is to identify the cooling rate that is least detrimental to yeast physiology and structure allowing yeast and beer quality to be maintained. The response of yeast to heat shock is investigated. Moreover, the relationship between cooling rates, osmotic strength of the media and cell wall flexibility is developed. Building on the literature review presented above, this research aims to provide further understanding of the effect of cold shock on yeast quality by addressing the following key questions:

- What cooling rate is least detrimental to brewing yeast in terms of viability, cell envelope integrity, cell flexibility, metabolic activity and, lastly, recovery following cold shock injuries?
- What is the effect of yeast consistency on the fermentation performance of cooled yeast?
- What is the effect of diluent on the fermentation performance of cooled yeast?
- What is the effect of ethanol on exposure to cold stress on the fermentation performance?

At the onset of this study, it was hypothesized that:

- Yeast cooled at a low cooling rate will yield better fermentation performance than yeast cooled at rates closer to the critical cooling rate, previously proposed as $2^{o}C.s^{-1}$ (Nkosi, 2001).
- Flexibility of the cell envelope increases with an increase in cooling and heating rate.
- Yeast structural strength is indirectly proportional to the cooling rate beyond a critical rate.
- Haze generation is aggravated at higher cooling rate.
- Lower cell viability, cell growth and fermentation performance are expected on yeast cooled in the presence of beer, compared to that cooled in a suspension where the beer is diluted.

CHAPTER 3: METHODOLOGY

This chapter defines assays selected to identify, characterize and quantify loss of yeast quality and the physiological stress indicators. A summary of experiments and their controls is also given. Section 3.1 describes the various physiological states of yeast (healthy yeast, physiologically stressed yeast, replicatively deactivated yeast, cells with minor damage to cell envelope, dead yeast cells and disrupted yeast cells) and their influence on fermentation profile. Section 3.2 covers the principle of the assays and motivates their selection for measuring yeast metabolic activity, physiological stress indicators and minor envelope damage (cell wall and membrane). Furthermore, the chapter describes the analytical techniques selected to quantify yeast quality and performance. Section 3.3 describes the experimental approach and design followed throughout the research project. Section 3.4 gives materials and methods applied to investigate the effect of cooling on fermentation performance and cell wall flexibility. This includes of yeast collection at the brewery, cooling experiment protocol in the laboratory, culture preparation, measuring the consistency and measuring the viscosity of the yeast suspension. Section 3.6 covers the experimental protocol used for cooling yeast in the laboratory and all the control experiments.

Loss of yeast quality can occur through loss of integrity, viability, vitality or a combination of these (Avis, 1990; Ormrod *et al*, 1991; Basson *et al*., 1997). Cell integrity is defined as the intactness of the cell envelope. Cell viability is defined as the ability of cells to grow and reproduce; losses of viability may occur via loss of structural integrity, cell death or replicative deactivation (Jones, 1987; Lentini, 1993 as cited in Basson, 1996). Cell vitality is a term used within the brewing industry to describe the metabolic activity of yeast. Loss of vitality is displayed by changes in flux through metabolic pathways. Further changes in partitioning between the metabolic pathways may occur.

In this study, the yeast flow rates through the cooling coil were varied to obtain cooling rates between 0.1 and $4^{o}C.s^{-1}$. Similarly the heating rates obtained for undiluted yeast ranged between 0.1 and $5^{o}C.s^{-1}$. In order to characterize loss of yeast quality accurately, it is important that the physiological states of yeast be defined and knowledge of the resultant yeast performance of each physiological state subsequent to various stresses is required.

3.1 PHYSIOLOGICAL STATES OF YEAST

Yeast cells respond differently to stress based on their physiological state as well as the type and extent of stress experienced. Basson (1996) defined the factors which determine the aspects of yeast quality as follows:

- The rate and extent of biomass growth

- The ability to reproduce
- The overall metabolic rate of yeast
- The integrity of the cell envelope
- The ability of yeast to withstand stress
- The rate of various metabolic pathways, influencing the flavour compounds formed
- The flocculation and sedimentation characteristics of the yeast

Loss of yeast quality is progressive, moving through differing degrees, as illustrated in Figure 3-1. The physiological states of yeast identified in Figure 3-1 are classified into the categories described below. Further, the nature of stress experienced together with the potential assay used to predict the impact of stress are shown.

Healthy yeast

The cells are metabolically active and able to withstand stress conditions. When inoculated into media, they have the ability to grow and reproduce. Cells are adequately intact so that intracellular or wall associated materials are not released into the product.

Physiologically stressed cells

The cells are intact, able to grow and reproduce, but show signs of reduced metabolic rates. Fermentation is slower and the flavour profile may be different. The cells may also be characterized by altered levels of intracellular storage compounds and stress resistance compounds, such as glycogen, trehalose and heat shock proteins (HSP).

Replicatively deactivated yeast

The cells do not have the ability to reproduce due to impaired membrane integrity. While this is the first in a number of steps leading to cell death, it may be reversible, permitting cells to return to a replicating state after a period of adaptation. These cells also have complete or partial metabolic activity.

Cells with minor damage to the cell envelope

Cells with the envelope damage appear intact and are not necessarily poorer in terms of fermentation performance. However, the presence of these cells may negatively affect beer owing to release of haze materials, protease and other intracellular compounds influencing the flavour profile. This represents the least extreme damage to yeast.

Dead yeast cells

These cells are unable to reproduce and show no metabolic activity. Given time, these cells go through autolysis.

Disrupted cells

These cells are partially (still visible as cellular entities under the microscope) or completely (visible only as fragments) disrupted. Metabolic activity as an integrated system has ceased and intracellular compounds are released into the beer. This represents the most extreme damage to yeast.

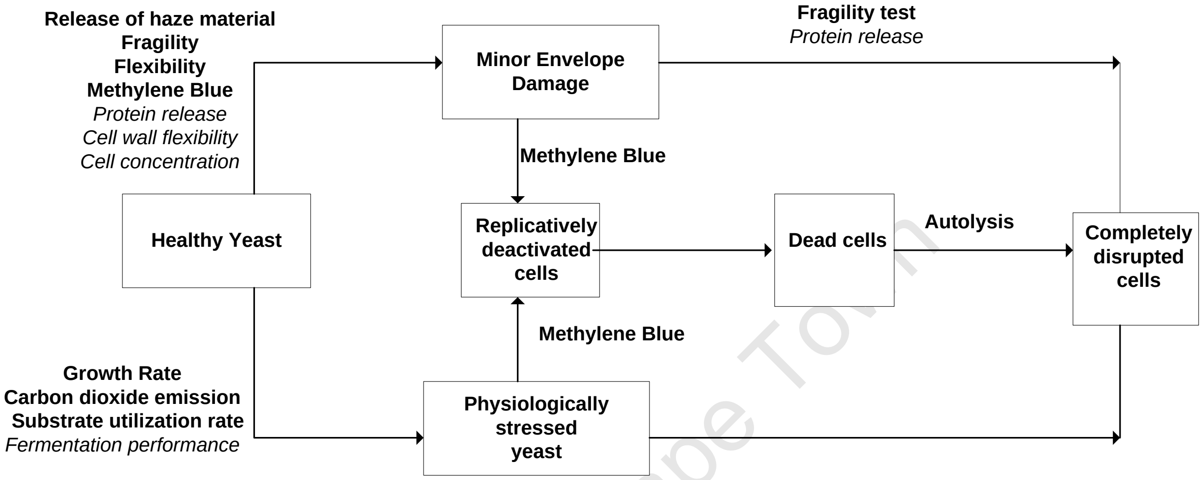


Figure 3-1: Proposed scheme for a loss of yeast quality (Basson et al., 1997) within the brewery yeast circuit. The blocks described physiological states of yeast identified. The text in italics indicates the manner in which the stress response is mediated. The text in bold indicates potential assay methods for quantification of the change experienced.

3.2 THE PRINCIPLE AND MOTIVATION FOR THE ASSAYS USED

3.2.1 Yeast metabolic activity

Cold and heat stress are likely to influence the fermentation performance in the context of yeast growth and substrate utilization upon inoculation. In this study, the accelerated fermentation method was chosen to monitor the changes in yeast viability and vitality due to cooling.

3.2.1.1 Accelerated small-scale fermentation

Large-scale brewery fermentation takes approximately 2 weeks to complete hence it is time consuming to monitor the fermentation performance of pitching yeast in the brewery (Henschke and Eglinton, 1991). The use of EBC fermentation tubes for laboratory fermentation requires a large sample volume and data generation requires 7 to 10 days (Basson, 1996).

Accelerated small-scale fermentation was developed by D’Amore *et al*. (1991) to measure the metabolic activity of yeast. The fermentation was conducted in Erlenmeyer flasks at optimum temperature for metabolic activity. The flasks were agitated throughout the fermentation period of 24 hours (Lentini, 1993). A similar approach was used successfully by Nkosi (2001). Lentini (1993) analysed progress of the small-scale fermentation by the loss of weight due to CO_2 production over the 24 hour period, as an indicator of the physiological condition and fermentative ability of yeast. At the University of Cape Town, the analytical approach was extended to include CO_2 evolution, yeast growth rate based on cell number, glucose utilization and biomass yield as cell dry mass per unit glucose (Robinson, 2001; Nkosi, 2001; Daramola, 2004).

For fermentation, sufficient inoculum ensures an acceptable rate of attenuation. Robinson (2001) reported the standard pitching rate of yeast at SAB Miller Newlands brewery as 14 g of 100% viable wet yeast per liter of wort. In small-scale fermentation, lower pitching rates were used in order to detect more subtle changes in yeast performance. The inoculation rate was maintained at 3.5 g of 100 % viable wet yeast per litre of wort i.e. a quarter of the standard pitching rate (Robinson, 2001).

In this study, subsequent to each stress experiment, the yeast consistency (**C**) and the viability (**V**) of the cold stressed yeast suspension were measured to determine the required inoculum for a specific volume of growth media. The pitching rate (**P**) was calculated using Equation 3.1

$$\mathbf{P} = [3.5 / (\mathbf{v} \times \mathbf{C})] \text{ X Volume of sterile growth media (L)} \qquad 3\text{-}1$$

where P is the pitching rate ($g.100ml^{-1}$), v is the viability of yeast following subjection to cold shock (%) and C is the yeast consistency (%).

3.2.2 Minor envelope damage (cell wall)

Damage to the cell wall may result in the release in glucans and mannan into beer (Klis, 1994). In this study, the two methods were used to measure damage to the yeast cell wall due to hydrodynamic stress: the release of haze and fragility analysis.

3.2.2.1 Haze analysis

Yeast can encounter shear stress when it is pumped, transferred, centrifuged or agitated. Shear is postulated to cause rearrangement or loss of surface molecules from the cell wall, resulting in haze. Such change in cell surface molecules was demonstrated by Robinson (2001). Haze is observed as the presence of sub-micron particles in beer. The presence of haze in beer results in loss of beer quality, sedimentation and altered flocculation.

Work done by Siebert *et al.* (1987) has shown a reduction in filtration performance of yeast following centrifugation owing to haze formation. Lewis and Poerwantaro (1991) showed haze release from the cell wall upon agitation. Haze resulting from cell wall damage is comprised of glucan and mannan particles. However, the presence of glucan in haze does not necessarily confirm damage incurred by yeast because glucan is also found in the husk of barley. Unlike glucan, the presence of mannan in beer is associated with a damaged cell wall because mannan does not form part of the wort. The release of surface molecules from the yeast cell wall into beer due to attrition has been studied extensively (Robinson, 2001; Lewis and Poerwantaro, 1991; Siebert et *al*., 1987). The presence of haze may be detected by measuring the particle size distribution, using dye staining to determine the nature of haze (proteinaceous or carbohydrate) and using the enzyme assay test to identify and quantify the carbohydrate.

After yeast was removed from suspension by centrifugation at 2500 rpm for 3 minutes (low speed used to ensure that no extra haze is generated by the centrifuge), the sample could be analysed for haze using Malvern mastersizer (long version 2). The size range of haze was shown to be between 0.08 µm and 2 µm (Robinson, 2001). The size distribution analysed in assessing the haze concentrate showed some trace yeast (3–13 µm) in the haze concentrate. Moreover, the particle size range of 13-500 µm observed was attributed to aggregate formation. When the size distribution data were analysed on a volume basis, the smaller haze particles were overshadowed by yeast cells. Valuable data were obtained by converting the particle size distribution from a volume basis into the particle number basis. Robinson (2001) used the percentage particle number to analyse haze.

In this study, in order to measure the presence of haze in beer, the size distribution of sub-micron particles over a broad range was examined using Malvern Mastersizer (long bed version 2), based on laser light scattering. Smaller particles were found to scatter light at a greater angle whereas larger particles allow light to pass through almost undeflected. Using this technique, it is possible to calculate the concentration of particles for a given size range by detecting the angle and intensity of scattered light. The Malvern Mastersizer (long bed version 2) was equipped with a lens of 300 mm diameter and can detect particles in the size range of 0.05 to 850 µm i.e. both yeast and small haze particles.

3.2.2.2 Yeast fragility quantified through ease of cell disruption

Shear stress on the yeast cell wall can be experienced during mechanical handling. Excessive shear was demonstrated to lead to damage to the cell wall or cell membrane, ultimately leading to a release of surface molecules or intracellular solutes due to rupture of the cell wall. The release of intracellular contents using the French Press pressure cell was shown to be dependent on the operating pressure, operating temperature and the initial cell concentration (Harrison, 1991).

Fragility analysis was assessed by means of the ease of disruption of the cell envelope. In this study, the French Press pressure cell was used to quantify the mechanical resilience against shear of cold shocked yeast. This equipment was operated by rapid release of pressure causing a shear environment. The French press can be operated over a range of cell damage conditions from sublytic to lytic by varying the operating pressure of the cell using a hand driven pump and flow control valve. In this study, the release of intracellular protein from yeast is quantified on exposure to conditions considered sublytic to high quality yeast.

3.2.2.3 Cell wall flexibility

Previously HSP12, located in the cell wall and cytoplasmic membrane, was shown to be one of the numerous factors contributing to the resilience of yeast at low temperatures. Further HSP12 is up-regulated on exposure to cold stress (Nkosi, 2001, Kandror *et al*., 2004). The various techniques used to induce HSP12 have been explained in Sections 2.5.2.4 and 2.5.2.5. HSP12 was demonstrated to protect yeast against desiccation. It is therefore reasonable to postulate that the flexibility of the cell wall plays an important role in enhancing post-cold stress survival. HSP12 was postulated to form a hydrophilic net around the membrane thus controlling the efflux of intracellular water when yeast is placed near freezing temperatures. This hydrophilic net is thought to be formed by the hydrogen bonding with proteins and glycolipids in the membrane (Sales *et al*., 2000). In this study the flexibility test was carried out using three different liquid solutions of varying osmolality; namely, hypertonic (650 mM NaCl and 10 mM Tris), isotonic (150 mM NaCl and 10 mM Tris) and hypotonic solution (water).

3.2.3 Minor envelope damage (cell membrane)

3.2.3.1 Methylene violet staining

Fermentation performance was found to depend largely on concentration of cells in the inoculum and their ability to grow (SAB, 1993). Cell concentration can be determined by direct cell count. Methylene blue is reported to be used industrially (in breweries) to measure viability (Boyd *et al*., 2003).

Methylene blue staining is relatively inexpensive, easy to perform, measures impermeability of the cell and it is suitable for the following physiological states: healthy (exponential and stationary phase population) and stressed yeast (i.e. starved in water). Commercially available methylene blue stain contains impurities (Conn, 1977) consisting of two or more dye molecules (azure B, a light blue dye, Bernthsen methylene violet, and, to a lesser extent, the lower azures). These impurities result from the oxidative demethylation of methylene blue in aged stock solution. Methylene blue was also reported to give inaccurate results when used in yeast which has been declared non-replicative by prolonged storage (Parkkinen *et al*., 1976).

Methylene violet (3 RAX) is preferred over methylene blue (Smart *et al.,* 1999). The advantages of using methylene violet are that it exhibits great resistance to oxidative demethylation and enhances a reduction in variation of color intensity for the dead and dying population, allowing clear differentiation between stained and unstained cells e.g. living and dead cells.

3.3 EXPERIMENTAL APPROACH AND EXPERIMENTAL DESIGN

Cooling rate is a function of the driving force and heat transfer resistance. The driving force can be defined in terms of the temperature difference between the coolant and the fluid being cooled. The physical property of the fluid influences the heat transfer coefficient.

When cooling or heating, yeast with a lower consistency (concentration) cools at a faster rate than its higher consistency counterpart due to resistance to heat transfer and influence on viscosity. In this study, yeast consistency was varied between 30 and 60 %. Diluents used to vary yeast consistency were beer, phosphate buffered saline (PBS) and a mixture of beer and PBS. The effect of diluent on yeast quality was studied by varying the type of diluent. PBS is an isotonic buffer hence the cell morphology is not expected to be affected by PBS. Prior to diluting yeast with PBS, the suspension was centrifuged to remove yeast from beer. The effect of the hydrodynamic shear on yeast due to centrifugation was analysed in terms of cell growth, carbon dioxide emission, residual substrate utilization and cell wall integrity. Damage on yeast due to hydrodynamic shear was shown previously to depend on the physiological state of yeast, cell growth phase and magnitude of the force applied (Basson, 1996; Robinson, 2001). The presence of extracellular fragments in beer was measured using the haze assay. The influence of cooling or heating on the flexibility of the cell wall was measured by quantifying the concentration of HSP12 expressed upon cooling the yeast and by measuring the variation in yeast cell size on suspension in buffers of varying osmolality.

The experiments were divided into three separate phases. The first phase studied the effect of cooling rate on yeast quality. The yeast consistency was kept constant whilst the flow rate of the yeast suspension through the cooling coil was varied. Cooling rates were varied using linear flow rates of 0.23 to 0.50 $m.s^{-1}$ corresponding to cooling rates of 1.02

and 2.15^{o}C.s^{-1}. The second phase of experiments studies the effect of heating rate or cooling rate on cell envelope flexibility. Heating rates were studied over the range of 0.5^{o}C.s^{-1} to 4.6^{o}C.s^{-1}. The third phase of experiments studied the effect of diluent and the effect of yeast consistency on yeast quality. For all cooled samples of yeast, the yeast quality was analysed in terms of the cell count, residual substrate assay, carbon dioxide emission, cell wall fragility and cell wall flexibility. Materials and methods of the above experimental design are laid out in Section 3.4.

3.4 MATERIALS AND METHODS

3.4.1 Strains used and culture condition

The commercial yeast strain SAB 5 was obtained from the SAB Miller, Newlands Brewery, Cape Town, South Africa. The yeast was obtained on cropping from vessels fermenting Castle lager and Black Label brands. The yeast was collected from the transfer lines subsequent to end of fermentation and prior to chilling using the brewery heat exchanger. The generation number of yeast used ranged from one to six. The yeast temperature at this point of cropping was routinely maintained between 14 and 17^{o}C.

3.4.2 Point of yeast collection

In order to minimize the influence of change in pressure during sampling on yeast collected, the brewery yeast was collected at a location of low pressure. At the end of the fermentation cycle, lager yeast collects at the bottom of the 3000 hl conical shaped fermentation vessel (FV) under a hydrostatic pressure of about 150 to 200 kPa owing to a liquid height of 18m. Yeast is cropped from the FV using a rotary lobe pump and passes through 85 to 145 m of pipework of internal diameter of 65 mm at a maximum linear velocity of 0.5 m.s^{-1} (Basson, 1996). During cropping at Newlands Brewery, the density, viscosity and Reynolds numbers of brewers yeast are estimated as 1050 kg.m^{3}, 88 cP and 260-340 respectively (Basson, 1996). Both the chiller and the yeast collection vessels are located in the yeast room maintained at 10^{o}C. There are two points at which one can collect unchilled yeast at the end of the brewery fermentation. The first location of collection is directly at the base of the FV where the yeast is at a hydrostatic pressure of about 260 kPa and the second location is in the yeast handling room prior to yeast being chilled. Here the yeast pressure has been reduced to 180 kPa. For this study, yeast was collected at the latter location aseptically into sterile sample bottles.

3.4.3 Culture preparation

Yeast was collected from the brewery and divided into three different aliquots for dilution with different diluents. The effect of diluent and shear on the quality of cooled yeast was compared. The first aliquot was diluted with PBS to lower the ethanol level (Condition 1). The second aliquot was diluted with beer (ethanol containing supernatant) (Condition 2).

The selection of this diluent allowed investigation of the effect of biomass consistency on yeast quality while maintaining constant ethanol toxicity. The third aliquot of yeast was centrifuged to remove all beer. This was replaced with PBS to investigate the effect of biomass consistency in the absence of ethanol and shear force on yeast quality (Condition 3). The effect of diluent or ethanol toxicity on yeast quality was compared using aliquots 1 and 2. The effect of shear and ethanol concentration on yeast quality compared using aliquots 1 or 2 and 3. For each aliquot, three different consistencies were prepared, namely; 30 %, 40 % and 60 %, to investigate the effect of biomass consistency on yeast quality.

3.4.4 Thermal shock of yeast

A 100ml aliquot of yeast suspension was pumped through a 2.75 m (8 mm internal diameter) copper coil at varying flowrate using a Watson- Marlow 313S peristaltic pump. The cooling coil was attached to silicone tubing (8 mm internal diameter) at both ends with flexible tubing running to each reservoir, as shown in Figure 3.2. An ethylene glycol solution (1 ethylene glycol:1 distilled water), set at -1°C, was used as the coolant in the cooling chamber in which the cooling coil was placed. Yeast reservoir 1 was a 500 ml glass beaker. This was kept covered using aluminum foil. Yeast reservoir 2 was a 100 ml measuring cylinder. The temperature probes were used to measure the temperature at the entry and exit of the yeast reservoirs.

Three forms of control experiment were used. As a control with respect to cooling, control yeast was maintained at about 14°C without being pumped through the laboratory cooling equipment. Cooling rates were varied by varying both pump speeds (15-100 % of maximum flowrate) and yeast consistency. The cooling rates achieved were between 0.1 and 4°C.s^{-1}.

Prior to cooling yeast, the yeast was diluted with three different diluents to investigate the effect of diluent on small-scale fermentation. The diluents used were PBS, beer (ethanol) and a mixture of PBS and beer. Ethanol concentration across all samples was between 8.0 % (v/v) to 4.0 % (v/v). The results obtained are used to relate ethanol toxicity to yeast quality subsequent to cooling. Prior to diluting the wet yeast pellet with PBS, the yeast was centrifuged. A control experiment with respect to hydrodynamic stress was included in the study in which the yeast suspension was pumped through the cooling equipment at ambient temperature. Where heating rates were investigated, the cooling bath was replaced by a water bath set at 30°C or 34°C. The heating rates achieved were between 0.5 and 5°C.s^{-1}.

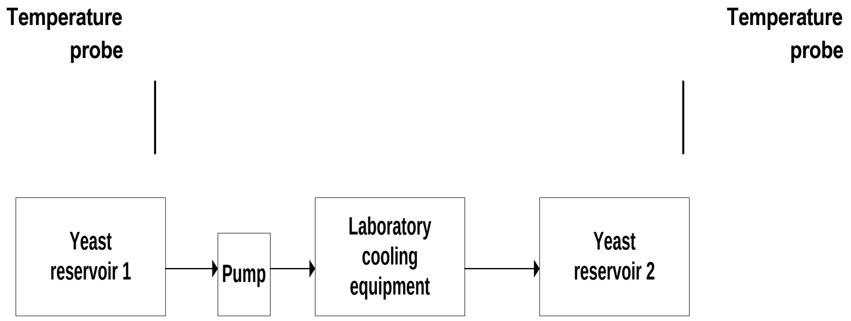


Figure 3-2: The laboratory yeast cooling apparatus. Glycerol was used as the coolant (50:50 dilution in water). The coolant temperature was set at -1°C. Yeast was cooled from 14°C by pumping from reservoir 1 via the laboratory cooling equipment to the reservoir 2.The temperature probes were inserted in each reservoir to measure the difference in temperature of the uncooled yeast (reservoir 1) and cooled yeast (reservoir 2).

Subsequent to carrying out each heating and cooling experiments, the following analyses were carried out. The cooled and uncooled yeast were used for small-scale fermentation. Assays carried out during the fermentation included cell counts, substrate utilization, carbon dioxide evolution and metabolic activity. Cell count results quantify the effect of cooling rate on cell growth during fermentation. The residual substrate assay was used to quantify the rate of substrate utilization and biomass yield. The carbon dioxide evolution was used as a measure of yeast vitality i.e. the metabolic activity of the cells during fermentation.

In the second experiment, the effect of yeast consistency on yeast quality during cooling was studied. The response was measured in terms of yeast viability, growth rate, substrate utilization, carbon dioxide evolution and metabolic activity. The list of all experiments and their controls is shown in Table 3 -1. The assays methods and their controls are shown in Table 3.2.

Studies have shown heat shock proteins to be induced by either cooling or heating of yeast within a specified temperature range (Nkosi, 2001; Miller *et al*., 1979 cited in a review by Mager and Moradas Ferreira, 1993; McAlister *et al*., 1979 cited in a review by Mager and Moradas Ferreira, 1993; Mtwisha *et al*., 1998). Furthermore, Motshwene *et al*. (2004) showed that cell wall flexibility is directly proportional to the concentration of heat shock protein 12. In this study, the effect of cooling and heating rates on cell wall flexibility was investigated. Cooling and heating rates in the range 0.1 to $5^{o}C.s^{-1}$ were used. Malvern diluents used were the hypertonic salt solution, isotonic salt solution and water. The effect of the temperature gradient between the Malvern diluent and the injected yeast sample on cooled yeast was investigated in order to determine whether the change in flexibility observed was due to the cold stress imposed in the experiment or whether this was dominated by the temperature gradient between the Malvern diluent and the injected sample. Moreover, the effect of cooling on the structural strength of the yeast cell is assessed through haze formation and the amount of protein released under sub-lytic conditions.

Table 3 -1: The main experiments and their controls.

Type of experiment	Objective	Control
Cooling and heating yeast	Investigate the effect of cooling or heating rates on yeast quality e.g. cell wall flexibility, metabolic activity etc.	Maintaining yeast at 14°C
Diluting yeast in various diluents prior to cooling. Diluents used beer, (PBS) and a mixture of PBS and beer.	Investigate the effect of ethanol toxicity on yeast quality	Yeast diluted in the Phosphate Buffered Saline (PBS)
Centrifuging yeast and re-suspending the pellet in ethanol (beer).	Investigate the effect of the hydrodynamic shear on the yeast quality e.g. Cell membrane integrity	Diluting yeast in ethanol (beer). Resuspending yeast in ethanol without prior introduction to shear.

Table 3-2: Assay methods and their controls

Type of experiment	Objective	Control
Small-scale fermentation of cooled yeast	Investigate the effect of cold stress on the metabolic activity of yeast, yeast vitality and yeast viability	Fermenting uncooled yeast
Vitality test during the small-scale fermentation	Investigate the effect of cooling on yeast vitality by measuring the rate of carbon-dioxide evolved.	Water evaporation
Fragility test	Investigate the effect of cooling or heating on the cell membrane fragility	Non-disrupted cells
Flexibility test	Investigate the effect of cooling or heating on cell wall flexibility.	Maintaining Malvern diluent at 14°C for yeast cooled < 10°C and maintaining a zero temperature gradient between the Malvern diluent and injected heated yeast.

3.4.5 Measuring consistency of the yeast suspension

An aliquot of 1 ml yeast suspension was pipetted into an Eppendorf microfuge tube, pre-weighed to a 4th decimal place (w_1). The yeast was sedimented using a laboratory bench micro-centrifuge for 10 minutes at 3144 x g. The combined mass of Eppendorf tube and yeast was measured prior to decanting the supernatant (w_2). The combined mass of Eppendorf tube and wet pellet of yeast was then measured (w_3). Consistency was determined as the biomass concentration of a yeast suspension expressed as the percentage wet mass as indicated by Equation 3-2. The consistency was measured in triplicate. The typical coefficient of variance was 0.001.

$$\textbf{Consistency} = (w_3-w_1)/\ (w_2-w_1) \times 100 \qquad \text{3-2}$$

3.5 ANALYTICAL TECHNIQUES

Two categories of analytical techniques were used to assess the yeast: those measuring loss of yeast quality in terms of yeast performance and those measuring the molecular response of yeast upon cold stress. The selected assays are shown in Table 3.3. The principle of each assay is given in Section 3.2

Table 3-3: Summary of the yeast quality assays to determine physiological states of brewers yeast.

Yeast Quality indicator	Selected Assays
Minor envelope damage (cell wall)	- Haze (Particle size distribution) - Yeast fragility (French Press pressure cell)
Minor envelope damage (cell membrane)	- Methylene violet viability stain (Direct cell count)
Cell reproduction and cell death	- Methylene violet stain (Direct cell count)
Yeast metabolic activity	- Small scale fermentation (Cell growth rate, Biomass yield, Carbon dioxide evolution, Substrate utilisation)
Physiological stress indicators	- Heat Shock protein (HSP) 12 - Cell wall flexibility test

3.5.1 Minor Envelope damage

3.5.1.1 Haze

Yeast from the control and stressed yeast suspensions were sedimented by centrifuging the yeast for 5 minutes at 489 x *g* in a Beckman J-25 Avanti refrigerated centrifuge. The supernatant was recovered and a portion analysed for the presence of haze. The supernatant was deflocculated in the ultrasound bath of the Malvern Mastersizer for 2 minutes at 50 % power. The sample was then pumped continually at 50 % of the maximum stirrer speed and 50 % of the maximum flowrate through the measuring chamber where the presence of haze was determined by laser light scattering (Malvern Mastersizer, long bed version 2 equipped with a lens of 300 mm diameter that can detect particles in the size range of 0.05 to 850 μm). Data of volume % as a function of size range was obtained. To analyse he presence of haze particles, the data were converted from a volume-based size distribution to a number–based size distribution. Haze was estimated by evaluating particles in the size range between 0 and 2 μm (Robinson, 2001). Figure 3-3 (a) and (b) show the typical data obtained from yeast collected from the brewery following cropping then and maintained at 14°C. The volume distribution depicted in Figure 3-3 (a) shows that smaller haze particles are overshadowed by the yeast. For clear visualization of haze, valuable data was extracted by conversion of the data from volume-based distribution to a particle number basis as shown in Figure 3-3 (b). The conversion of particle size distribution by volume to number-based size distribution is shown in Equation 3-3.

$$f_M(x) = k_3 x^3 f_N(x) \tag{3-3}$$

where f_M (x) denotes the particle size distribution by mass (volume)

k_3 denotes a scaling factor used to normalize the area under the graph to 100 %.

x denotes the particle diameter

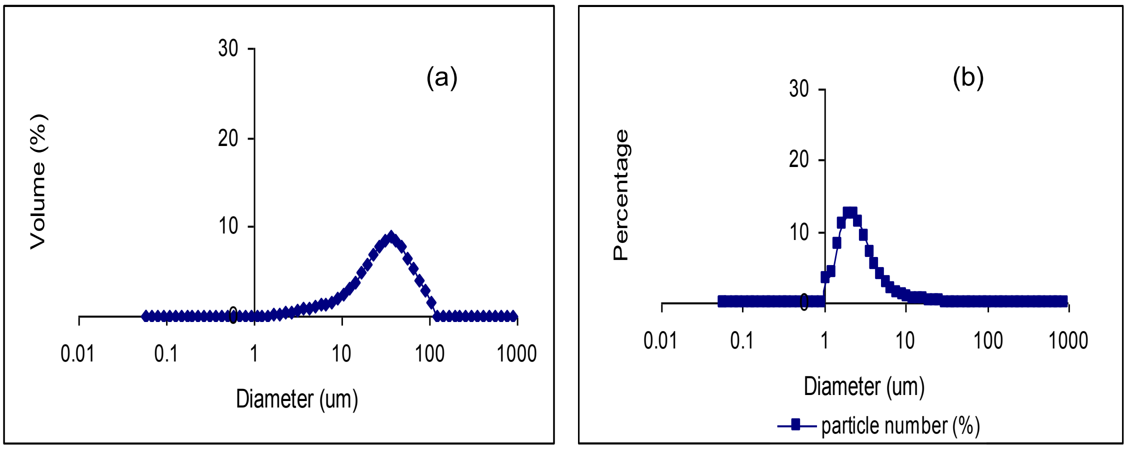


Figure 3-3: Size distribution of control yeast and haze measured through laser light scattering, presented on (a) a volume basis (b) a number basis. The control yeast sample was maintained at 14°C. k_3 was 4.04.

3.5.1.2 Fragility

The yeast was re-suspended in Phosphate Buffered Saline (PBS) at a mass ratio of 1 part yeast to 4 parts PBS within 1 hour of pumping yeast through the laboratory cooling rig. The temperature of PBS used was adjusted to the average final exit temperature of the thermally stressed yeast suspension prior to use. The yeast suspension was vortexed to ensure homogeneity.

An aliquot of 20 ml of the yeast suspension was required for cell damage using the French Press. The yeast sample was pressurized at 6 MPa (sublytic), 10 MPa and 16 MPa. Rapid pressure release was allowed through a narrow orifice. The treated cell suspensions were collected on release from the pressure cell.

Following exposure to rapid pressure drop, the sample of yeast was put on ice to prevent protein denaturation due to heating. Cell debris was sedimented in a micro-centrifuge for 10 minutes at 3144x *g*. The amount of intracellular protein released into the supernatant was measured at A_{280}, relative to the BSA standard curve. The standard deviation and coefficient of variance for duplicate samples were calculated to be 0.001 and 4 % respectively. The results of the standard curve for protein assay using Bovine Serum Albumin (BSA) quantified by absorbance at 280 *nm* is shown in Figure 3-4.

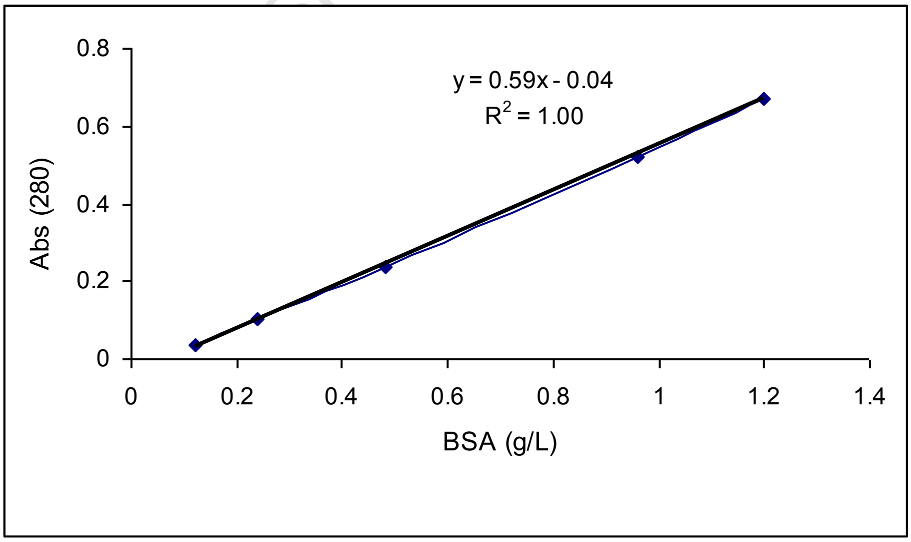


Figure 3-4: The standard protein curve based on A280. The protein standard BSA was dissolved in PBS. The y-error bars are not visible because the average standard deviation was 0.5 %.

3.5.1.3 Methylene violet viability

A suitable dilution of cells in methylene violet solution was prepared. The cells were counted differentially using a haemocytometer (Improved Neubauer, BS. 748, Weber Scientific International, England) viewed under a light microscope (Model: BX40F, Olympus Optical co. Ltd, Japan) using the 10x optical lens and 40x objective lens thus giving (400x magnification) with brightfield optics. Cells counted are classified into three: viable yeast cells not budding (transparent); viable budding cells (transparent) and dead cells (violet). The counting chamber (depth 0.1 mm, area 1/400 mm^2) was comprised of 25 identical blocks. In each block, there are 16 small squares. Cells falling on the left and top grid perimeter lines are included in the cell count along with all cells enclosed by the perimeter lines. Cells were typically counted across 5 of 25 blocks, counting an average of 200 cells to reduce statistical error. When used in conjunction with methylene violet staining (Sigma) (0.25 g.l^{-1} methylene violet), the cell concentration, budding index and viability may be determined simultaneously.

$$\text{Viability (\%)} = \mathbf{(N_{total} - N_{violet})/N_{total} * 100} \qquad 3\text{-}4$$

The total number of cells counted over the 25 blocks was used to determine the cell concentration in the 0.1 mm depth and 0.0025 mm^2 of the counting chamber.

$$\text{Cell concentration (Cells.ml}^{-1}\text{)} = \mathbf{N_{total}\ x\ dilution\ factor\ x1000/\ [0.0025\ x\ 0.1]} \qquad 3\text{-}5$$

$$\text{Budding index} = \mathbf{[N_{budded} / N_{total}]} \qquad 3\text{-}6$$

3.5.2 Yeast metabolic activity by small-scale fermentation

The inoculum taken from the treated yeast suspension (or control) was placed into 250 ml Erlenmeyer flasks containing the appropriate volume of media (120 ml total working volume) and incubated. The quantity of inoculum used was determined using Equation 3.1. The flask was stoppered using a silicone stopper. Gas evolved during fermentation was released from the flask via glass tubing filled with silica gel. The silica gel was used to trap in moisture from the flasks. Cotton wool was inserted at both ends of the glass tubing to provide an air filter. The flasks were incubated in the orbital shaker (160 rpm) for a period of 24 hours at 30°C.

3.5.2.1 Biomass measurement of growth

In order to determine growth of the yeast culture in the reactor, the population size was measured by a total cell count every second hour during the first 8 hours of fermentation.

The last sample was taken at the end of the 24 hour period. The cell count was used to determine the growth rate of the culture in the conical flasks. The standard deviation and coefficient of variance for replicate samples were calculated to be 0.004 and 2.42 % respectively. Biomass was also measured as dry mass. The wet pellet was washed three times to remove any salts before drying to constant mass at 80°C for 48 hours.

3.5.2.2 Carbon dioxide measurement and metabolic activity

On sampling, the mass of the flasks before and after sampling was recorded. Any mass loss observed between sampling was attributed to carbon dioxide evolution as control flasks illustrated minimal mass loss due to evaporation. The average amount of water evaporated in the control was less than 0.012 % of the initial volume. Moreover, the average mass loss due to water evaporated was 0.05 % of the mass loss due to carbon dioxide evolution, hence it is considered insignificant. The standard deviation and the coefficient of variance for evaporation in control flasks were 0.04 and 2.83 % respectively. The CO_2 emission rate provided a measure of metabolic activity over the 24 hour period.

3.5.2.3 Substrate utilisation

Supernatants collected from small scale fermentation were diluted accordingly. An aliquot of 200µL of the standard glucose solution or diluted sample and 600µl DNS were pipetted into a test tube. The mixture was heated in a water bath at 95°C for exactly 5 minutes to develop the red-brown colour, and then cooled on ice. An aliquot of 3200 µl of distilled water was added to each test tube and the absorbance measured against the blank at A_{510}. Glucose concentration was determined using a standard calibration curve generated using glucose concentrations from 0.002 to 0.1 $g.l^{-1}$. Residual reducing sugar concentration was measured by the dinitrosalicyclic acid (DNS) assay after incubation for various time intervals. The standard deviation and coefficient of variance for duplicate samples were calculated to be 0.01 and 6 % respectively.

3.5.3 Physiological stress indicators

3.5.3.1 Cell envelope flexibility test

The cell envelope flexibility test was carried out within 1 hour of pumping yeast through either the laboratory cooling equipment or the hot water bath to limit recovery from stress. In this study, the flexibility test was carried out using three different liquid solutions of varying osmolality: namely, hypertonic (650 mM NaCl and 10 mM Tris), medium salt solution (150 mM NaCl and 10 mM Tris) and hypotonic solution (water). An aliquot of 200 µL of yeast suspension was injected into 1 litre of the specified solution in Malvern bowl chamber. The effect of solution temperature of the assay on cell wall flexibility results was explored by altering both the exit temperature (T_{final}) of the temperature-stressed yeast and the temperature of the solution in the bowl. The sample was stirred and pumped continually at 70 % of the maximum stirrer speed and 70 % of the maximum flowrate through the measuring chamber. The particle size was analysed by laser light scattering of yeast samples using the Malvern Mastersizer (long bed version 2) fitted with a 63 mm lens. The particle size distribution measurements were taken every 5 seconds until steady state was achieved after 16 readings. Data collected were used to determine the rate of change of cell size immediately on changing suspension osmolarity. In addition, the extent of the change in cell size was also measured.

CHAPTER 4: COOLING RATES AND FERMENTATION PERFORMANCE

4.1 INTRODUCTION

Cooling rate is a function of numerous factors including, amongst others, the nature, velocity and viscosity of the fluid or suspension cooled; material of construction of the pipe and the temperature driving force. The influence of cooling rate on the fermentation performance of yeast has been reviewed in Chapter 2. The rate at which a yeast cell is cooled affects its metabolic activity (Dumont *et al.,* 2003), the cell membrane and the induction of cryoprotectants (Kandror *et al*., 2004; Dumont *et al*., 2004, Nkosi 2001).

The temperature to which the yeast is cooled was reported to have an influence on the fermentation performance of yeast in terms of cell vitality and viability (Kandror *et al*., 2004; Dumont *et al*., 2003; Nkosi, 2001). Vitality is a term used to describe the metabolic activity of yeast; in some cases, overall fermentation capacity may be implied. Viability describes the ability of cells to grow and reproduce. Loss of viability may occur via loss of the structural integrity, cell death or replicative deactivation.

This study investigated the effects of yeast flow rate, yeast consistency and the type of diluent used on the cooling rate. The relationships between the cooling rate and the flow rate of yeast and between cooling rate and the type of diluent developed are presented in Section 4.2. In Section 4.3, a base case study of small–scale fermentation is presented (Section 4.3.1). Thereafter the influence of three variables investigated in the cooling studies: cooling rate, consistency and diluent, on small-scale fermentation is investigated (Section 4.3.2 to 4.3.4). Cooling rates were varied between $0.5^{o}C.s^{-1}$ and $2.15^{o}C.s^{-1}$. The yeast consistencies were varied over the range 30 to 64 % using the following diluents: PBS, beer and a blend of PBS and beer.

4.2 THE EFFECT OF FLOW RATE ON THE COOLING RATE OF YEAST

Yeast, collected from the brewery, following cropping from the fermentation vessel and prior to heat exchanger, was pumped through the cooling coil at varying flow rates defined by the maximum pump capacity. Typical consistencies of yeast suspensions collected were 62 ± 2.6 %. The consistencies of the yeast used to determine the relationship between pump speed and flow rate for both run 1 and run 2 were 64 %. Figures 4-1 and 4-2 show cooling rate increasing linearly with an increase in flow rate given in terms of pump settings and linear velocity respectively. Linear velocities obtained at 15 %, 25 %, 50 %, 75 % and 100 % of the maximum flow rate were $0.10\ m.s^{-1}$, $0.20\ m.s^{-1}$, $0.57\ m.s^{-1}$, $0.91\ m.s^{-1}$ and $1.02\ m.s^{-1}$ respectively.

Data presented in Figure 4-3 represent yeast cooling rate as a function of consistency. Three diluents were used to vary consistency: a mixture of PBS and beer (Condition 1), beer (Condition 2) and PBS (Condition 3). A summary of all the conditions applied is given in Section 3.4.3. The yeast consistencies of 30, 40 and 60 % were prepared under each set of yeast preparation conditions. Data are compared at 50 % and 100 % of the maximum flow rate. Yeast pumped at lower flow rates gave lower cooling rates compared to yeast pumped at higher flow rates. At lowest yeast consistencies, the cooling rates obtained were the highest owing to the lower viscosity and the higher linear velocities achieved (Figure 4-4).

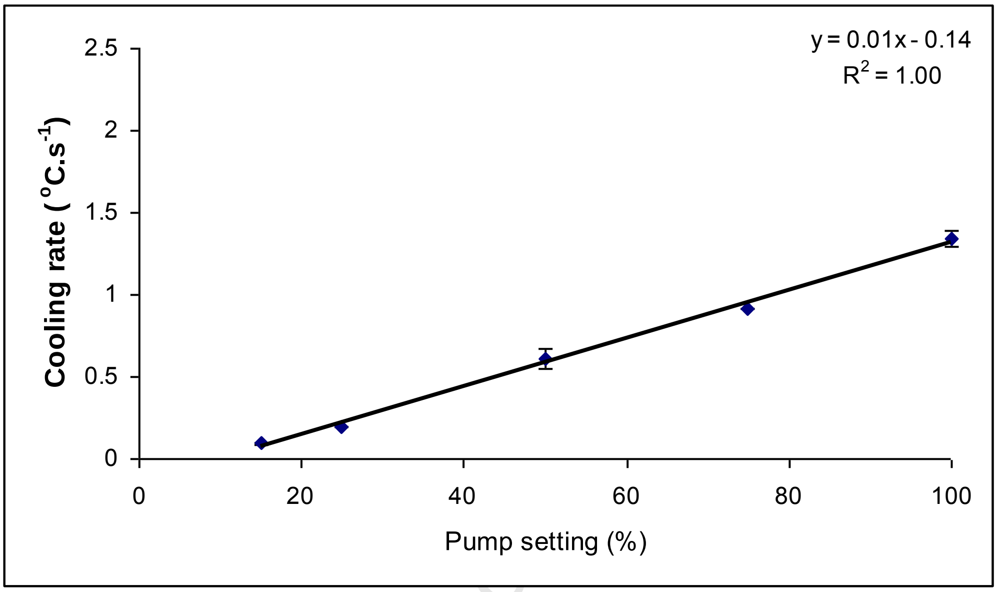


Figure 4-1: Cooling rates as a function of pump setting. The experiment was conducted in duplicate with two different yeast suspensions harvested from SA Breweries, having 64 % consistency. The pump setting ranged from 15 % to 100 % of the maximum flow rate.

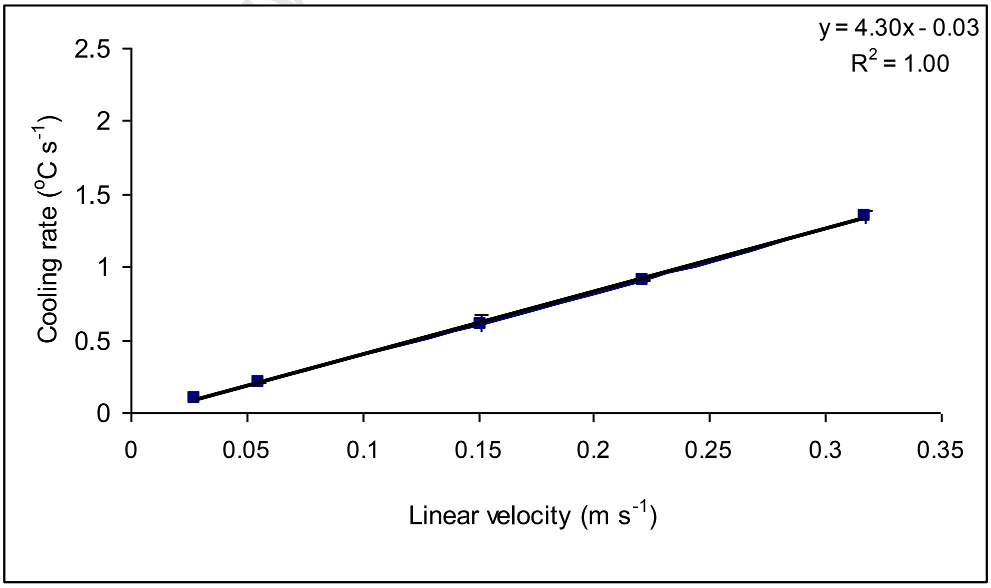


Figure 4-2: Cooling rate as a function of linear velocity of yeast having 64 % consistency. The experiment was conducted in duplicate with 2 different yeast suspensions harvested from SA Breweries. The pump settings ranged from 15 % to 100 % maximum flow rate.

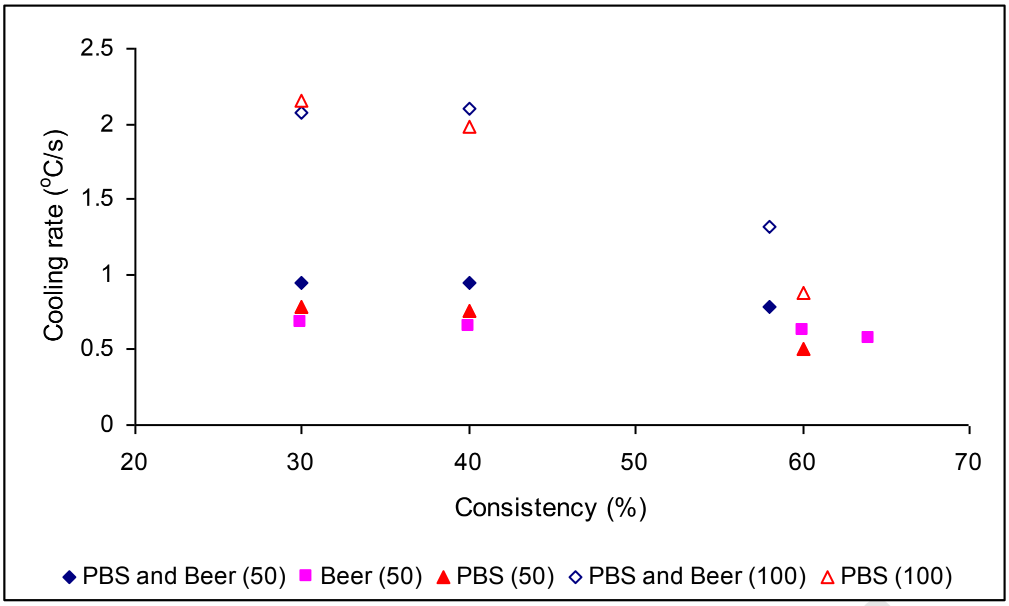


Figure 4-3: Cooling rate as a function of yeast consistency. The yeast concentrations were varied using three diluents; namely, a mixture PBS and beer; beer and PBS. Closed symbols refer to data collected at 50 % maximum flow rate while open symbols refer to data collected at 100 % maximum flow rate. For each diluent, the yeast consistencies prepared were 30, 40 and 60 %.

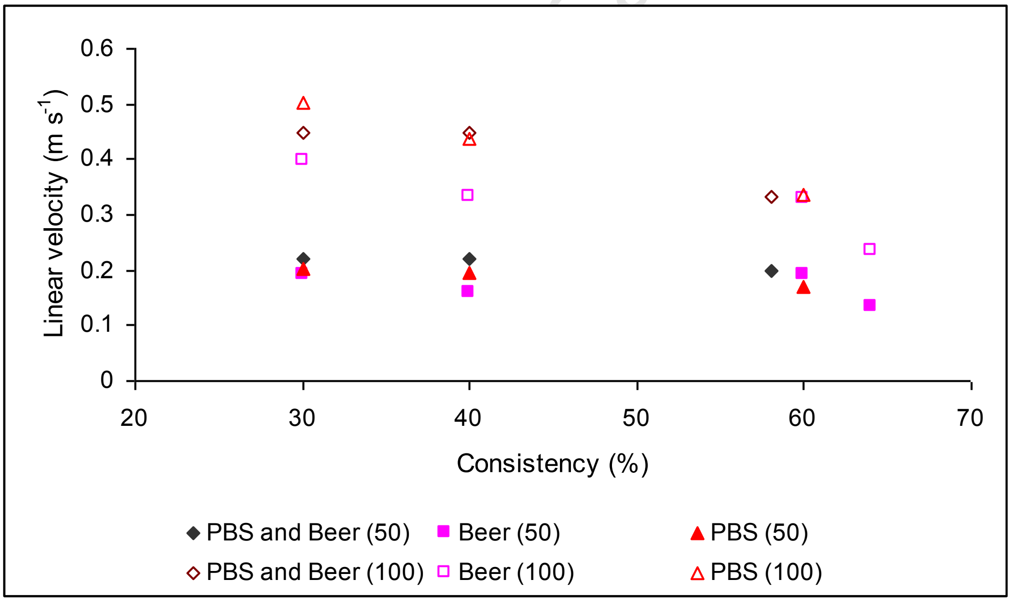


Figure 4-4: Linear velocity as a function of yeast consistency. The yeast concentrations were varied using three diluents; namely, a mixture PBS and beer; beer and PBS. The closed and open symbols refer to yeast pumped at 50 % and 100 % maximum flow rate respectively. Yeast concentrations prepared were 30 %, 40 % and 60 % consistency respectively.

4.3 THE EFFECT OF COOLING RATE ON THE FERMENTATION PERFORMANCE OF YEAST

4.3.1 Base case of cell growth during the small-scale fermentation

In all experimental sets, control yeast collected from the brewery and maintained at a temperature of 14^{o}C was used to provide a base line. Typical baseline data are presented for comparison and to demonstrate reproducibility. The small-scale fermentation experiment was carried out in duplicate with yeast of 58 % ± 0.002 consistency. Fermentation performance was quantified as a function of time by cell concentration, mass of carbon dioxide evolved, and the residual substrate.

An inoculum of 0.76 ml was injected into 120 ml of sterile growth media and was incubated at 30^{o}C with agitation at 160 rpm. Cell growth was monitored by total microscopic cell count at 2 hourly intervals over 8 hours. The data, presented in Figure 4-5, show cell concentration increasing with time. At the start of fermentation period, cell concentration was 2.7x 10^{8} cells.ml^{-1}. Cell concentration decreased slightly during the first two hours of fermentation due to cell adaptation. Between the 2nd and 6 hour, the cell number increased from 2.3 x 10^{8} to 8.2 x 10^{8} cells.ml^{-1}. Thereafter the growth rate decreased. The overall cell concentration recorded after 24 hours of fermentation was approximately 1.1x10^{9} cells.ml^{-1}. The increase in cell concentration over the course of fermentation (X_{max} /X_{o}) was approximately 4. There was a rapid increase in dry biomass during the first 8 hours of small-scale fermentation (Figure 4-6). The final dry biomass obtained at the end of the fermentation was 1.9 milligrams per millilitre of yeast sample. The difference in average biomass obtained between the 8th and the 24th hour of fermentation shows that cell growth slowed down after 8 hours of small-scale fermentation. This confirms that the cell concentration data obtained at the 24 hour period was an outlier (overestimate).

The residual glucose concentration, during the small-scale fermentation monitored by the DNS assay is given in Figure 4-7 for control yeast diluted with a blend of PBS and beer. The glucose concentration decreased from 2.4 g.l^{-1} to approximately 0.19 g.l^{-1} in the first 8 hours of fermentation. At the end of the fermentation, the residual substrate concentration was 0.16 g.l^{-1}. The biomass yield was calculated as the quotient of the change in cell concentration and the change in glucose concentration (Figure 4-8).

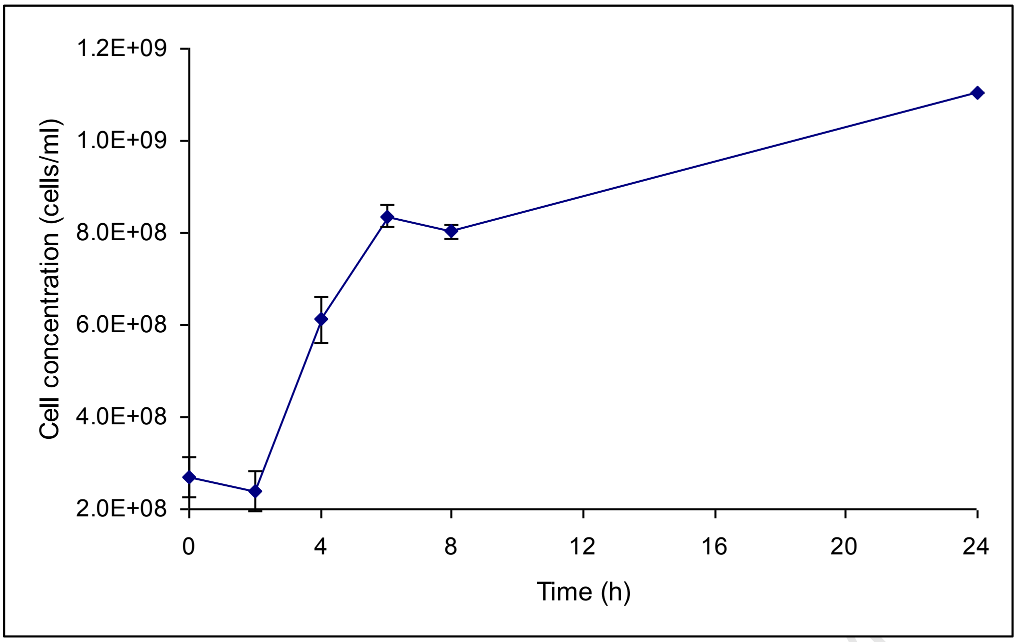


Figure 4-5: Final cell concentration in small-scale fermentation as a function of time where control yeast was suspended in a blend of PBS and beer. The consistency of the control yeast was 58 %. The temperature of the yeast was maintained around 14°C prior to inoculation. Small-scale fermentation was run at 30°C.

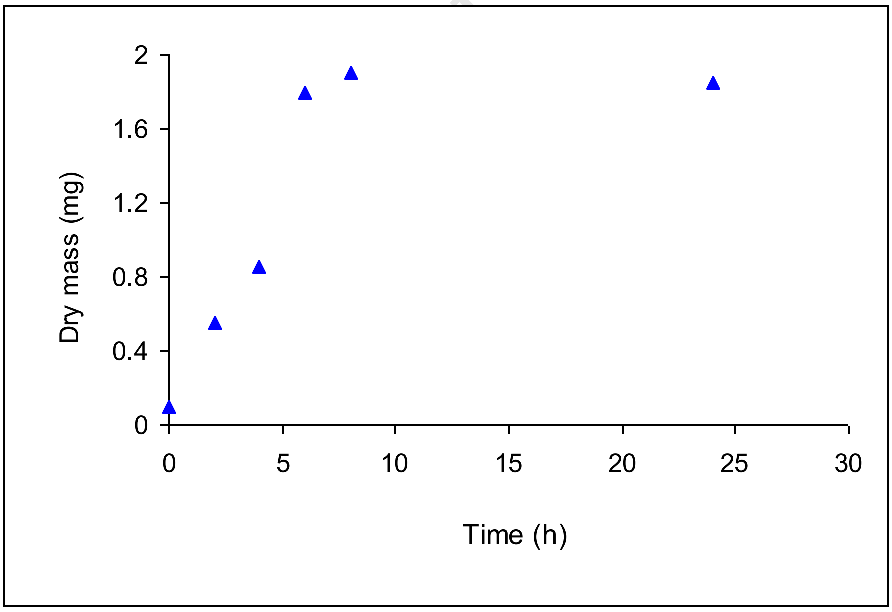


Figure 4-6: Dry mass yield in small-scale fermentation as a function of time where control yeast was suspended in a blend of PBS and beer. The yeast consistency was 58 %. The yeast was maintained at 14°C prior to inoculation. Small-scale fermentation was run at 30°C. Yeast was dried in the 80°C oven for 48 hours.

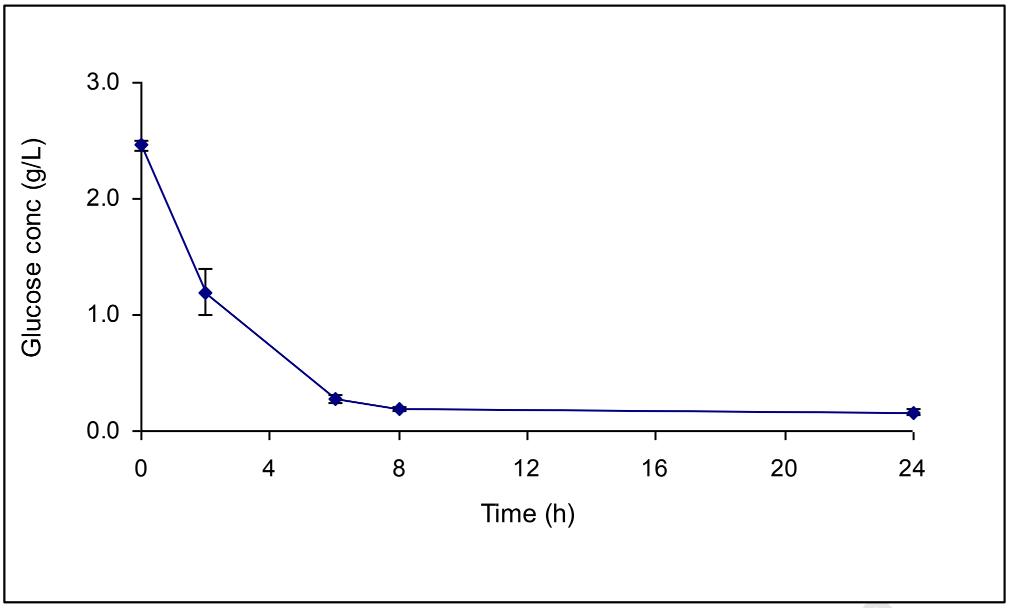


Figure 4-7: Substrate utilisation in small-scale fermentation as a function of time where control yeast is suspended in a blend of PBS and beer. The yeast concentration was 58 %. The temperature of the control yeast was maintained at 14°C prior to running the small-scale fermentation.

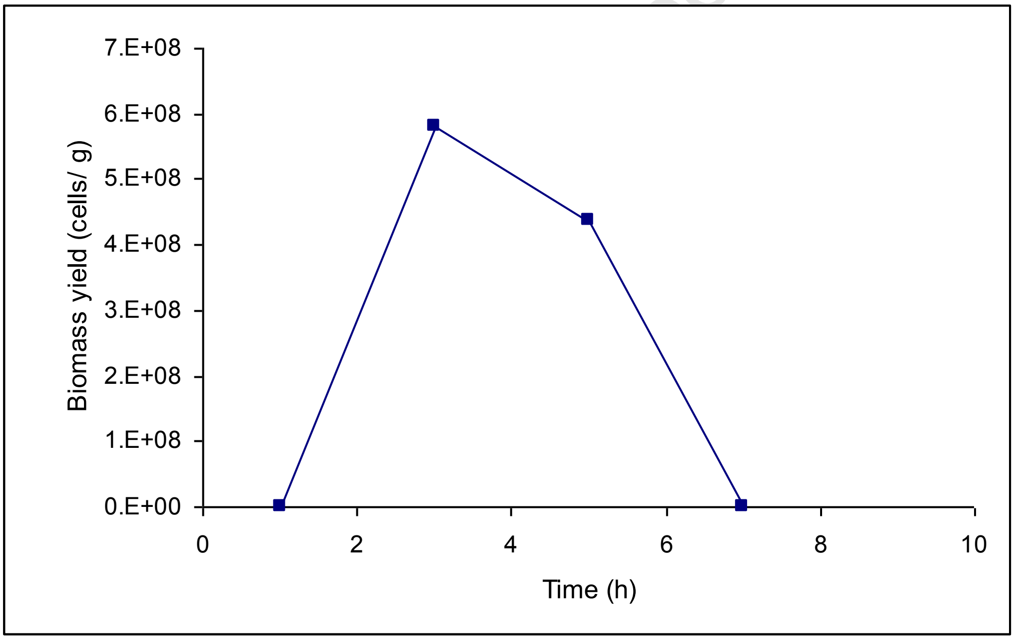


Figure 4-8: The overall biomass yield as a function of time in fermentation of control yeast. The yeast concentration was 58 %. The yeast was maintained at 14°C prior to inoculation. The biomass yield was determined based on the change in the number of cells obtained per change in substrate concentration during fermentation.

The evolution of CO_2 was measured by the change in mass of flask and its contents over selected time intervals. This provided a measure of catabolism and thereby vitality of the cell. Data are presented in Figure 4-9. Figure 4-9 shows that the mass of flask and contents decreased by approximately 0.3 g during the first eight hours of fermentation and a further 0.02 g between 8 and 24 hours. The rate of carbon dioxide evolved thus mimicked the growth of yeast. The method was not sufficiently sensitive to quantify carbon dioxide evolved due to maintenance.

To confirm that evaporation was prevented by the use of a silica water trap, sterile water was incubated in the volumetric flask under the identical conditions to the small-scale fermentation. This was to ensure that the mass loss measured could be attributed to CO_2 evolved only (Figure 4-10). The flasks were weighed at each time interval. To demonstrate the typical levels of evaporation, the results of these evaporation controls are given in Figure 4-10. The average mass of water loss ranged between 0.00 g and 0.02 g. Evaporation controls were carried out across small-scale fermentation experiments using the starting liquid volume of 120 ml. The average amount of water evaporated was less than 0.012 % of the initial volume. Moreover, the average amount of water evaporated was 0.05 % of the carbon dioxide loss, hence it is considered insignificant.

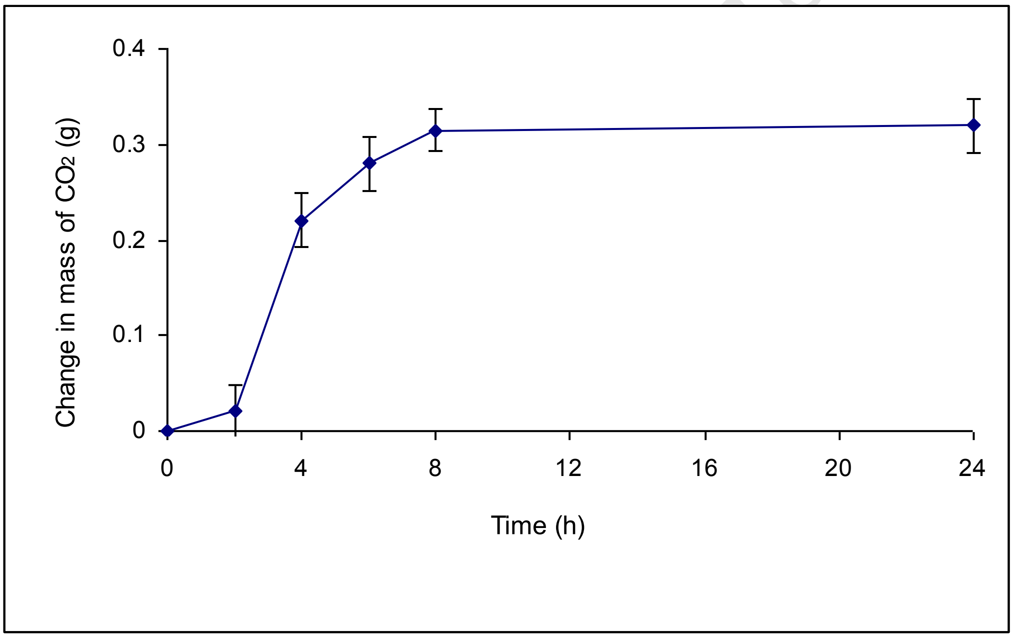


Figure 4-9: The change in mass of carbon dioxide evolved during the small-scale fermentation following control yeast cooling at 0°C.s-1 and a consistency of 58 %. The yeast suspension was diluted with a blend of PBS and beer. Yeast temperature was maintained at 14°C prior to running the fermentation.

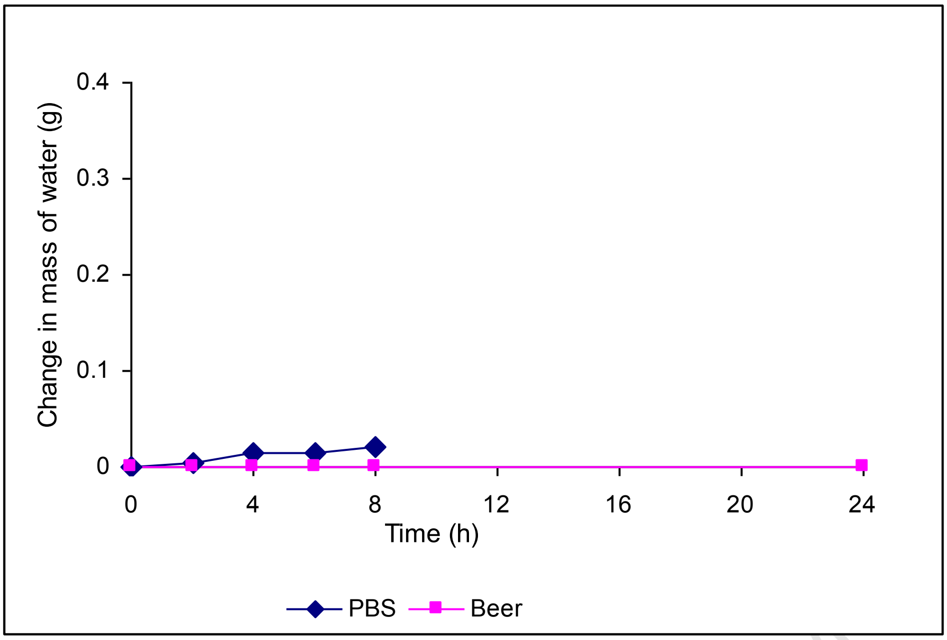


Figure 4-10: The evaporation control for the quantifying of carbon dioxide formed during small-scale fermentation. Liquid water was sterilized prior to incubation under small-scale fermentation conditions.

4.3.2 The effect of consistency on the small-scale fermentation

4.3.2.1 Cell growth

Yeast suspensions of different concentration or consistency were subjected to cooling prior to analysis through small-scale fermentations. Consistent inoculum size was ensured using a basis of ratio of wet pellet to growth media (Appendix C.3). Cell counts were carried out from samples collected at each time interval during the small-scale fermentation (raw data shown in Appendix E.9). The error bars displayed on Figure 4-11 show that the final cell concentration of the control yeast was higher than the cooled yeast. Further, the final cell concentration was the highest at the lowest yeast consistency (30 %). There was a small difference in the final concentration obtained from cooled and control yeast at the lowest yeast consistency (30 %). The final cell concentration obtained at 40 % yeast consistency was approximately equal to the one at 60 % consistency. The relationship shown between cell concentration and yeast consistency is not well understood hence further information is shown in Section 4.3.4.

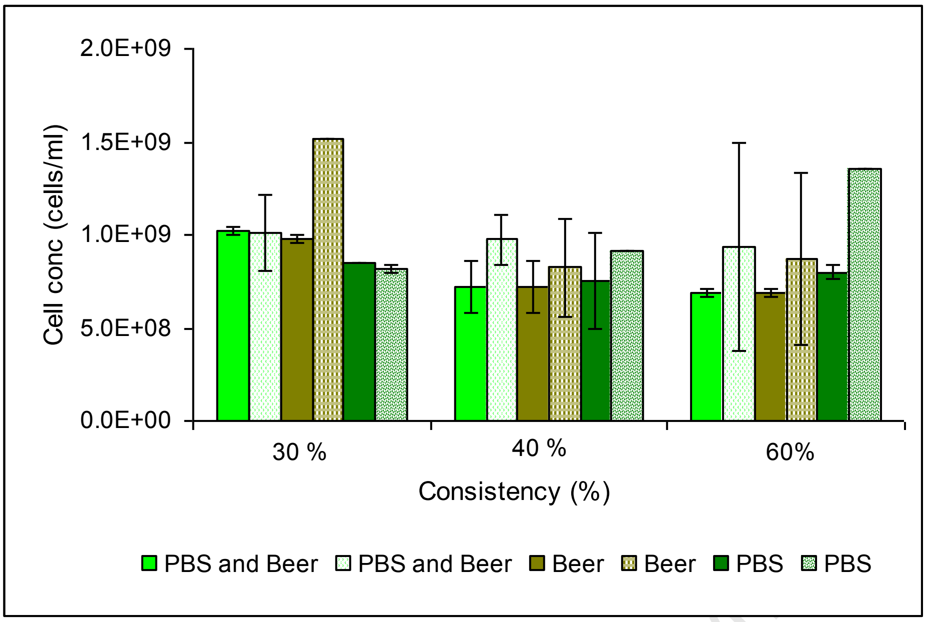


Figure 4-11: Final cell concentration following small-scale fermentation as a function of yeast concentrations (30%, 40% and 60%) during cooling. The solid and hatched colour represent cooled (0.88 to 2.15^{o}C.s^{-1}) and control yeast respectively. Cooled yeast was pumped through the cooling equipment whilst the control yeast was maintained at 14^{o}C subsequent to dilution. The diluents used to prepare various yeast concentrations are PBS only, Beer only and a blend of PBS and Beer. Cell count data shown represent conditions at the end of the 24 hour small-scale fermentation.

4.3.2.2 Substrate utilisation

Substrate utilisation in small-scale fermentation as a function of time following inoculation subsequent to cooling of yeast suspensions of varying concentrations are displayed in Figures 4-12, 4-13 and 4-14. The substrate concentration decreased rapidly over the first 6 hours of small-scale fermentation. This is attributed to the exponential growth during which carbon is assimilated into biomass. There is no significant difference between the rates of substrate utilisation at various yeast consistencies. The rate of decrease in substrate concentration was approximately the same in control and cooled yeast (Figure 4-6 and 4-12). The final residual substrate concentrations of all the three yeast suspensions were the same (below 0.5 g.l^{-1}). The error bars are not visible on the graphs because the standard deviations obtained are typically less than 0.05, corresponding to a coefficient of variance at end of fermentation between 1 and 10 % (Table 4-1). During the first 7 hours of fermentation, the initial specific substrate utilisation of yeast diluted with the three diluents was 3.3x10^{-9}. Similarly, the final specific substrate utilisation was 7.3x10^{-11}.

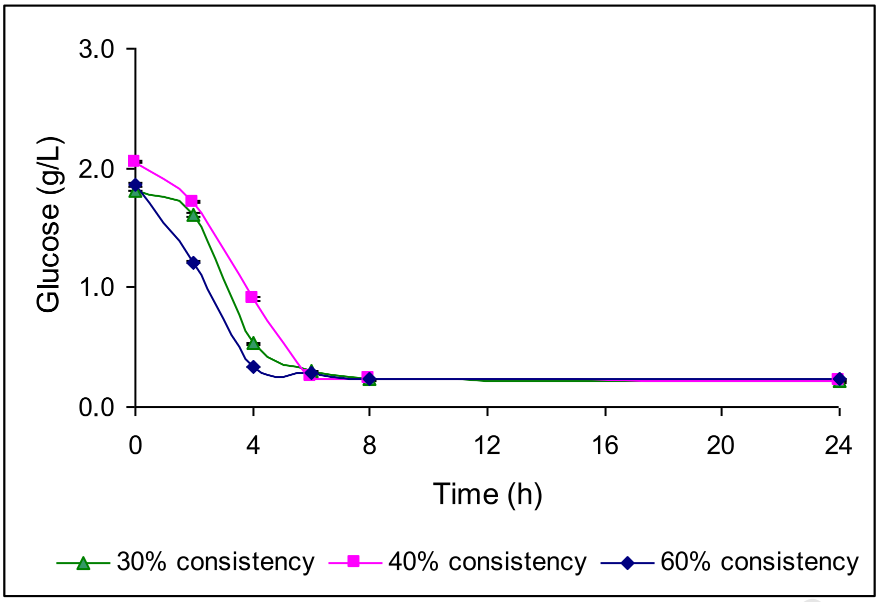


Figure 4-12: Substrate utilisation in small-scale fermentation as a function of consistency of yeast cooled where yeast is suspended in beer. The final consistencies prepared were: 30 %, 40 % and 60 %. Subsequent to dilution, the yeast was cooled (0.88 to 2.15°C.s^{-1}) prior to running the small-scale fermentation.

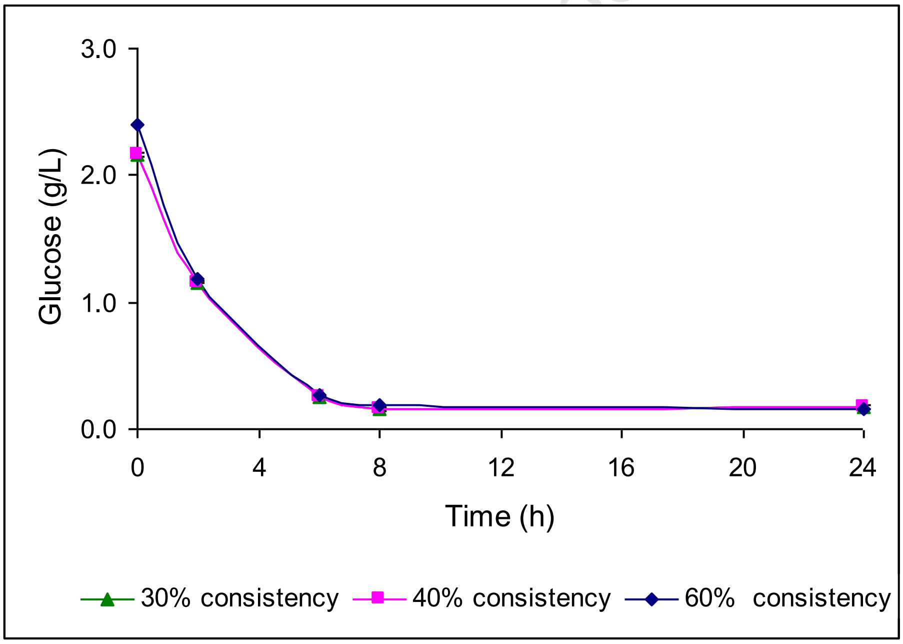


Figure 4-13: Substrate utilisation in small-scale fermentation as a function of consistency of yeast cooled where yeast is suspended in a blend of PBS and beer. The final consistencies prepared were: 30 %, 40 % and 60 %. Subsequent to dilution, the yeast was cooled (0.88 to 2.15°C.s^{-1}) prior to running the small-scale fermentation.

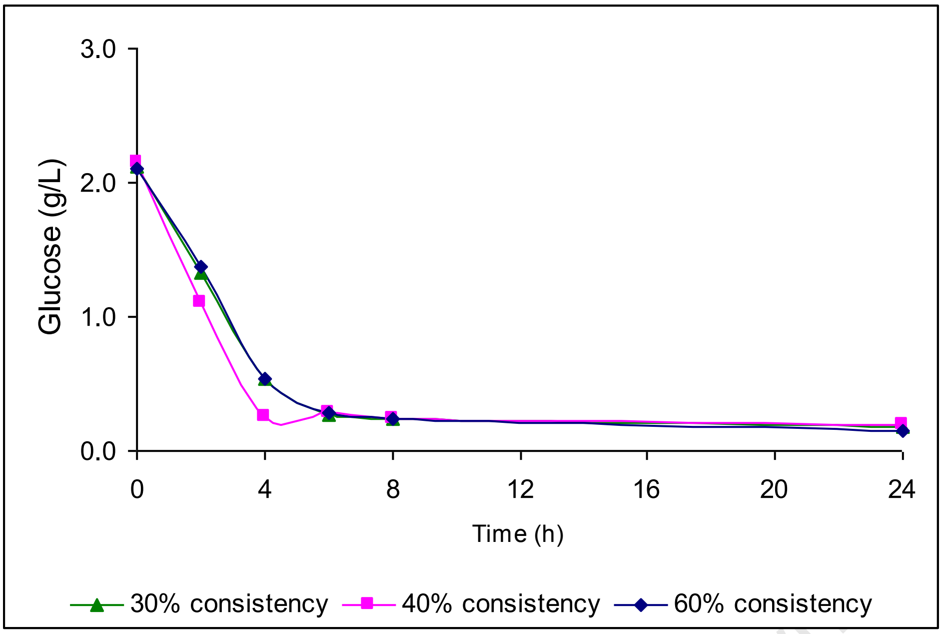


Figure 4-14: Substrate utilisation in small-scale fermentation as a function of consistency of yeast cooled where yeast is suspended in PBS. The final consistencies prepared were: 30 %, 40 % and 60 %. Subsequent to dilution, the yeast was cooled (0.88 to 2.15°C.s^{-1}) prior to running the small-scale fermentation.

Table 4-1: The average standard deviation of the residual substrate concentration on small-scale fermentation of yeast diluted with PBS only, Beer only and a blend of PBS and Beer.

	30%	**40%**	**60%**
PBS and beer	0.01	0.08	0.02
Beer	0.04	0.01	0.008
PBS	0.01	0.006	0.009

4.3.2.3 Cell viability

The cell viabilities determined for cooled yeast slurries diluted in each of the three diluents are shown in Table 4-2. At the beginning of the small-scale fermentation, the majority of samples had cell viability in excess of 95 %. At the end of the 24 hour fermentation period, the average cell viability obtained was above 90 %. The decrease in viability resulted from prolonged nutrient limitation at the end of the exponential phase. There was no particular trend in cell viability followed subsequent to cooling at various yeast consistencies.

Table 4-2: The range of cell viabilities obtained following yeast cooling and during subsequent small- scale fermentation. Yeast was cooled prior to running the fermentation. Cell viability at the beginning and end of the small-scale fermentation.

Yeast concentration (%)	**Diluent**	**PBS and Beer**	**PBS**	**Beer**
30 % consistency	**beginning**	97 %	98 %	97 %
	end	96 %	95 %	92 %
40 % consistency	**beginning**	96 %	98 %	95 %
	end	94 %	98 %	94 %
60 % consistency	**beginning**	95 %	83 %	95 %
	end	90 %	89 %	90 %

4.3.2.4 The change in mass of carbon dioxide

The change in mass of carbon dioxide evolved during small-scale fermentation was used as a measure of vitality. Vitality results of the control yeast and cold stressed yeast prepared by diluting with PBS only, beer only and a blend of PBS and beer are displayed in Figure 4-15. The results displayed are for samples collected at the end of the 24 hour small-scale fermentation period. For all diluents, there was no significant difference in the amount of carbon dioxide evolved at varying yeast consistencies.

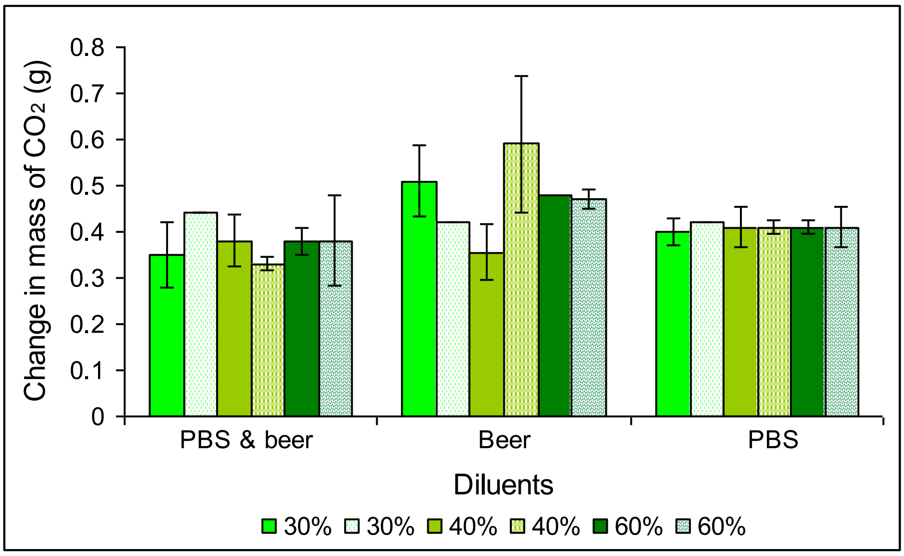


Figure 4-15: The change in mass of carbon dioxide evolved during the small-scale fermentation following yeast cooling at 0.88 - 2.15°C.s^{-1} and varying consistency. The solid and hatched colours represent cooled and control yeast respectively. Cold stressed yeast was pumped at 100 % of the maximum flow rate. Control yeast was maintained at 14°C. Both the control and the cooled yeast slurries were diluted in beer only, PBS only and a blend of PBS and beer.

4.3.2.5 Conclusions

Generally, the final cell concentration of the control yeast was higher than the cooled yeast. Yeast having 30 % consistency gave the highest final cell concentration. In addition, the difference between the final cell concentration obtained from cooled and control yeast was small when using yeast having 30 % consistency. However, the final cell concentration obtained at 40 % yeast consistency was approximately equal to the one at 60 %. The relationship shown between cell concentration and yeast consistency was not well defined. The substrate utilisation and the cell vitality were not sensitive to varying yeast consistency. The final substrate concentration and specific substrate utilisation were 0.5 g.l^{-1} and 7.3x10^{11} respectively.

4.3.3 The effects of diluent on small-scale fermentation

The change in the fluid in the external environment of yeast affects availability of nutrients, inhibiting compounds and transport of molecule into the cytoplasm. The suspending fluid also influences the osmotic potential experienced by the yeast. Osmotic stress around the yeast can be brought about by various factors (as discussed in Section 2.3.2.2). Changing the external environment using alcohol can expose yeast to osmotic stress and alcohol toxicity. Yeast is exposed to varying levels of alcohol in its surrounding environment during the natural fermentation. It has been proposed that yeast quality can be improved by diluting the ethanol to which yeast is exposed on handling. In this study, the effect of diluent on the fermentation performance of brewers yeast was investigated. Yeast slurries were collected from the brewery and diluted with three diluents: PBS only, beer only and a mixture of PBS and beer.

The yeast consistencies prepared were 30 %, 40 % and 60 %. For each experiment carried out, the cooling rate of the yeast was varied between 0.5°C.s^{-1} and 2.15°C.s^{-1}**.** The effect of a diluent on fermentation performance was based on the following assays: cell concentration and the residual substrate concentration and the amount of carbon dioxide evolved.

4.3.3.1 Cell growth

Figure 4-16 shows that irrespective of the type of diluent, the final cell concentration of the control and cold stressed yeast were approximately the same. The two protruding peaks of yeast suspended in either beer or PBS are outliers and will be later supported by the findings in Section 4.3.4.

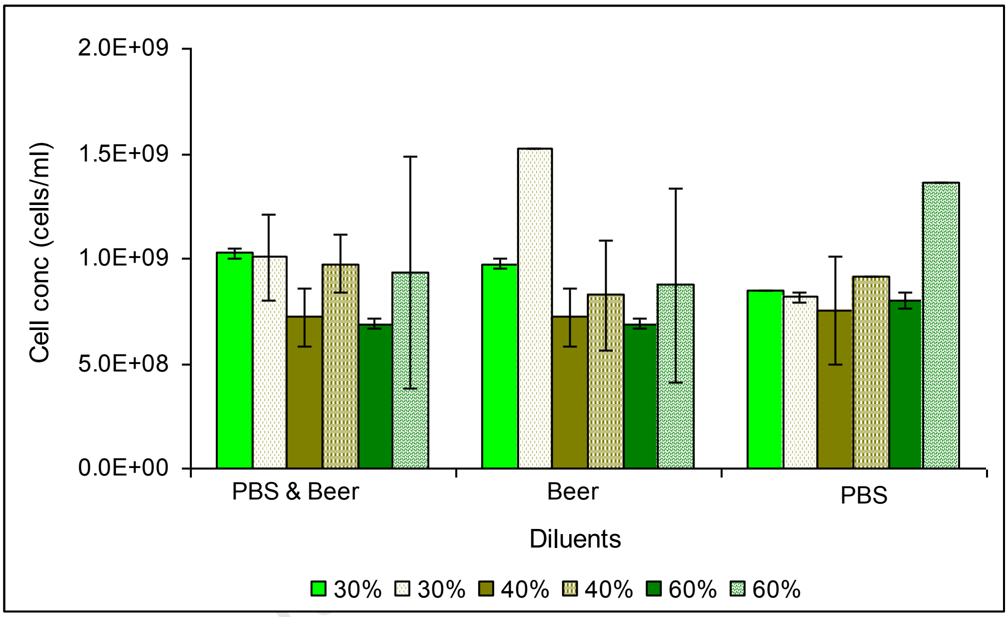


Figure 4-16: Final cell concentration in small-scale fermentation following cooling of yeast suspensions as a function of diluents (PBS only, beer only and a blend of PBS and beer). The solid and hatched colours represent cooled (0.88 to 2.15°C.s^{-1}) and control yeast respectively. Yeast slurries with consistencies of 30, 40 and 60 % respectively were pumped through the cooling equipment whilst the control yeast was maintained at 14°C subsequent to dilution.

4.3.3.2 Substrate utilisation

Figures 4-17 to 4-19 illustrate glucose utilisation in small-scale fermentation subsequent to yeast cooling. Initial glucose concentrations of 2.0 to 2.4 g.l^{-1} were recorded while the final substrate concentration was 0.5 g.l^{-1} in all cases. There was no significant difference between the residual substrate utilization profiles of yeast diluted with different diluents. During the first 4 hours of fermentation, the rate of substrate utilisation decreased at approximately 0.5 g.h^{-1}.

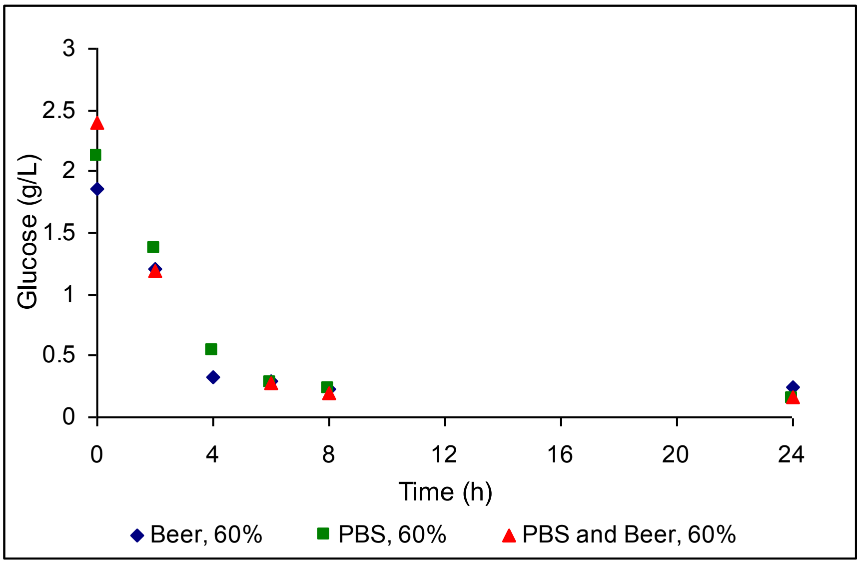


Figure 4-17: Substrate utilisation in small-scale fermentation subsequent to yeast cooling (0.88 to 1.4°C.s^{-1}) using the diluents PBS, beer and a mixture of PBS and beer at a yeast consistency of 60 %. During cooling, yeast was pumped at 100 % maximum flow rate.

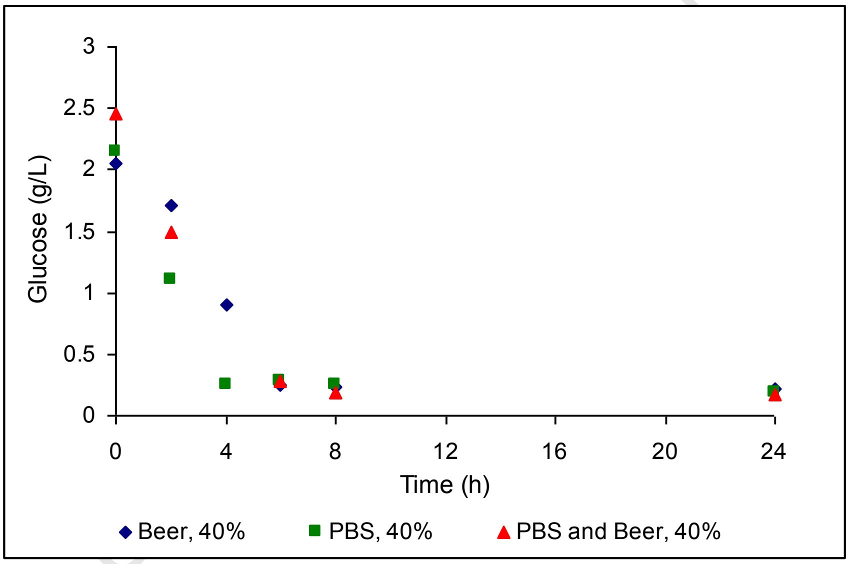


Figure 4-18: Substrate utilisation in small-scale fermentation subsequent to yeast cooling (1.98-2.1°C.s^{-1}), using the diluents PBS, beer and a mixture of PBS and beer at a yeast consistency of 40 %. During cooling, yeast was pumped at 100 % maximum flow rate.

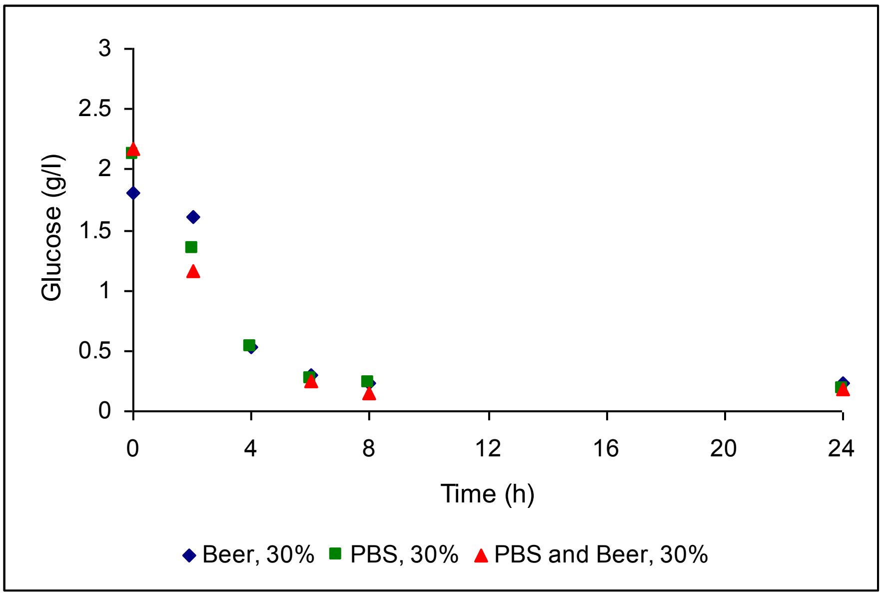


Figure 4-19: Substrate utilisation in small-scale fermentation subsequent to yeast cooling (2.07-2.15°C.s^{-1}), using the diluents PBS, beer and a mixture of PBS and beer at a yeast consistency of 30 %. During cooling, yeast was pumped at 100 % maximum flow rate.

4.3.3.3 The change in mass of carbon dioxide

Vitality was determined by measuring the amount of carbon dioxide released at each time interval. Figure 4-20 shows that for all diluents used, there was no significant difference in the amount of carbon dioxide evolved.

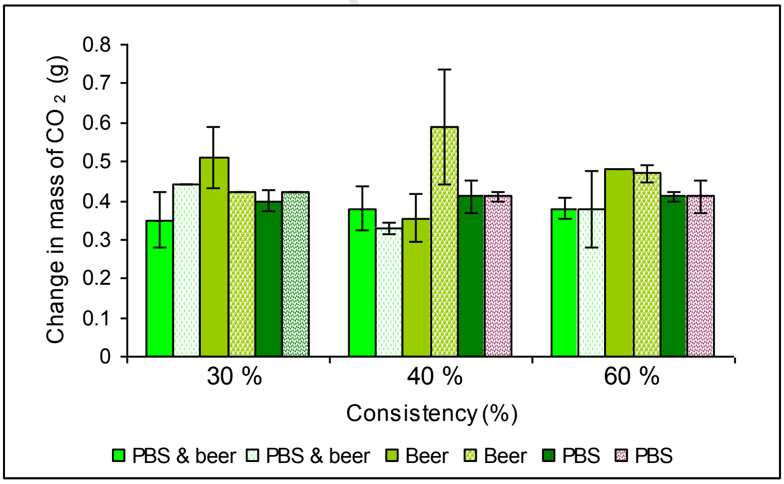


Figure 4-20: The change in mass of carbon dioxide evolved during the small-scale fermentation following yeast cooling at 0.88 - 2.15°C.s^{-1} and varying consistency. The solid and hatched colours represent cooled and control yeast respectively.

4.3.3.4 Conclusion

Data show that the cell concentration, cell vitality and residual substrate utilisation were not sensitive to varying diluent (2-8 % v/v alcohol). This is supported by zero effect of diluent on cooling rates (Figure 4-3).

4.3.4 The effect of cooling rate on the small-scale fermentation performance

4.3.4.1 Cell growth

Cooling rate can affect the yeast fermentation in many different ways (Section 2.4). Figure 4-21 shows the cell concentration at the end of the 24 hour small-scale fermentation following cooling at different rates. The lowest cooling rates were obtained from yeast with 60 % consistency and the highest cooling rates from yeast with 30 % consistency. The effect of cooling rate on small-scale fermentation was more pronounced at lowest yeast consistency (30 % consistency). The final cell concentrations obtained using yeast suspensions at 40 % and 60 % consistency were equal.

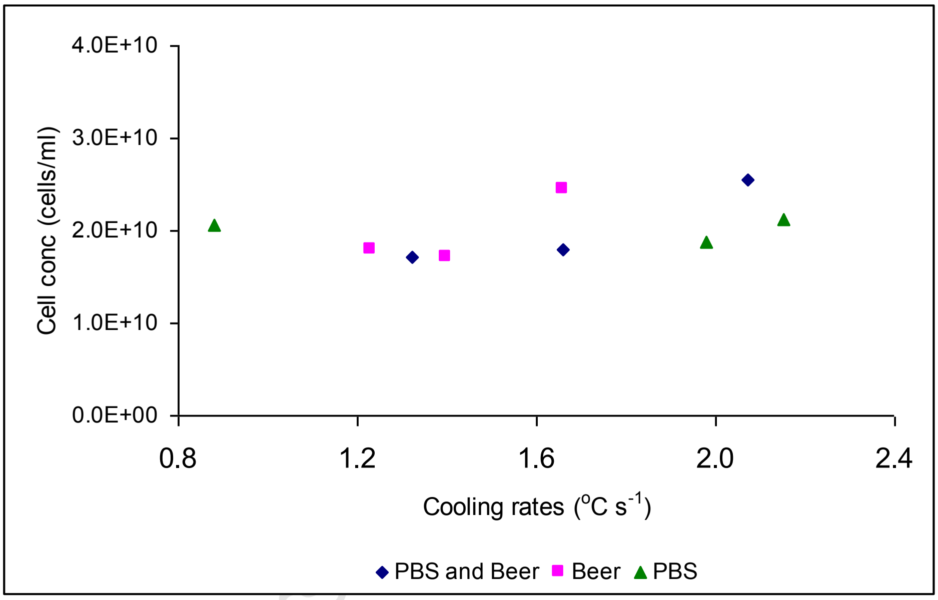


Figure 4-21: Cell concentration at 24 h in small-scale fermentation as a function of cooling rates for cooled yeast. Yeast was diluted with different diluents prior to cooling. The coolant was set at -1°C. Yeast was pumped at 100 % of the maximum flow rate. Yeast consistencies prepared were 30 %, 40 % and 60 %.

4.3.4.2 Mass of carbon dioxide evolved

The increase in the mass of carbon dioxide evolved with an increase in cooling rate was more pronounced when yeast was diluted with beer (Figure 4-22). As more cells were produced, more substrate was utilized and the rate of carbon dioxide evolved increased. For other yeast suspensions, the CO_2 evolution was not sensitive to varying cooling rate. The lowest change in mass of CO_2 evolved was 0.4 g compared to the highest amount of CO_2 evolved in the base case (0.3 g).

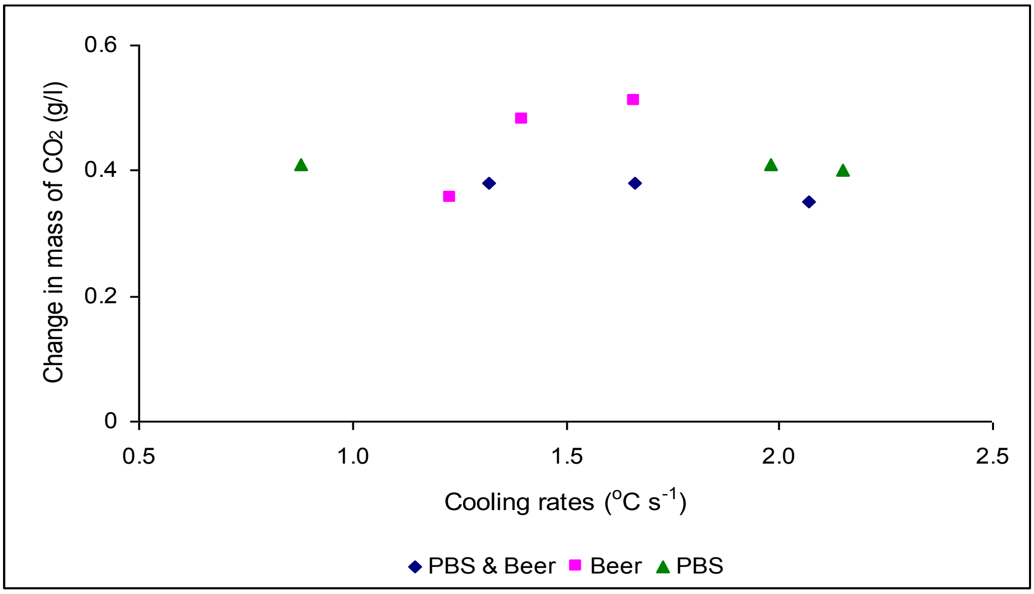


Figure 4-22: The change in mass of carbon dioxide evolved during the small-scale fermentation as a function of cooling rate. Yeast was diluted with different diluent, namely: PBS only, beer only and a blend of PBS and beer. Yeast concentrations were 30 %, 40 % and 60 %.

4.3.4.3 Substrate utilization

Figure 4-23 shows the final glucose concentration of cold stressed yeast at various cooling rates. A decrease in residual substrate concentration is directly proportional to the increase in cell concentration. In this study, the increase in cell concentration with cooling rate was more pronounced at the highest cooling rate obtained (lowest yeast consistency of 30 %), however, there was no significant difference in the residual substrate concentration of yeast at various cooling rates. The average rate of substrate utilisation during the first 4 hours was the highest at 0.37 $g.l^{-1}$. The average final substrate concentration obtained was approximately equal to the base case (0.15 $g.l^{-1}$). Similarly, the yield was the highest and more pronounced at the highest cooling rate.

4.3.4.4 Conclusions

The highest final cell concentration was obtained at the highest cooling rate (lowest yeast consistency). Cell vitality of yeast diluted with beer increased with an increase in cooling rate. This value is an outlier because the final cell concentration was not sensitive to varying diluent. In addition, there was no significant difference in the residual substrate utilisation of yeast across all other conditions at various cooling rates. Carbon dioxide evolved was not sensitive to varying cooling rate.

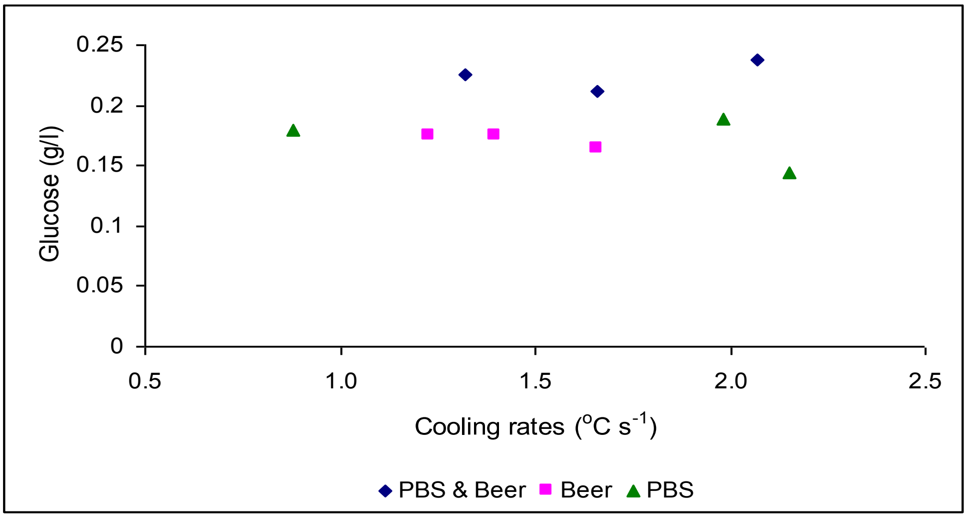


Figure 4-23: Substrate utilisation in small-scale fermentation as a function of cooling rate of yeast suspended in three diluents (PBS only, beer only and a mixture of PBS and beer). Glucose samples were collected at the end of the 24 hour small-scale fermentation. The coolant was set at -1°C.

4.4 CONCLUSIONS

From the above findings, it can be concluded that cooling rate is directly proportional to the flow rate. The cooling rates obtained using yeast (64 % consistency) varied between 0.02 $m.s^{-1}$ and 0.235 $m.s^{-1}$ and the corresponding cooling rates were 0.1$^{o}C.s^{-1}$, and 1$^{o}C.s^{-1}$ respectively. Cooling rates obtained were not sensitive to varying diluent; namely, PBS only, beer only and a blend of PBS and beer. In addition, varying the diluent did not have any effect on the final cell concentration, cell vitality and residual substrate utilisation of the control and cooled yeast. The cooling rate was indirectly proportional to the yeast consistency.

The base case showed the cell concentration and cell vitality increasing during the first six hours of fermentation. The final cell concentration obtained was 8.2 x 10^{8} $cells.ml^{-1}$. In addition, the amount of carbon dioxide evolved was slightly greater than 0.3 g. Cell vitality and substrate utilisation rate were also active during the first six hours of fermentation. At the end of the fermentation, the substrate concentration decreased by 94 %.

The effect of cooling rate on cell concentration was more pronounced at highest cooling rate (30 % yeast consistency). There was no significant difference between the final cell concentration obtained at 40 % and 60 % yeast consistencies. At all yeast consistencies, there was a small difference between the concentrations obtained from the control and cooled yeast hence the relationship shown between cell concentration and yeast consistency was not fully understood. There was no significant difference between the rates of substrate utilisation at various yeast consistencies for control and cooled yeast. The CO_2 evolution was not sensitive to varying cooling rates however the total amount of CO_2 evolved was higher for cooled (0.4 g) than for base case (0.3 g).

CHAPTER 5: CELL ENVELOPE FLEXIBILITY

5.1 INTRODUCTION

The structure of the cytoplasmic membrane is composed of the lipid bilayer with embedded proteins (Williams, 1990; Alberts *et al*., 1998). The phospholipid bilayer is maintained in a fluid lamella, liquid–crystalline phase at physiological temperatures and levels of hydration. The fluidity of the plasma membrane is crucial since it enables embedded proteins to interact with one another for cell signalling and to diffuse rapidly in the plane of the bilayer (Alberts *et al*., 1998). The ability of the cell to preserve its fluidity under osmotic pressure or low temperature is influenced by the rate at which the cell membrane can sense and respond to the stress positively as well as the ductility of the cell wall. This rate is quantified through the cell envelope flexibility. Cell envelope flexibility was studied by immersing yeast in media of varying osmotic potential. Mille *et al*. (2002) showed that immersing cells in an environment of high osmotic potential (depending on the level of osmotic stress) can induce a mechanical effect on the cell envelope leading to cell volume decrease which leads to an increase in surface area-to-volume ratio. It can also lead to disruption of the cell in extreme cases.

The influence of temperature on cell envelope flexibility was reported in Section 2.4.2.5. Cooling was reported to lead to desiccation effects at the membrane and changes in the membrane sol to gel state. Furthermore, the effects of exposing yeast to low temperature are reported to include changes to the cell membrane and induction of cryoprotectants such as trehalose and Heat Shock Proteins (HSP) (Kandror *et al*., 2004; Dumont *et al*., 2004, Nkosi 2001). Previously HSP12, located in the cell wall and cytoplasmic membrane (Motshwene *et al*., 2004), was shown to be one of the numerous factors contributing to the endurance of yeast at low temperatures. HSP12 is up-regulated on exposure to cold stress (Kandror *et al*., 2004; Nkosi 2001). The presence of HSP12 was correlated with cell envelope flexibility and the phenotype resistant to pressure drop (Motshwene *et al*., 2004). Cell envelope flexibility of the wild type and the knock-out strain were measured by observing the response of yeast to rapid changes in the osmotic strength of the environment. After 1 minute, a rapid decrease in cell volume of the wild type yeast was observed upon its addition to the high osmolarity buffer (Motshwene *et al*., 2004). The % volume decrease of the wild-type and knock-out yeast cells were 13 % and 5 % respectively. After 10 minutes, the volumes of the wild-type and knock-out strain had decreased by 17 % and 8 % respectively.

The response of yeast to varying cooling rates was shown to depend on the cooling rate and the extent of cooling (Nkosi, 2001; Dumont *et al*., 2004). Nkosi (2001) detected expression of the heat shock protein HSP12 by SDS PAGE electrophoresis using the fast cooled (4.3^{o}C.s^{-1}, after 1 hour at 4^{o}C) and slow (1^{o}C.s^{-1}, after 1 hour at 4^{o}C) cooled Brewers' yeast, following a 4 hour incubation after stress. The results showed a 37 % increase in HSP12 in fast cooled yeast compared to the slow cooled yeast. The difference in the amount of HSP12 induced was attributed to the variation in stress to which yeast is exposed.

Motshwene *et al.* (2004) used the laboratory strain and showed that an increase in HSP12 led to an increase in cell envelope flexibility. Nkosi (2001) showed that rapid cooling increased the level of HSP12. In this study, it is hypothesized that an increase in cooling rate will increase the flexibility of the cell envelope. The effect of cooling on the cell envelope was explored in terms of cell envelope flexibility using three different osmotic media. The experimental protocol of this assay is detailed in Section 3.3 and Section 3.4. In Section 5.2, the assay protocol is assessed through observing the effect of temperature difference between the sample and assay diluent. Thereafter the effect of changes in osmolarity and cooling rate on the cell envelope flexibility is quantified.

The flexibility test was carried out using four solutions of varying osmotic strength; namely, hypertonic (650 mM NaCl and 10 mM Tris, for cooled yeast), (866 mM NaCl and 10 mM Tris, for heated yeast), low salt (150 mM NaCl and 10 mM Tris) and hypotonic (water) buffers. An aliquot of 200 µL of yeast suspension was injected into 1 litre of the diluent inside the Malvern bowl chamber and flexibility analyzed according to Section 3.5.3.3.

Cell envelope flexibility was measured in terms of the rate and extent of change in the size of yeast upon injection into the Malvern diluent. The data were used to determine the:

- initial (after 5 seconds) rate of change in size of yeast upon injection in the Malvern diluent
- the extent of change in size of yeast at steady state as a percent of the untreated yeast size

To calculate the rate of change of cell size, the volume-based size distribution of the cell was determined as a function of time. Data was collected every 5 seconds until steady state was achieved after 16 readings. From a volume-based size distribution, the percentage change in cell size was determined (Appendix D). From the delta size change (%), the slope was determined during the first 10 seconds of yeast being injected into the osmotic medium. The extent of change in cell size was recorded after steady state was reached.

5.2 THE EFFECT OF THE DIFFERENCE IN TEMPERATURE (ΔT) BETWEEN THE YEAST SAMPLE AND THE MALVERN DILUENT ON CHANGE IN YEAST CELL VOLUME

In order to measure response to cold shock through cell flexibility, no further temperature shock should be introduced in the analytical approach. In this study, the effect of change in temperature (ΔT) by dilution in the assay protocol is considered to determine the maximum ΔT to be used within the assay that will not influence the cell envelope flexibility results. To minimise the change in temperature, the effect of running the assay at low temperature or high temperature was also explored.

Yeast was collected from the brewery and divided into three aliquots to provide three temperature fractions for study. The coolant and the hot water bath were set to -1°C and 30°C respectively. The average exit temperature of the cooled and heated yeast samples were 7°C and 25°C respectively. The first aliquot of control yeast sample was maintained around 14°C, and the second portion was cooled slowly on ice from 14°C to 7°C. The third portion was slowly heated from 14°C to 25°C. All the control samples were not pumped through the coil. Instead, the yeast sample was placed in a beaker, then slowly cooled or heated with gentle mixing. The effect on flexibility of temperature change by dilution into buffers of 7°C, 14°C and 25°C was investigated.

Brewery yeast was collected at 14°C following cropping. On reaching the laboratory, the experiments are usually carried out at 14°C ± 10°C. Injecting yeast at 14°C into Malvern diluent at 14°C ± 10°C, is expected to give the same results. Figure 5-1 shows the cell envelope flexibilities of the controlled yeast suspensions exposed to the temperature differences of 0°C and 11°C. The yeast suspensions were injected into the Malvern diluent at 14°C or 25°C. Similarly, Figure 5-2 shows the controlled yeast suspensions at 7°C, exposed to the temperature differences of 0°C and 18°C. The data from Figure 5-1 and Figure 5-2 were extracted into Table 5-1 to give the rate and extent of change in cell size. Table 5-1 shows that when ΔT between the Malvern diluent and sample was zero, the extent of change in cell size was 24 or 28 ± 2.2 % with an absolute error of ± 15 % and 12 % respectively. In addition, when ΔT between the Malvern diluent and sample was 11°C, the extent of change in cell size was 24 % with an absolute error of 15 %. It can be concluded that the cell envelope flexibility is the same as long as the ΔT is equal or less than 11°C. When a higher ΔT was introduced (18°C), a larger cell envelope flexibility of 32 % was obtained. It can be concluded that the larger the temperature difference between the Malvern diluent and the yeast sample, the larger the cell envelope flexibility. It is recommended that when running cell envelope flexibility tests, ΔT should be a minimum and must be kept constant. Introducing a large temperature difference between the yeast sample and the Malvern diluent results in interference in the cell envelope flexibility test.

From experimental observation, collection of data at a low temperature (<10°C) had an effect on the results obtained. When cooled yeast (7°C) was injected into a Malvern diluent set below 10°C, the data obtained from replicate samples were more scattered. The scattered data were seen by numerous peaks on the particle size distribution curve (Figure 5-3). It was postulated that low temperature caused condensation on the lens of the Malvern Mastersizer. Size ranges greater than an average size of yeast were recorded. Knowing the size range of brewers' yeast, aberrant peaks were detected (outside the yeast size range of 7-10 µm). After deletion of these peaks (assumed to result from condensation on the lenses), the normal distribution curve was re-constructed. Figure 5-3 shows the percentage size change of yeast using the original and the reconstructed data. The % size change was obtained by comparing the rate and extent of change in size of yeast relative the original yeast when it was first injected into the Malvern diluent.

When the cooled sample (7°C) was injected into the Malvern diluent at a temperature above 10°C, the aberrant peaks were not obtained in the yeast size distribution. It was decided that the temperature of the Malvern diluent set at 14°C for cold stressed sample to prevent interference with the assay and avoid the need for this correction. This resulted in a ΔT of 7 to 10°C, depending on the final temperature on cooling. From the data presented in Table 5.1, this is recognized to introduce a coefficient of variance for the assay with the maximum absolute error of 15 %. When heated sample (> 25°C) was injected into Malvern diluent, the aberrant peaks were not obtained. The difference in temperature between the Malvern diluent and the heated sample was kept at a minimum (ΔT =0).

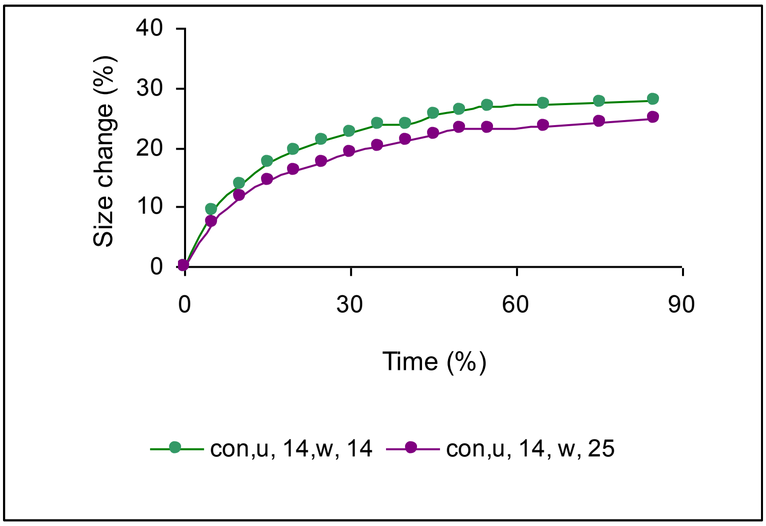


Figure 5-1: Cell envelope flexibility of control yeast following its maintenance at 14°C. The legend used: con, c, 14, w, 25: control yeast, maintained at 14°C, injected into water at 25°C; con, u, 14, w, 14: control yeast, maintained at 14°C, injected into water at 14°C.

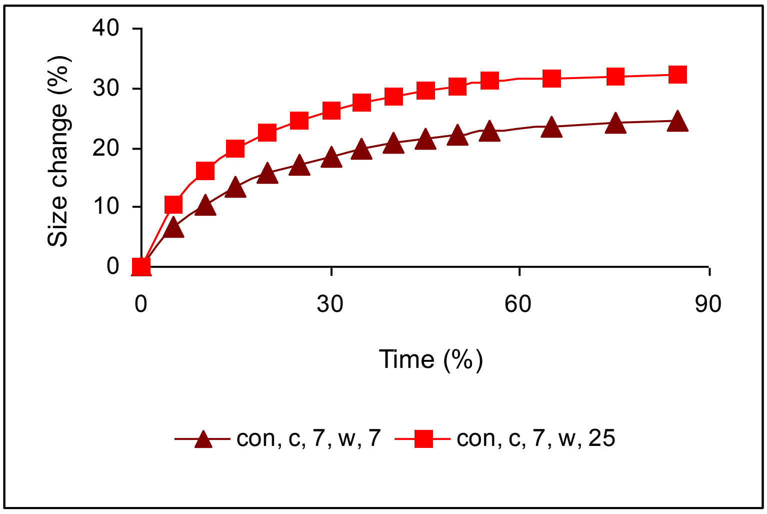


Figure 5-2: Cell envelope flexibility of control yeast following gradual cooling to 7°C. The two yeast suspensions were injected into water at 7 and 25°C. The legend used: con, c, 7, w, 25: control yeast, gradually cooled to 7°C, injected into water at 25°C; con, u, 7, w, 7: control yeast, gradually cooled to 7°C, injected into water at 7°C.

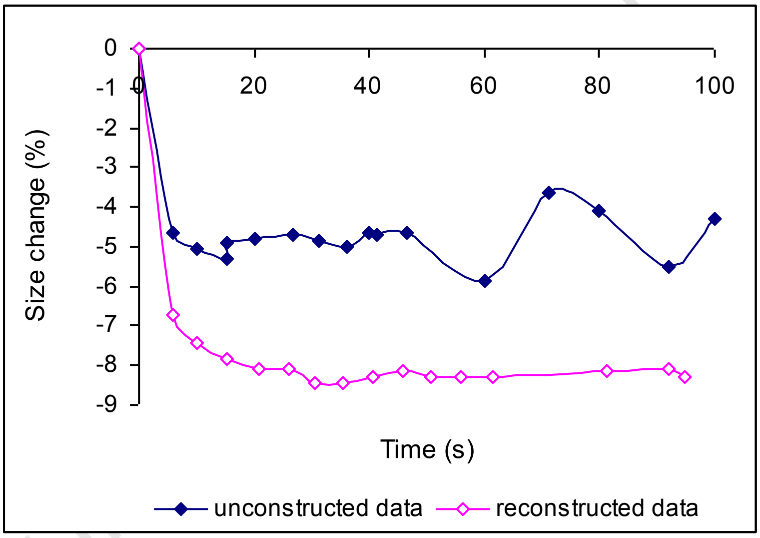


Figure 5-3: Cell envelope flexibility of yeast following cooling to 7°C at 0.5°C.s^{-1}. Yeast having consistency of 30 %, was injected into the Malvern diluent (low salt solution) at 7°C.

Table 5-1: The rate and extent of change in cell size of control yeast as a function of difference in diluent and sample temperatures following either gradual cooling to 7°C or maintenance 14°C. The yeast suspensions were injected into water at 7, 14 and 25°C. The legend used: con, c, 7, w, 7: control yeast, gradually cooled to 7°C, injected into water at 7°C; con, c, 14, w, 14: control yeast, maintained at 14°C, injected into water at 14°C; con, u, 14, w, 25: control yeast, uncooled, maintained at 14°C, injected into water at 25°C; con, c, 7, w, 25: control yeast, gradually cooled to 7°C, injected into water at 25°C.

Yeast treatment	Temperature difference (°C)	Initial rate of change in the size of the cell [Δ Size change (%)]/ [Δ time (s)]	Extent of change in cell size (%)
con, c, 7, w, 7	0	1.35	24.50
con, u, 14, w, 14	0	1.88	28.04 ± 2.20
con, u, 14, w, 25	11	1.46	24.50
con, c, 7, w, 25	18	2.09	32.38

5.3 THE EFFECT OF COOLING RATE ON FLEXIBILITY

Yeast harvested on cropping from the brewery fermentation vessel was split into three aliquots. Different diluents were used to prepare the final consistencies of 30 %, 40 % and 60 %. The cooling rate was varied by varying the pump-settings for passage through the cooling coil between 50 % and 100 %. Figure 5-4 shows the size change of yeast (at 60% consistency) diluted with a blend of PBS and beer following cooling. Data from Figure 5-4 was extracted into Table 5-2. During the first 5 seconds of injection yeast into water, the rate of change in cell size of yeast was smaller for moderately cooled (0.78°C.s^{-1}) yeast than for the fast cooled yeast (1.32°C.s^{-1}). Similarly, the extent of change in cell size was higher for fast cooled yeast compared to the moderately cooled yeast (Figure 5-4). It can be concluded that the cell size change of the fast cooled yeast was slightly greater than the slow cooled yeast.

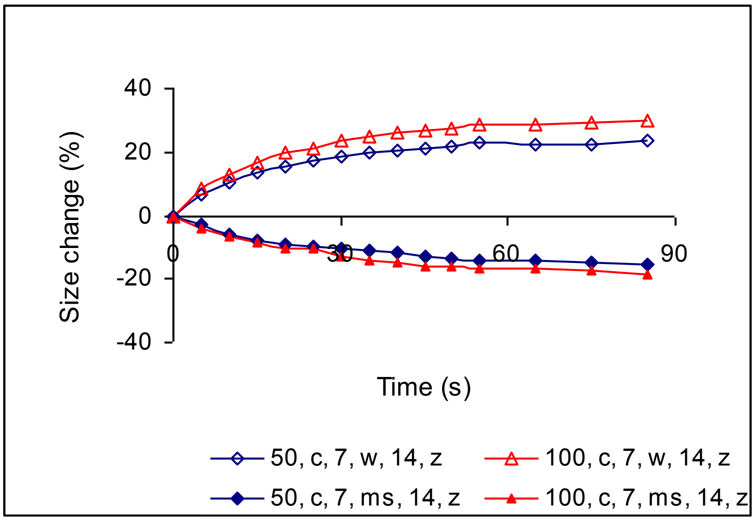


Figure 5-4: The cell envelope flexibility of yeast at 60% consistency (z) suspended in a blend of PBS and beer following moderate and rapid cooling to 7°C at 50 % (0.78°C.s^{-1}) and 100 % (1.32°C.s^{-1}) of the maximum flow rate respectively. Cell envelope flexibility was tested by injecting the yeast into water and into low salt solution at 14°C.

Table 5-2: The rate and extent of change in size of yeast at 58- 60% consistency (z) suspended in a blend of PBS and beer following moderate and rapid cooling to 7°C at 50 % and 100 % of the maximum flow rate respectively. Data was taken from Figure 5-4.

Yeast treatment	**Initial rate of change in the size of cell [Δ Size change (%)]/ [Δ time (s)]**	**Extent of change in cell size (%)**
50, c, 7, ms, 14, z	- 0.52	- 15.30
100, c, 7, ms, 14, z	- 0.79	- 18.40
50, c, 7, w, 14, z	1.34	23.00
100, c, 7, w, 14, z	1.71	30.20

5.4 THE EFFECT OF HEATING RATE ON FLEXIBILITY

In Figures 5-5 and 5-6 the flexibility of yeast is demonstrated on change in osmotic strength following heating to about 25°C. These data are further analysed in Table 5-3. Yeast, collected from the brewery following cropping, was heated at a moderate and rapid rate by varying the flow rate through the heating coil with the wall temperature set at 30°C. The average exit temperature of heated yeast was 25°C. The heating rates obtained when yeast was pumped at

50 % and 100 % of the maximum flow rate were $0.9^{o}C.s^{-1}$ and $1.7^{o}C.s^{-1}$ respectively. The Malvern diluents, water and hypertonic buffer solution, were kept at $25^{o}C$. The hypertonic buffer solution used in testing flexibility of the heated cells (Figure 5-6) was comprised of 866 mM NaCl and 13 mM Tris-HCl. The concentration was made stronger because of inadequate amount of warm water during the test period. It is important to note that the concentration of the hypertonic buffer solution used in testing flexibility of the heated cells was different to the rest of the cell envelope flexibility experiments. The control yeast used was heated from $14^{o}C$ to $25^{o}C$ gradually over extended time (without pumping through the copper coil) at approximately $0.036^{o}C.s^{-1}$. Irrespective of the Malvern diluent used, Figure 5-5 and Figure 5-6 show that the percentage size change of cells was the greatest in rapidly heated yeast. Furthermore, Table 5-3 shows an increase in both rate and extent of change in cell size with an increase in heating rate. As error of analysis was not carried out, the confidence interval within which this conclusion is drawn cannot be quantified.

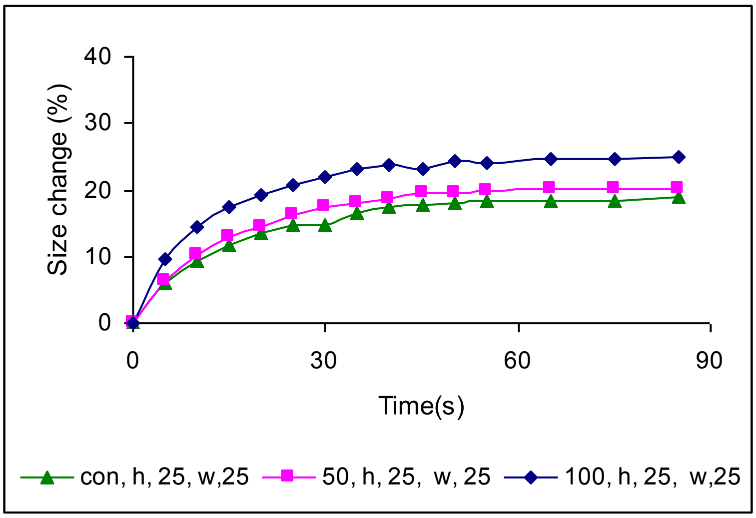


Figure 5-5: Size change (%) of yeast at 61 % consistency on dilution into water following moderate heating to $25^{o}C$ at 50 % and 100 % of the maximum flow rate respectively through the heating coil using a wall temperature of $30^{o}C$. Yeast was injected into warm water maintained around $25^{o}C$. Control yeast was slowly raised to $25^{o}C$ ($0.036^{o}C.s^{-1}$).

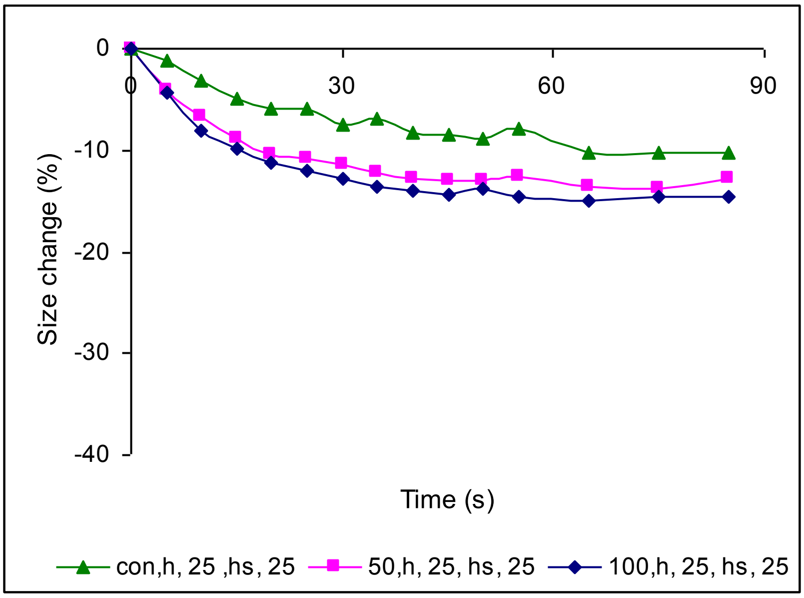


Figure 5-6: Size change (%) of yeast at 61 % consistency on dilution into the hypersomal salt (866 mM NaCl and 13 mM Tris) solution following moderate and rapid heating to 25°C at 50 % and 100 % of the maximum flow rate respectively through the heating coil using a wall temperature of 30°C. Yeast was injected into hypersomal salt solution control yeast was slowly raised to 25°C (0.0367°C.s^{-1}).

Table 5-3: **The rate and extent of change in size of yeast at 61 % consistency following moderate and rapid heating to 25°C at 50 % (0.9°C.s^{-1}) and 100 % (1.7°C.s^{-1}) of the maximum flow rate respectively through the heating coil using a wall temperature of 30°C.** Yeast was injected into water (w) (Figure 5-5) and the hypertonic salt solution (hs) (Figure 5-6).

Yeast treatment	**Rate of change in the size of the cell [Δ Size change (%)]/ [Δ time (s)]**	**Extent of change in cell size (%)**
con, h, 25, hs, 25	- 0.25	- 10.00
50, h, 25, hs, 25	- 0.84	- 13.00
100, h, 25, hs, 25	- 0.87	- 15.00
con, h, 25, w, 25	1.19	19.00
50, h, 25, w, 25	1.26	20.17
100, h, 25, w, 25	1.90	24.91

5.5 THE EFFECT OF THE OSMOTIC STRENGTH OF THE MEDIA ON FLEXIBILITY OF HEATED CELLS

Yeast, collected from the brewery following cropping, was heated on flow through the heating coil with the wall temperature of 35°C to yield an average exit temperature of heated yeast of 30°C. The average heating rate obtained from yeast suspension pumped at 50 % of the maximum flowrate was 2.1°C.s^{-1}. Figure 5-7 shows the cell envelope flexibility response of yeast upon injection into diluents of varying osmotic strength. Yeast enlarged and became smaller when injected into the hypotonic and hypertonic salt solution respectively. The size change of yeast immersed in the water was greater than when yeast was injected into the hypertonic salt solution. Table 5-4 shows that the rate and extent of size change (%) of yeast injected into water was the faster than in the hypersomal salt solution. It is concluded that yeast will shrink when injected into the environment of high osmotic potential.

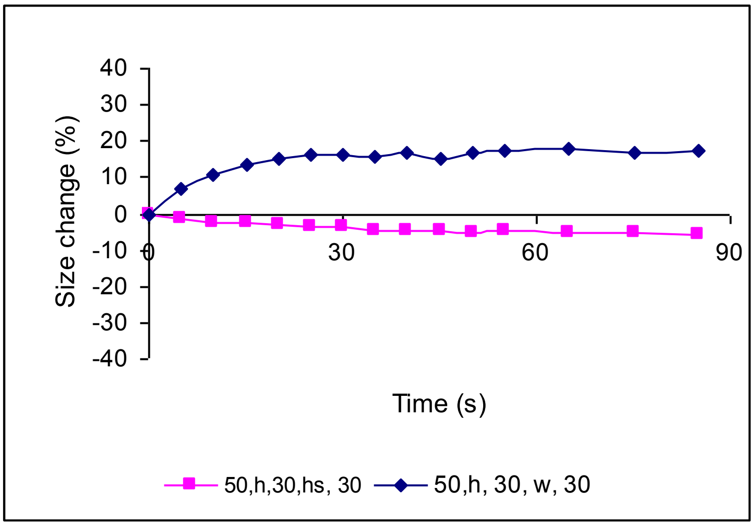


Figure 5-7: Cell envelope flexibility of yeast following moderate heating to 30°C at 50 % of the maximum flow rate through the heating coil using a wall temperature of 35°C. Yeast flow rate and heating rate were 0.38 m.s^{-1} and 2.1°C.s^{-1} respectively. Yeast was injected into water (w) and hypersomal salt solution (hs). The legend used: 50, h, 30, hs, 30: yeast pumped at 50% of the maximum flow rate, heated at 30°C, injected into hypersomal salt solution at 30°C; 50, h, 30, w, 30 : yeast pumped at 50% of the maximum flow rate, heated at 30°C, injected into water at 30°C.

Table 5-4: **The rate and extent of change in size of yeast at 61 % consistency following moderate (2.1^{o}C.s^{-1}) and rapid (4.5^{o}C.s^{-1}) heating to 30^{o}C at 50 % and 100 % of the maximum flow rate respectively.**

Yeast treatment	**Initial rate of change in the size of the cell [Δ Size change (%)]/ [Δ time (s)]** **(50 % max flowrate)**	**Extent of change in cell size (%)** **(50 % max flow)**
50, h, 30, hs, 30	- 0.32 ± 0.01	- 5.56
50, h, 30, w, 30	1.35	17.30

5.6 THE NATURE OF SUSPENSION ON FLEXIBILITY

The aim of the experiment was to compare the effect of the suspension on the cell envelope flexibility of yeast following cooling. The three suspending media used were a blend of PBS and beer; beer only and PBS only. Figure 5-8 shows the influence of the nature of yeast suspension on the extent of change in yeast cell size during the cell flexibility test. In all cases, the rate at 60 % consistency was greater than other rates. Generally, the rate obtained when using PBS was greater than other rates. The effect seen at 60 % yeast consistency is not due to cooling rate effect instead of a dilution effect. The effect may result from yeast modifying the surrounding environment of a concentrated suspension. Figure 4-3 showed that there was no difference cooling rates obtained when varying the nature of yeast suspension. The rates of change in size of yeast suspensions diluted with the three diluents were equal at all yeast consistencies.

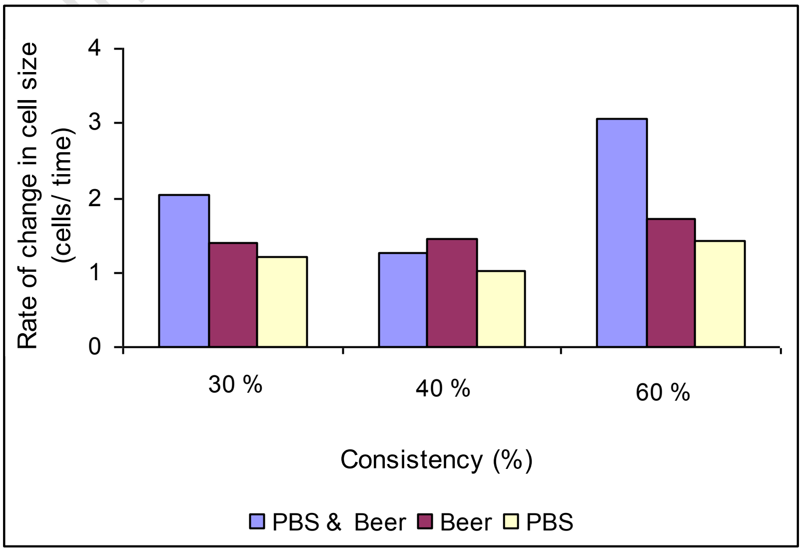


Figure 5-8: **The rate of change in the size of yeast at 30 %, 40 % and 60 % consistency in PBS only, beer only and a blend of PBS and beer following rapid cooling to 7^{o}C at 100 % of the maximum flow rate.** Yeast was injected into water (Malvern diluent).

5.7 CONCLUSIONS

On examining the effect of the difference in the temperatures of the yeast sample and the Malvern diluent on the measured flexibility of yeast, it was shown that it is crucial to minimise any temperature shock within the assay when determining the relation between the yeast sample and cell envelope flexibility. However, for cooled yeast sample, it is difficult to maintain the diluent temperature at the same temperature as the sample because condensation on the lens reduced the accuracy of data. When carrying out cell envelope flexibility for yeast cooled below 10^{o}C, the temperature shock was reduced to a minimum while avoiding clouding of the lens by keeping the Malvern diluent at 14^{o}C. The size of the error introduced was approximately 15 %.

The increase and decrease in the size of heated yeast cell was observed when injected into the hypotonic and hypertonic solution respectively. Moreover, the cell envelope flexibility of yeast injected in water was greater than for hypersomal salt solution. Yeast injected in water displayed the highest change in cell size due to high influx of the surrounding water into the cell owing to the differing osmotic pressure. Yeast cooled at a rapid rate displayed a higher cell envelope flexibility compared to yeast cooled at a slower rate. Similarly, yeast heated at a rapid rate displayed a higher cell envelope flexibility compared to the less heated yeast. The results obtained could be related to the concentration of HSP in fast and slow cooled yeast. The rate of change in cell size of yeast was the highest when yeast was suspended in PBS. Furthermore, the highest rate of change was obtained at 60 % yeast consistency due to yeast modifying the surrounding environment in the concentrated suspension.

CHAPTER 6: THE EFFECTS OF COOLING RATE ON THE STRUCTURAL STRENGTH OF BREWERS YEAST

6.1 INTRODUCTION

The cell envelope plays a crucial role in yeast integrity and viability. The cell integrity of brewers yeast may be weakened through mechanical handling of yeast such as centrifuging, transferring of yeast throughout the yeast circuit, passage through pumps etc (Section 2.4.1). Weakened cell integrity can be observed by the presence of external cell wall materials like mannan and glucan in beer (Lewis and Poerwantaro, 1991; McCaig and Bendiak, 1985; Pickerell *et al.,* 1991), the presence of intracellular compounds in the beer (Mochaba *et al*., 1993; Basson et al, 1997), and reduced cell envelope strength. Pumping yeast within the brewery yeast circuit exposes the yeast cell wall to shear, thus providing potential to compromise the cell integrity. Damaged yeast cell walls may result in the release of compounds into beer. This may lead to the production of off - flavours (McCaig and Bendiak, 1985) and haze formation (O'Connor-Cox, 1994), resulting in reduced beer quality and shortened shelf life. Hazy beer increases the costs of clarifying beer, leads to reduced beer quality and a shortened shelf life.

The aim of the study was to investigate the effect of cooling rate on the structural strength of brewers yeast. The yeast was pumped through the cooling coil immersed in a coolant set at -1^{o}C to achieve cooling. Cell integrity was determined both as haze generated and the fragility of the cell envelope. Haze generation was quantified by determining the presence of fine particles in the supernatant recovered from centrifuged beer using the Malvern laser light scattering to quantify particle size distribution. To test cell fragility, the yeast slurry was subjected to sublytic pressure using a French Press cell. The operating pressures used were 6, 10 and 16 MPa. Typically, 25 to 100 MPa pressure is used to disrupt yeast. In Section 6.2, haze measurement for a certain generation of control yeast maintained at 14^{o}C is presented, whereafter haze generated as a function of the cooling rate and yeast consistency is also presented. In Section 6.3, cell wall fragility is presented as a function of cooling rate.

6.2 THE CELL WALL HAZE GENERATION FROM YEAST

6.2.1 Analysis of haze generated on cropped yeast

The control yeast in this study was *Saccharomyces cerevisiae*, generation 6 yeast from SAB Newlands brewery cropped at the end of fermentation and maintained at 14^{o}C. The haze analysis was carried out as described in Section 3.2.3.1. Following low speed centrifugation to remove yeast particles, particle size analysis was performed by laser light scattering. Prior to yeast removal from the beer by a low speed centrifugation, a sample of yeast at 14^{o}C was analysed for haze. Figure 6.1 shows a typical volume-based yeast size distribution with an average particle size diameter of 8 µm. Robinson (2001) determined the size range of haze in yeast by centrifuging the yeast sample at 2500 rpm for 3 minutes to remove yeast cells, followed by analysis of the sample for haze using Malvern Mastersizer (long version 2). The size range for haze was shown to be between 0.08 µm and 2 µm (Robinson, 2001). The size distribution analysed in assessing the haze concentrate showed some trace yeast (3–13 µm) in the haze concentrate. Further, the particle size range of 13–500 µm observed was assumed to be caused by aggregate formation. In this study, in Figure 6.1, protrusion on the left shoulder of the volume-based yeast size distribution indicates the presence of haze in beer supernatant.

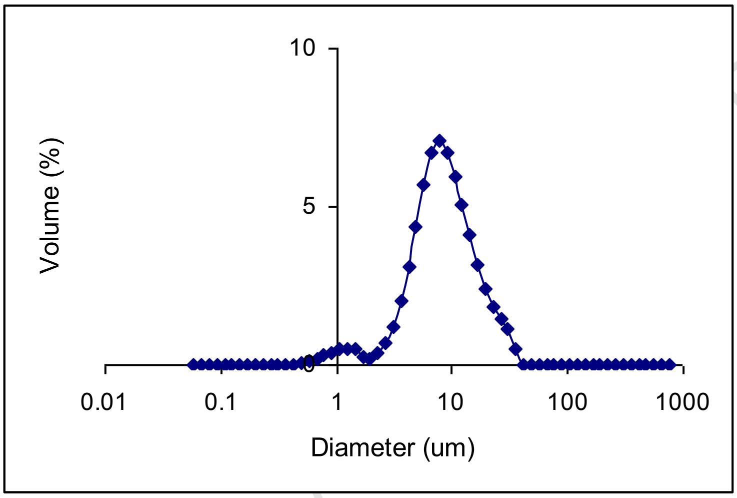


Figure 6-1: **Size distribution of control yeast without centrifuging.** The supernatant used for haze was collected following yeast sedimentation.

Yeast cropped from the brewery was diluted with beer. The beer supernatant used for haze test was collected after the yeast had sedimented at the bottom of the sample. For all the haze tests, the size distributions collected are presented as both volume distribution (a) and number distribution (b). Data collected for (a) and (b) were generated from the same sample. The volume-based size distribution is shown for undiluted control yeast (Figure 6.2 (a)). Clearly not all the yeast cells (average diameter between 7 and 10 µm) were removed when preparing the haze concentrate. Further, some flocculated yeast particles in the range 10.5-104 µm remain. The volume distribution depicted in Figure 6.2 (a) shows that smaller haze particles are overshadowed by the yeast. For clear visualization of haze, valuable data was extracted by conversion of the data from volume-based distribution to a particle number basis as shown in Figure 6.2 (b). The conversion of particle size distribution by mass (equivalent to volume) to particle size distribution by number is shown in Equation 6.1.

$$f_M(x) = k_3 x^3 f_N(x) \qquad 6\text{-}7$$

where f_M (x) denotes the particle size distribution by mass (volume)

k_3 denotes a scaling factor used to normalize the area under the graph to 100%.

x denotes the particle size diameter

f_N (x) denotes the particle size distribution by number

Figure 6.2(b) shows the presence of both haze material and yeast in the size range 0.9 µm to 16.6 µm. The median of the particle number was 1.95 µm with a maximum number frequency of 13%. Cropped yeast experienced the hydrodynamic shear damage upstream of where the yeast sample was collected at the brewery. The yeast was not introduced to further shear in the laboratory instead transferred into a container and maintained at 14°C.

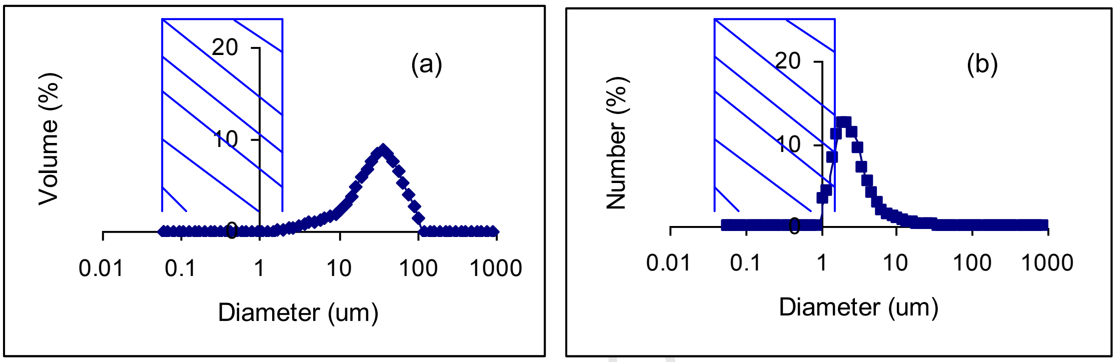


Figure 6-2: Size distribution of (a) control yeast and (b) haze. The control yeast sample was maintained at 14°C. k_3 was 4.04. Shaded region illustrates the size range 0.08 to 2um attributed to haze material.

6.2.2 The influence of cooling rate on haze generated

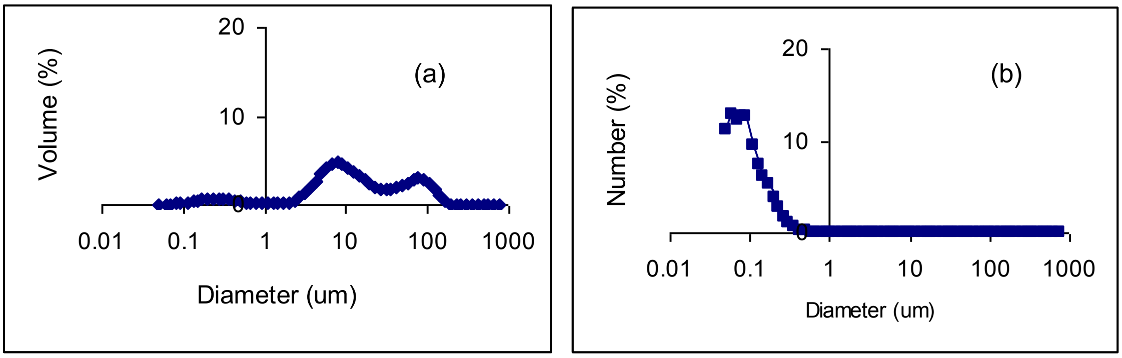
The increase in flow rate of the yeast suspension exposes the yeast to higher shear. Excessive shear damage to the cell envelope results in increased haze particles in beer. Generation 1 yeast (64% consistency) from SAB Newlands brewery at the end of fermentation was introduced to different cooling treatments. Some of the yeast suspension was maintained at 14°C as control (neither exposed to shear nor cooled). A separate control in which yeast was exposed to shear but not cooled was not carried out. The remaining fractions were cooled by pumping through the laboratory cooling equipment at the pump setting 15 %, 25 %, 50 %, 75 % and 100 % corresponding to cooling rates of 0.1, 0.2, 0.57, 0.91 and 1.02°C.s^{-1} respectively. The coolant was set at -1°C. The aim of the experiment was to observe the influence of cooling rates on shear damage experienced by yeast in the heat exchanger. The haze test was carried out for the following cooling rates: control (14°C), 0.2°C.s^{-1} and 1.02°C.s^{-1}. The size distributions under these conditions are shown in Figure 6.3 (I), (II) and (III) respectively as both volume distribution (a) and number distribution (b).

Figure 6.3 (I a) shows a resultant volume size distribution of haze particles collected in the absence of cooling without passage through the cooling coil. Some flocculated yeast particles in the range 10.5 to 190.8 µm remained in the sample. The presence of yeast particles with a median of 7.7 µm and flocculated yeast with the median of 89 µm are clearly seen. While the smaller haze particles are overshadowed by the yeast in the volume distribution; a clear shoulder is seen below 1 µm. On conversion of the data from a volume to a particle number basis as shown in Figure 6.3 (I b), a clear peak representing haze material was observed in the size range 0.05 to 1.44 µm. From Figure 6.3 (I a), the area under the volume size distribution was determined in the range between 0 and 2 µm, indicating 0.6 % haze material was obtained. In Figure 6.3 (I b), it is clear that small haze particles dominate the suspension following low speed centrifugation. The median of the particle number was 0.27 µm with a maximum number frequency of 12.9 %.

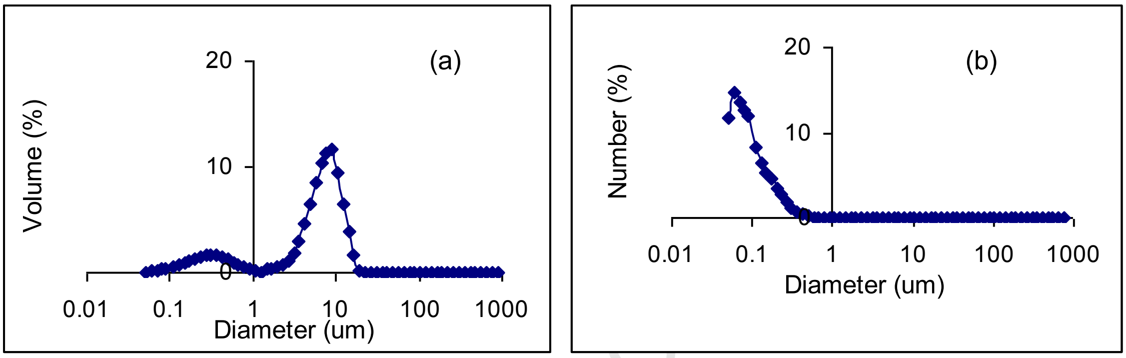
Figure 6.3 (II a) shows a resultant volume size distribution of haze particles collected following cooling at $0.2^{o}C.s^{-1}$. Subsequent to centrifuging the yeast sample to prepare a haze concentrate, some traces of yeast were left. The two peaks shown represent haze (median of 0.31 µm) and unicellular yeast material (median 9 µm). Some flocculated yeast particles in the range 10.5–22.49 µm remain. No larger flocs beyond 100 µm were observed. While the smaller haze particles are overshadowed by the yeast in the volume distribution; a clear shoulder is seen below 1µm accounting for 1.15 % suspended as haze. On conversion of the data from % volume to a particle number basis as shown in Figure 6.3 (II b), a clear peak representing haze material was observed in the size range 0.05 to 1.06 µm. The median was 0.31 µm with a maximum number frequency 14.6 %. Yeast cooled at $0.2^{o}C.s^{-1}$ released more haze particles as indicated by the greater contribution of the area of the haze peak on a volume basis. The median of the size distribution of haze for control and the fast cooled yeast are approximately the same.

Figure 6.3 (III a) shows a resultant volume size distribution of haze particles collected following cooling at $1.02^{o}C.s^{-1}$. Not all of the yeast particles were removed when preparing the haze concentrate. Maxima in the distribution at 0.58 µm represent haze; 7.72 µm represents unicellular yeast and 56.23 µm and 163.77 µm represent flocculated yeast. On conversion of the data from a volume to a particle number basis as shown in Figure 6.3 (III b), the peak representing haze material in the size range 0.05 to 1.95 µm was dominant. From Figure 6.3 (III a), the area under the volume size distribution correlating to haze, was determined in the range between 0 and 2 µm, accounted for 1.38 % of the suspended material. In Figure 6.3 (III b), the median of 0.58 µm is observed with a maximum number frequency of 16.2 %.

(I) Control ($0^oC.s^{-1}$)



(II) Cooled at $0.2^oC.s^{-1}$



(III) Cooled at $1.02^oC.s^{-1}$

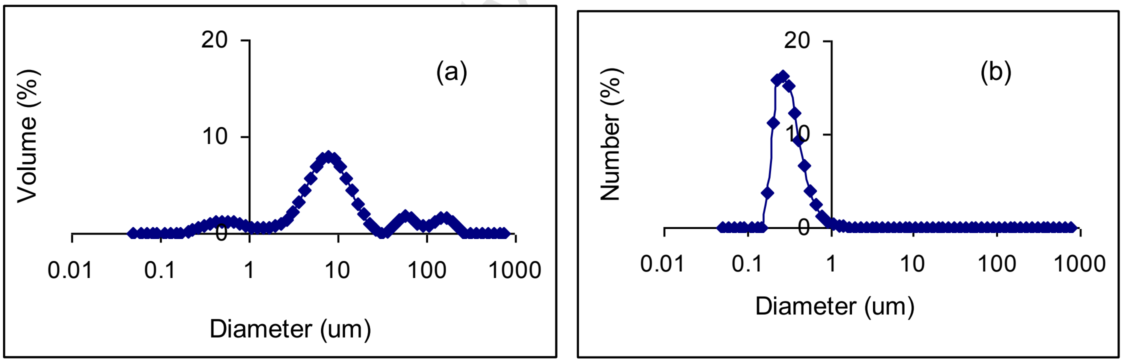


Figure 6.3: Size distribution of yeast and haze suspensions of 64% consistency, generation 1 yeast suspension (I) control yeast, (II) cooled at $0.2^0C.s^{-1}$ and (III) cooled at $1.02^0C.s^{-1}$.In (a) data are presented on a volume basis and in (b) on a number basis. The scaling factors (k_3) used to normalize the area under the graph to 100% were 0.03, 6 x 10^{-2} and 2 x 10^{-4} for control yeast, cooled at $0.2^0C.s^{-1}$ and $1.02^0C.s^{-1}$ respectively.

6.2.2.1 Conclusions

In this study, haze increased with an increase in flow rate and cooling rate on flow through the cooling coil. The relative contribution of flow rate or cooling rate was not determined separately. More fine haze material was generated on rapid cooling. The volume based area under the peak attributed to haze (0.05 to 2 μm) showed that haze generation increased as cooling rate increased. It is recommended that further tests should be carried out to determine the rate of fluid flow per se on haze generated.

6.2.3 The influence of yeast consistency (concentration) in haze generated following cooling

An increase in the consistency of the yeast suspension imposes additional resistance to transfer of yeast through the pipework owing to increased apparent viscosity and shear thinning behaviour (Robinson, 2001). The higher the yeast consistency, the greater the amount of yeast particles that are exposed to the pipe wall however the rate of shear experienced by yeast of a lower consistency is greater than of yeast of a higher consistency due to higher velocity of the fluid. In order to observe the influence of yeast consistency on shear damage experienced by yeast following cooling, generation 5 yeast (60 % consistency) taken from SAB Newlands brewery at the end of fermentation was used to prepare the yeast suspensions of three different yeast consistencies, namely: 30 %, 40 % and 60 %, using beer as diluent. The effect of cooling rate on haze generation across the different consistencies was studied. The control yeast was maintained at 14°C without passage through the coil. Yeast was cooled by pumping through the laboratory cooling equipment at the pump setting of 50 % of the maximum flow rate with the coolant set at -1°C. The cooling rates obtained from lower (30 %) to higher (60 %) yeast consistencies were 0.79, 0.76 and 0.54°C.s^{-1} respectively. The haze test was carried out for all the three samples. Size distributions are presented as both volume distribution (a) and number distribution (b). The size distribution of haze fraction following cooling at 0.79°C.s^{-1}, 0.76°C.s^{-1} and 0.54°C.s^{-1} are shown in Figure 6.4 (I), (II) and III) respectively.

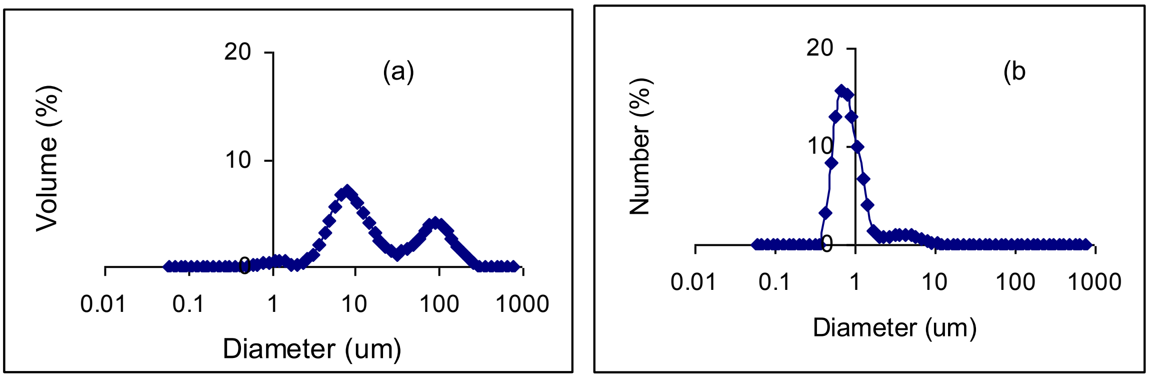
Figure 6.4 (I a) shows the resultant volume size distribution of the haze concentrate collected on cooling the 30 % yeast suspension at 0.79°C.s^{-1}. A clear shoulder is seen below 1.95 µm, median of 1.06, accounting for 0.51 % suspended as haze. Two large peaks shown represent unicellular yeast (7.7 µm) and flocculated yeast (88.9 µm) respectively. On conversion of the data from % volume to a particle number basis (Figure 6.4 (I b)), a clear peak height representing haze material was observed having a median of 0.67 µm with a maximum number frequency of 15.78 %.

Figure 6.4 (II a) shows the resultant volume size distribution of the haze concentrate collected on cooling the 40 % yeast suspension at 0.76°C.s^{-1}. Two shoulders are seen on the left and right of the volume size distribution. The right shoulder shows that not all of the yeast particles were removed when preparing the haze concentrate. The left shoulder (extending below 1.67 µm) shows that some smaller haze particles were overshadowed by the yeast in the volume distribution. The median of the unicellular yeast was 7.7 µm respectively. On conversion of the data from % volume to a particle number basis as shown in Figure 6.4 (II b), a clear peak representing haze material was observed in the size range 0.05 to 1.95 μm. From Figure 6.4 (II a), the area under the volume size distribution correlating to haze, was determined in the range between 0 and 2 μm, accounted for 0.61 % of the suspended material. In Figure 6.4 (II b), the median of 0.67 μm is observed with a maximum number frequency of 18.16 %.

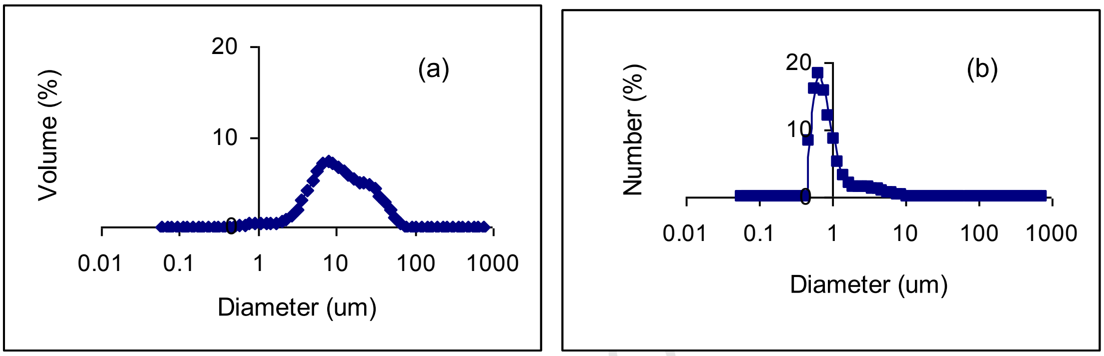
Figure 6.4 (III a) shows a resultant volume size distribution of the haze preparation following cooling of the yeast suspension of 60 % consistency at $0.54^{o}C.s^{-1}$. Three peaks are seen corresponding to unicellular and flocculated yeast particles. A clear shoulder is seen below 1.95 μm, representing haze material. Maxima in the distribution at 1.23 μm represent haze; 6.6 μm represents unicellular yeast and 56 μm and 90 μm represent flocculated yeast. On conversion of the data from % volume to particle number basis as shown in Figure 6.4 (III b), a clear peak representing haze material in the size range 0.31 μm to 1.95 μm was dominant. From Figure 6.4 (II a), the area under the volume size distribution correlating to haze, was determined in the range between 0 and 2 μm, accounted for 0.98 % of the suspended material. In Figure 6.6 (II b), the median of 0.49μm is observed with a maximum number frequency of 17.14 %.

From Figures 6.4 (I b), (II b) and (III b), it can be seen that the medians of the haze size distribution were 0.67 μm, 0.67 μm and 0.49 μm respectively. In addition, the medians of the unicellular yeast were equal for the 30 % and 40 % yeast suspensions. The cooling rates obtained for the 30 % yeast suspension was slightly lower (less 4 %) than at 40 % yeast consistency. The median of the unicellular yeast decreased with an increase in consistency. The area of haze peak on the volume basis increased following cooling of yeast with an increase in yeast consistency. The maximum number frequency of the haze obtained ranged between 15 % and 19 %.

(I) 30 % consistency, 0.79°C.s^{-1}



(II) 40 % consistency, 0.76°C.s^{-1}



(III) 60 % consistency, 0.54^{0}C.s^{-1}

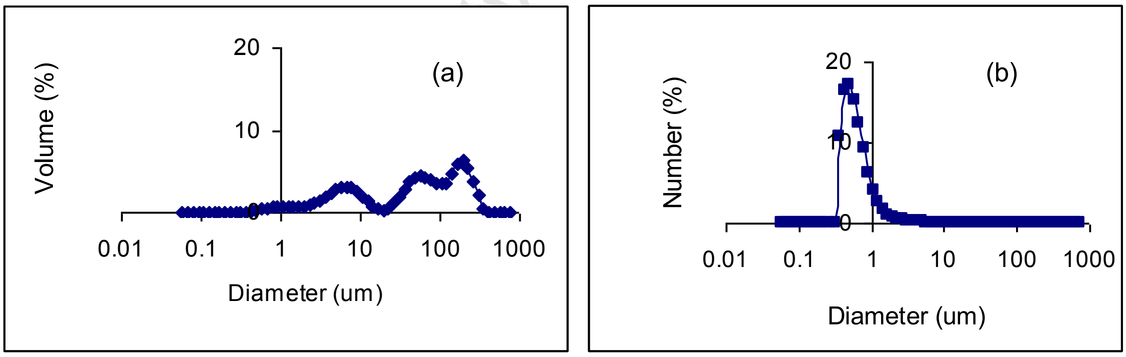


Figure 6.4: Size distribution of yeast and haze suspensions of 30 %, 40 % and 60 % consistency, generation 5, cooled at (I) cooled at 0.79^{0}C.s^{-1} , (II) cooled at 0.76°C.s^{-1} and (III) cooled at 0.54^{0}C.s^{-1}. Data presented on a volume basis (a) and on a number basis (b). The scaling factors (k_3) used to normalize the area under the graph to 100% were 0.25, 0.2 and 0.07 for the yeast cooled at 0.79^{0}C.s^{-1}, 0.76°C.s^{-1} and 0.54^{0}C.s^{-1} respectively.

6.2.4 Integration of generation number, cooling rate, peak height and median of the haze size distribution

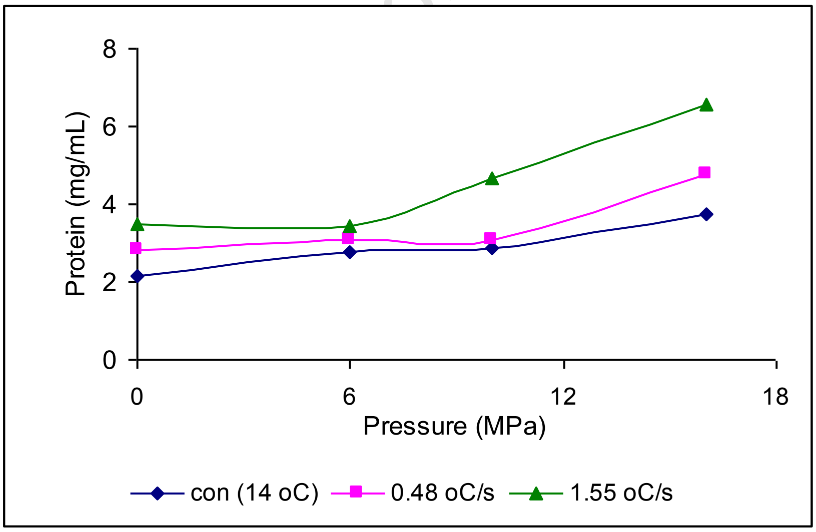
A summary of the relationship between cooling rate and the fraction of haze found in each sample is shown in Table 6.1. Table 6.1 shows differing haze in different control samples. This may arise from how yeast was treated before collection, so cannot be attributed to differing consistency. An increase in cooling rate at the same consistency did not change haze much. However, an increase in consistency on cooling increased haze although cooling rate decreased. This can be linked to changes in viscosity and shear rate. Without control yeast on generation 5, it is difficult to say whether haze generated was due to flow rate or cooling rate or the combined effect of flowrate and cooling rate.

Table 6.1: **The integration of cooling rate, peak height and percentage area of haze per area of yeast**

Generation number	Linear velocity (m.s^{-1})	Consistency (%)	Cooling rate (^{o}C.s^{-1})	Peak height (%)	Median (μm)	Vol. [area < 2 μm/ area btw 7-9μm]
6	0(control)	60 %	0	13	1.95	0.125
1	0(control)	64 %	0	12.9	0.27	0.93
1	0.055	64 %	0.2	14.62	0.31	0.82
1	0.235	64 %	1.02	16.21	0.58	0.81
5	0.201	30 %	0.79	15.78	0.67	0.22
5	0.194	40 %	0.76	18.16	0.67	0.26
5	0.169	60 %	0.54	17.14	0.49	1.14

6.3 THE EFFECTS OF COOLING RATES ON THE FRAGILITY OF THE CELL ENVELOPE

An increase in fluid flow rate exposes yeast to more shear thus compromising cell wall integrity. In this study, yeast was cooled at different cooling rates and its cell wall integrity was measured by carrying out the cell wall fragility test. Generation 2 yeast (61 % consistency) taken from SAB Newlands brewery on cropping at the end of fermentation was divided into three portions. Two yeast suspensions were cold stressed by pumping through the laboratory cooling equipment and the other portion of yeast was left on ice (control yeast). The fragility test was carried out on both the control and the cold stressed yeast using the method defined in Section 3.5.1.2. The amount of protein released on damage in the French Press as a function of cooling rate is shown in Figure 6.5. Soluble protein released was measured by absorbance at A_{280} . Figure 6.5 shows that the yeast cooled at 1.55°C.s^{-1} released greater amount of protein compared to the yeast cooled at 0.48°C.s^{-1} and the control yeast. These findings are consistent with increased cell envelope damage occurring on increased rate of cooling in the range studied, resulting in increased envelope fragility. In a second experiment using generation 6 yeast at a consistency of 64 %, the yeast was cooled in the laboratory cooling equipment at rates of 0.9°C.s^{-1} and 1.9°C.s^{-1}. Figure 6.6 shows the fast cooled yeast (1.9°C.s^{-1}) to have released the most protein compared to the slow cooled and the control yeast. The amount of protein released increased with an increase in sublytic pressure.



. **Figure 6-5**: **Measure of cell envelope fragility in terms of protein release on sudden pressure drop in the French Press, as a function of cooling rate.** Yeast shown are for control yeast (maintained at 14°C), yeast cooled at 0.48°C.s^{-1} and yeast cooled at 1.55°C.s^{-1}. Yeast consistency was 61 %.

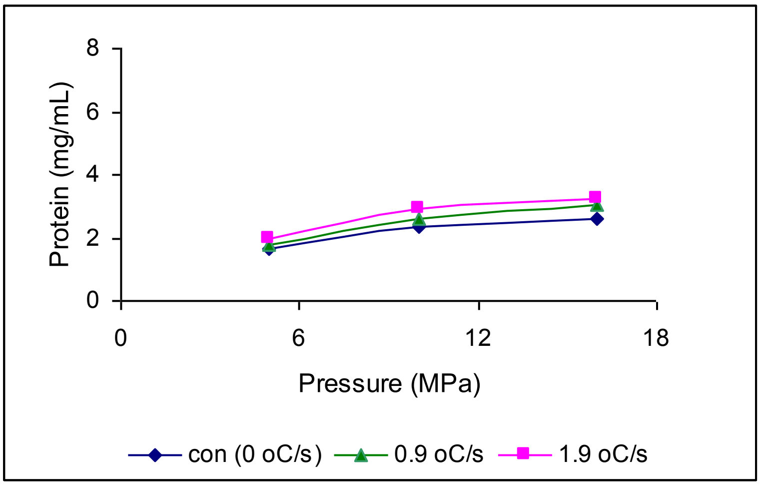


Figure 6.6: Measure of cell envelope fragility in terms of protein release on sudden pressure drop in the French Press, as a function of cooling rate. The coolant was set at -1°C.s^{-1}. PBS was used as a blank to zero the standard protein curve. Sublytic pressures used were 6MPa, 10MPa and 16MPa.

6.4 CONCLUSIONS

The number-based size distribution of the haze preparations from which yeast was removed by low speed centrifugation provided a suitable measure of haze formation on cold stress. Formation of haze in the size range less than 2 µm was clearly observed. The mode of the haze peak was observed in the range 0.05 to 1.95 µm. Haze found in different control samples was attributed to the previous yeast handling history prior to collection instead of yeast consistency. The amount of haze generated increased with an increase in consistency on cooling however an increase in cooling rate at the same consistency did not change haze much. When cooled yeast was subjected to sublytic pressure using a French Press, the cell wall fragility increased with an increase in cooling rate due to increased cell envelope damage damage.

CHAPTER 7: GENERAL DISCUSSION

7.1 INTRODUCTION

In this chapter, the findings reported in Chapters 4 to 6 are discussed in order to fully understand the effects of cooling rate on the quality of brewers yeast. The yeast is exposed to a range of thermal and shear stresses during its transfer within the brewery cellar and yeast handling operations. The response of cold stressed yeast was assessed in terms of fermentation performance, metabolic activity, cell envelope flexibility and structural strength.

Brewers yeast is exposed to various types of stress during processing in the brewery. These stresses are classified as physiological and mechanical. Proper handling of yeast in the brewery is required to prevent the deterioration of yeast quality as a result of exposure to excessive stress. Basson (1996) reported that the typical yeast handling regiment entails:

- Transfer of yeast from propagation vessels into the aerated wort for fermentation
- Transfer of yeast from the storage vessel into aerated wort for further fermentation
- Pumping settled yeast from bottom of cylindroconical fermentation vessel at the end of fermentation
- Flow of yeast through pipework (of specified diameter and length), constrictions and fittings
- Cooling to approximately 3°C by passage through heat exchanger
- Agitation in the storage tank
- Possible re-circulation through heat exchanger to maintain temperature

Yeast experiences mechanical stress while being agitated, transferred and separated. The potential sources of mechanical handling stresses are hydrodynamic shear and hydrodynamic cavitation as turbulent energy dissipation is low in viscous suspensions. Hydrodynamic shear may arise during flow of the yeast suspension through pipework, fittings, constrictions and expansions as well as other units of process equipment (pumps, heat exchangers, centrifuges and agitated vessels).

This study investigated the factors that affect the maintenance of yeast quality during cooling and identified the cooling rate that has the least detrimental effect on the yeast physiology such that subsequent fermentations are not adversely affected. It investigated the effect of diluent on fermentation performance, thereby assessing potential ethanol stress. It also investigated the cell envelope flexibility response upon heating, and lastly studied the relationship between cooling rates, osmotic strength of the media and cell envelope flexibility.

At the onset of this study, it was hypothesized that:

- Yeast cooled at a low cooling rate will yield better fermentation performance than yeast cooled at cooling rates close to the critical cooling rate of 2°C.s^{-1}.
- Flexibility of the cell envelope increases with an increase in cooling and heating rate.
- Yeast structural strength is indirectly proportional to the cooling rate beyond a critical rate.
- Haze generation is aggravated at higher cooling rate.
- Lower cell viability, cell growth and fermentation performance are expected on yeast cooled in the presence of beer, compared to that cooled in a suspension where the beer is diluted. Yeast fermentation performance is expected to decrease on increased exposure to ethanol concentration in the brewers yeast suspension. The presence of ethanol was shown to enhance permeability of the cell membrane. High ethanol concentration has also been shown to lead to decreased cell viability (Sales *et al*., 2001; D'Amore *et al*., 1990).

7.2 THE EFFECT OF FLOW RATE, DILUENT, AND YEAST CONSISTENCY ON THE COOLING RATE AND SMALL- SCALE FERMENTATION PERFORMANCE OF YEAST

When a fluid is cooled by pumping through the cooling coil, the driving force for heat transfer is determined by the temperature difference between the two fluids, while resistance to transfer is a function of the fluid velocity, pipe wall thickness, thermal conductivity of the pipe, and the physical properties of the fluids involved. In this study, the geometry of the pipe, the temperature of the coolant and the thermal conductivity of the coil were kept constant.

The linear velocity of yeast and the fluid viscosity determine the residence time and the extent of shear to which yeast is exposed and thus influence the cooling rate. In this study, the linear velocity of the fluid suspension and the yeast consistency were varied. A linear relation between linear velocity (altered through pump setting) and cooling rates was shown to exist at all flow rates (Figure 4-1). Yeast cooled at greater linear velocities displayed higher cooling rates compared to the yeast cooled at lower linear velocities (Figure 4-2). Results obtained by Nkosi (2001) displayed a similar relation for the cooling rate and linear velocities.

Cooling rate is directly proportional to the flow rate. The cooling rates obtained for yeast at a consistency of 64% and linear velocities of 0.02 m.s^{-1}, 0.05 m.s^{-1}, 0.13 m.s^{-1}, 0.22 m.s^{-1} and 0.235 m.s^{-1} were 0.1°C.s^{-1}, 0.2°C.s^{-1}, 0.6°C.s^{-1}, 0.9°C.s^{-1} and 1°C.s^{-1} respectively. There was no significant difference between cooling rates obtained with the type of diluent used; namely, PBS only, beer only and a blend of PBS and beer.

With other variables constant, an increase in yeast consistency, and thereby apparent viscosity and shear thinning behavior is expected to lead to an increase in residence time of yeast inside the cooling coil. When both the linear velocity and the yeast consistency were varied, the cooling rate decreased with increasing yeast consistency (Figure 4-3). The decrease in cooling rate was due to a decrease in fluid velocity and an increase in fluid viscosity; hence a decrease in heat transfer rate across the cooling coil. The lowest and highest cooling rates were obtained from yeast at 60 % and 30 % consistencies respectively. Cooling rate was indirectly proportional to the yeast consistency.

A base case for small-scale fermentation was established using the control yeast with 58 % consistency. The yeast was collected from the brewery and maintained at a temperature of 14°C. The yeast suspension was diluted with a blend of PBS and beer. Evaporation controls showed that the average amount of water evaporated during small scale fermentation was 0.05 % of the mass loss due to carbon dioxide loss, hence it is considered insignificant. The total amount of carbon dioxide evolved was greater than 0.3 g. The rate of carbon dioxide evolved mimicked the growth of yeast. The method was not sufficiently sensitive to quantify carbon dioxide evolved due to maintenance. Cell concentration increased rapidly between the 2nd and 6th hour of the small-scale fermentation. At the end of the small-scale fermentation, the final cell concentration was 4 times the starting cell concentration. The overall cell concentration recorded after 24 hours of fermentation was approximately 1.1×10^9 cells.ml^{-1} and a corresponding dry biomass of 1.9 mg.ml^{-1} yeast sample. The difference in average biomass obtained between the 8th and the 24th hour of fermentation shows that cell growth slowed down after 8 hours of small-scale fermentation. Further, the production of carbon dioxide was rapid during the first 6 hours of fermentation thus mimicking cell growth. Similarly, the concentration of the substrate decreased rapidly during the first 8 hours of fermentation. At the end of the small-scale fermentation period, the residual substrate concentration had decreased by 94 %.

Nkosi (2001) investigated the behavior of yeast cell performance upon cooling at different cooling rates, maintaining the yeast concentration constant (30 %). Cooling rates employed were between 1.03 to 4.03°C.s^{-1}. Rapid cooling of yeast (2 to 4°C.s^{-1}) through the heat exchanger resulted in greater stress than that experienced at slower cooling rates less than 1°C.s^{-1}, with a simultaneous increase in damage to the cell envelope and loss of yeast vitality. Cooling rates above 2°C.s^{-1} aggravated damage to the cell envelope and showed the most detrimental effect on the subsequent small-scale fermentation. A faster cooling rate was shown to impair fermentation performance by decreasing carbon dioxide evolved and the biomass yield (Nkosi, 2001). The results were attributed to the negative effect that shear can impose on yeast and to cold shock. Furthermore, the brewery experiments showed yeast growth rate, biomass and CO_2 yield decreased with increasing cooling rates in the range of 2 to 4°C.s^{-1}.

In this study, the effect of cooling on yeast quality was studied using yeast suspensions ranging in consistency between 30 and 60 %, equivalent to 75 to 150 g yeast dry weight per litre. The cooling rates obtained ranged between 0.1 and 2.15°C.s^{-1}. The extent of growth in small-scale fermentation following cooling was less than that of the control, but consistency did not have a dominant effect except when the yeast consistency was 30 %. There was a small difference in the final concentration obtained from cooled and control yeast at 30 % consistency. The final cell concentration obtained at 40 % yeast consistency was approximately equal to that at 60 % consistency. The relationship shown between cell concentration and yeast consistency is not well understood.

Cell vitality, measured as the rate and extent of carbon dioxide evolved during small-scale fermentation, increased following cooling of the cropped yeast relative to the control yeast. These results, together with the small-scale fermentation results, suggest a change in metabolic flux of carbon with rapid cooling. The CO_2 evolved was not sensitive to varying cooling rate and yeast consistency. The lowest change in mass of CO_2 evolved during fermentation following cooling was 0.4 g as compared to the highest amount of CO_2 evolved in the base case (0.3 g). The average final substrate concentration obtained for the cooled yeast was approximately equal to the base case (0.15 g.l^{-1}). The final glucose concentration was not sensitive to varying cooling rate.

Ethanol is known to exhibit several inhibitory effects on yeast cells. Previous studies have shown that ethanol toxicity can inhibit yeast cell growth (Mager and Varela, 1993, Walker, 1998) by causing membrane leakage (Sajdidor and Grego, 1992). This is due to its toxicity and the osmotic environment created by ethanol. D'Amore and co-workers (1990) have shown ethanol inhibiting cell growth, loss of nutrient uptake, loss of viability and a decrease in proton flux. The results were as a result of ethanol inhibition altering the cell membrane structure and permeability thus leading to impaired fermentation performance.

The response of yeast to different alcohol concentrations was studied by Sales *et al.* (2000) and Pina *et al.* (2000). Sales *et al.* (2000) showed that the fermentation of the HSP12 knock-out mutant and the wild type yeast grown in different alcohol concentrations. The YPD media was supplemented with ethanol to a concentration up to 15 % (v/v). No difference in growth rate of the two strains was detected after 24 hours when the ethanol concentration was 5 % (v/v). The growth of the knock out strain was affected by ethanol concentration up to 10 %

(v/v). The growth rate of the knock-out strain was reduced by about 75 % compared to the growth of this strain without ethanol. When ethanol concentration was increased to 12 % (v/v), a total inhibition of the growth of the knock-out strain was observed whereas growth of the wild type strain was only reduced to 75 % (compared to the control wild type strain grown in YPD media with no alcohol). The wild type strain was unable to grow in YPD media supplemented with 15 % ethanol. The above results suggested the role of HSP12 in yeast tolerance against ethanol.

Pina *et al.* (2000) studied the effect of ethanol concentration on yeast by suspending wet yeast pellets in ethanol following fermentation. In the first set of experiments, the tolerance to ethanol by one *Saccharomyces* and five non-*Saccharomyces* yeasts subsequent to growth by progressive inactivation was carried out. Cells were harvested by centrifugation. The cell pellet was resuspended in yeast malt (YM) broth at different ethanol concentrations. The measure of ethanol tolerance was defined as the concentration of ethanol at which yeast growth is just prevented (Mishra & Kaur, 1991 as cited in Pina *et al.,* 2004). The ethanol concentrations used for progressive inactivation were 20, 22.5 and 25 % (v/v). For *S. cerevisiae*, there was no effect of ethanol inactivation at 20 % (v/v) ethanol. However, the effect of ethanol inactivation was seen at 22.5 % and 25 % ethanol. Significant decrease in cell viability was observed at 22.5 % ethanol (Actual data on viability not shown). When yeast was exposed to 25 % ethanol, greater reduction in cell viability was observed. The second set of experiments was carried out with yeast grown in YM broth in the presence of various ethanol concentrations. For yeast grown in ethanol supplementation, alcohol concentrations of 4, 6, 8 and 10 % (v/v) were used. The lag phase increased and the final biomass decreased with an increase in ethanol concentration across all concentrations. *S. cerevisiae* was shown to be the most ethanol resistant strain.

In this study, the influence of yeast diluent on fermentation performance of brewers yeast was investigated. The diluents used were PBS only, beer only and a mixture of PBS and beer (containing ethanol). Unlike Sales *et al*. (2001) and Pina *et al.* (2000), the brewery yeast cropped after fermentation was used, not the laboratory grown yeast. Further, in this study yeast harvested was suspended at different alcohol concentrations prior to inoculation of the fermentation. The ethanol concentrations used in this study are in the same range as those used by Pina *et al*. (2000) in their second experiment and Sales *et al*. (2001).

In this study, the ethanol concentrations across all samples ranged between 2.0 % (v/v) and 8.0 % (v/v). Further, no external alcohol was added to the growth medium. Irrespective of the type of diluent used, the final cell concentration in the small-scale fermentation following inoculation with control and cooled yeast were approximately the same. There was no significant difference between the residual substrate utilization profiles of yeast diluted with different diluents. For all diluents used, there was no significant difference in the amount of carbon dioxide evolved. Based on the findings of this study, it can be concluded that when brewery yeast is suspended in alcohol (pre-inoculation), the yeast performance is not compromised at low alcohol concentrations (below 10 % v/v). The toxicity of alcohol in yeast cells was more prominent when yeast was grown in a growth media injected with alcohol (Sales *et al*., 2001 and Pina *et al.,* 2000), than when alcohol remains present during handling.

7.3 THE EFFECT OF COOLING RATE AND YEAST CONSISTENCY ON STRUCTURAL STRENGTH OF YEAST

The potential effects of hydrodynamic shear on yeast are rearrangement or loss of molecules (phosphomannan, proteins and lipids) from the cell wall surface (Basson, 1996; Prokop and Bajpai, 1992), loss of cell wall structure, and loss of membrane integrity (Basson, 1996). The degree of damage depends on the physiological state of yeast, cell growth phase and magnitude of the applied force (Basson, 1996). Nkosi (2001) investigated the behavior of yeast cell performance upon cooling at different cooling rates (1.03 to $4.03^{o}C.s^{-1}$). Over the range of cooling rates tested in the laboratory using cooling coils and online in the brewery using a plate and frame heat exchanger, the amount of protease released and haze generated increased with the increase in cooling rate. Fast cooled yeast was more susceptible to breakage. Evidence of the cold injury observed during fast cooling was dependent on the extent and the rate of cooling employed.

In this study, fragility test results showed that yeast cooled at higher cooling rates were most susceptible to breakage using the French Press. The final amount of protein detected from the fast cooled yeast and the control yeast was about 4 $mg.ml^{-1}$ and 2 $mg.ml^{-1}$ respectively. The same sublytic pressures were applied in this study and the experiments of Nkosi (2001) and the results obtained followed the same trends. The increased fragility of the cells when cooled at higher cooling rates compared to slower cooling rates may be attributed to either a higher degree of shear stress or rate of temperature drop during yeast cooling. Exposure to shear compromises cell wall integrity thus weakening the cell wall by removing the structural layers. Temperature decrease leads to structural changes in wall and membrane, thus compromising the cell strength.

Motshwene *et al.* (2004) investigated the sensitivity of the wild type yeast (with HSP12 gene) and the HSP12 knock-out mutant to cell breakage. Both yeasts were exposed to a pressure of 25 MPa using a French Press pressure cell for a period of 120 seconds, and then rapidly returned to atmospheric pressure. The wild type yeast, expressing HSP12, experienced approximately half the breakage experienced by the knock-out strain. This trend was the same irrespective of the pressure applied. Similarly, when the abrasion resistance for the wild type yeast was compared to the HSP12 knock-out mutant (HSP12 gene disrupted), it was shown that the knock-out strain was 10% less resistant than the wild type (Motshwene *et al*., 2004), thus it was concluded that the presence of HSP12 improves resistance of the wild type yeast to mechanical damage. The studies of Motshwene *et al*. (2004) showed HSP12 playing a critical role in protecting yeast against shear. Nkosi (2001) showed cell fragility increasing with an increase in cooling rate due to cell envelope damage. In this study, control yeast was neither cooled nor passed through the cooling coil but maintained at 14^{o}C. At the same sublytic pressure, it was shown that cell wall fragility increased with increasing cooling rate.

The presence of intracellular compounds in beer has been attributed to loss of cell wall integrity thus leading to the production of off- flavours (McCaig and Bendiak, 1985), resulting in reduced beer quality and shortened shelf life. An example of reduced beer quality is an unstable foam head which is believed to be caused by the presence of proteinase A (Stewart *et al.* 2006). A centrifuged yeast sample was tested for the activity of proteinase A and it was found that proteinase A was released from damaged yeast cells. This enzyme diminished the hydrophobicity of foam-positive polypeptides in beer thus making the beer foam unstable.

Robinson (2001) showed the particle size diameter of haze particles to be within the range 0.08-2 μm. In this study, the same methodology was used to monitor haze formation on cooling as a function of cooling rate and yeast concentration. The average particle size of haze particles was also found to be in a range of 0.05–2 μm. Robinson (2001) reported haze generation due to shear whilst Nkosi (2001) showed haze generation due to cooling on flow through a cooling coil or heat exchanger.

Similarly to Nkosi (2001), this study showed haze was generated on cooling by flow through the cooling coil. The relative contribution of flow rate or cooling rate was not determined separately in either study. More fine haze material was generated on rapid cooling. The volume based area under the peak (0.05 to 2 μm) of the particle size distribution measured by laser light scattering was used as a guideline to show haze generated. It is recommended that further tests should be carried out to determine the rate of fluid flow *per se* on haze generated. Differing haze concentrations were obtained from different control samples. This may arise from yeast treatment before collection, so cannot be attributed to differing consistency. An increase in cooling rate of yeast at the same consistency resulted in little change in haze generation. However, an increase in consistency of the yeast suspension cooled increased haze generation although the cooling rate decreased. This can be attributed to changes in viscosity and shear rate. Furthermore, the cell wall fragility following cooling increased with an increase in cooling rate due to increased cell envelope damage.

7.4 THE INFLUENCE OF COOLING RATE, DILUENT USED TO PREPARE DIFFERENT YEAST CONSISTENCIES AND THE OSMOLALITY OF THE MALVERN DILUENT ON CELL ENVELOPE FLEXIBILITY

Changing solute concentration of the environment affects living cells, since membranes are permeable to water. Osmotic stress is brought about by changes in solute concentration in a particular environment. These changes can either decrease (hyper-osmotic) or increase (hypo-osmotic) free water activity (a_w) or water potential (φ) of the medium (Mager and Varela, 1993). The effect of osmotic stress on yeast can be seen expressed as cell dessication. The cell envelope desiccation can be observed by the reduction in cell size (Sales *et al*., 2000) and sometimes by the expression of HSP12 (Sales *et al*., 2004). Placing a cell into a hypotonic solution drives water into the cell, causing the cell to swell and possibly burst. An isotonic (same strength) solution is osmotically balanced with the cell, hence placing a cell into this solution is not expected to cause net change in solution flow. When the cell was exposed to a hypertonic solution, the osmotic flow through the cell membrane was proportional to the change in osmotic pressure (Δπ) thus leading to a reduced cell size (Wood, 1999).

Cell envelope flexibility was shown to be influenced by an increase in HSP12 (Motshwene *et al.,* 2004). HSP12 was shown to be located at the cell membrane (Sales *et al*., 2000). The heat shock response, found to be triggered when the cells were exposed to increased temperatures, was the first stress response observed (Varela *et al*., 1995). Further studies showed that the heat shock response can also be induced by a variety of stress inducers, including ethanol (Birch and Walker, 2000; Sales *et. al*, 2000), metal concentration (Birch and Walker, 2000; Hohmann and Mager, 1997), low temperature (Praekelt and Meacock, 1990; Nkosi, 2001; Kandror *et al*., 2004; Palhano *et al*., 2004), osmotic stress (Mtwisha *et al*., 1998), water deficit (Mundy and Chua, 1988 as cited in Sales *et al*., 2000), pressure (Fernades *et al*., 2004) and on entry into the stationary phase (Praekelt and Meacock, 1990; Mtwisha *et al*., 1998). A selection of heat shock proteins (HSP104, HSP42, HSP12, Ssa 4) were found to accumulate in yeast subjected to temperatures below 10°C (Kandror *et al*., 2004; Nkosi, 2001). The presence of HSP12 was correlated with cell envelope flexibility and the pressure–resistant phenotype (Motshwene *et al*., 2004).

In this study, different degrees of cell flexibility were observed upon injecting yeast slurries into media of different osmotic potential. Yeast slurries were given three pre-treatments, namely: cooled (at 0.78 to $1.32^{o}C.s^{-1}$), heated (at 0.9 to $1.7^{o}C.s^{-1}$) and control yeast (maintained at 14°C for cooled samples and gradually heated to 25°C at $0.036^{o}C.s^{-1}$ for heated samples). The resultant effect of osmolarity on cell size was measured using Malvern particle size analyzer operated using the principle of laser light scattering. The temperature differential between the sample and the Malvern diluent had potential to interfere with the assay. The temperature of the Malvern diluent was shown to play a major role in the estimation of the cell envelope flexibility (Figure 5.1). On examining the effect of the difference in the temperatures of the yeast sample and the Malvern diluent on the measured flexibility of yeast, it was shown that it is crucial not to introduce a temperature shock during the assay when determining the relation between the yeast sample and cell envelope flexibility. However, for cooled yeast sample, the diluent temperature could not meaningfully be maintained at the cooled sample temperature because condensation on the lens reduced the accuracy of data. When carrying out cell envelope flexibility measurements for yeast cooled below 10^{o}C, the temperature shock was reduced to a minimum while avoiding clouding of the lens by keeping the Malvern diluent at 14^{o}C. The size of the error introduced was approximately 15 %.

Heated yeast cell increased in size when injected into the hypotonic solution and decreased in size in the hypertonic solution. The cell envelope flexibility of yeast injected into water was greater than of hypersomal salt solution due to high influx of the surrounding water into the cell owing to the differing osmotic pressure. The nature of the yeast suspension on cell envelope flexibility was observed when using PBS. This was shown by the highest rate of change in cell size compared to the other diluents.

For both heated and cooled yeast, rate and extent of change in size increased as heating or cooling rate to which yeast was exposed increased. Increased flexibility was expected to arise from an increase in HSP expression shown by Nkosi (2001) on similar handling (of brewers yeast SAB5). Motshwene *et al.* (2004) showed an increase in flexibility with an increase in HSP12 using laboratory grown yeast. This suggests potential to pre-stress yeast to induce improved flexibility for yeast handling.

CHAPTER 8: CONCLUSIONS

8.1 APPROACH TO STUDY

This thesis aimed to investigate the effects of cooling rate on the quality of brewers yeast by addressing the extent of cooling, the effects of yeast consistency on the cooling rate, yeast quality and fermentation performance of cold stressed yeast. In particular, the response of yeast to cold stress was studied in the context of cell envelope flexibility and cell envelope integrity through haze generation and fragility. Moreover, the relationship between cooling rates, heating rate, osmotic strength of the media and cell envelope flexibility was explored.

In general, this study focuses on maintenance of yeast quality during the cooling carried out at the end of the brewery fermentation process. Proper yeast handling will result in beer quality not being compromised. Maintenance of yeast quality allows serial re-use of yeast as inoculum in the pitching of subsequent fermentations, thereby maximizing the conversion of carbohydrate substrate to the desired product.

At the onset of this study, it was hypothesized that:

- Yeast cooled at a low cooling rate will yield better fermentation performance than yeast cooled at rates closer to the critical cooling rate, previously proposed as $2^{o}C.s^{-1}$ (Nkosi, 2001).
- Flexibility of the cell envelope increases with an increase in cooling or heating rate. Cell envelope flexibility was shown to be dependent on the level of HSP12 (Motshwene *et al.,* 2004) in yeast. The level of HSP12 was shown to increase with an increase in cooling rates (Nkosi, 2001); hence in this study yeast cooled or heated at faster rates is postulated to display the most flexible cell wall.
- Yeast structural strength is indirectly proportional to the cooling rate beyond a critical rate.
- Haze generation is aggravated by higher cooling rates.
- Lower cell viability, cell growth and fermentation performance are expected on yeast cooled in the presence of beer, compared to that cooled in a suspension where the beer is diluted.

8.2 CONCLUSIONS

From the findings presented in the thesis, the following conclusions were drawn and are discussed in terms of the hypothesis.

(1) Cooling rate is directly proportional to the flow rate. A linear relationship between linear velocity and cooling rate was shown to exist at all flow rates. The cooling rates obtained using yeast at a consistency of 64 % at 0.02 m.s^{-1}, 0.05 m.s^{-1}, 0.13 m.s^{-1}, 0.22 m.s^{-1} and 0.235 m.s^{-1} were 0.1^{o}C.s^{-1}, 0.2^{o}C.s^{-1}, 0.6^{o}C.s^{-1}, 0.9^{o}C.s^{-1} and 1^{o}C.s^{-1} respectively. Moreover, there was no significant difference between cooling rates obtained as a function of linear velocity with the type of diluent used, namely; PBS, beer and a blend of PBS and beer.

(2) Across all pump settings used, the cooling rates obtained were an indirect function of the yeast concentration. The highest cooling rate was obtained when using the least concentrated yeast suspension. The lowest and highest cooling rates were obtained from yeast at 60 % and 30 % consistencies respectively. This was accredited to an increase in viscosity of the suspension, decrease in suspension velocity thus decreasing the rate of heat transfer.

(3) A base case small-scale fermentation showed the final cell concentration having increased to four fold the original concentration with rapid cell growth during the first six hours of fermentation. The residual substrate concentration decreased by 94 %.

(4) High cell vitality was observed following cooling of the yeast. The CO_2 evolution was not sensitive to varying cooling rate across the range 0.1 to 1^{o}C.s^{-1}. The substrate utilisation and the cell vitality were not sensitive to varying yeast consistency across this cooling rate range, suggesting no change in metabolic activity.

(5) Irrespective of the type of diluent used, the final cell concentration of the control and cooled yeast following small-scale fermentation were approximately the same. The ethanol concentrations across all samples ranged between 2.0 % (v/v) and 8.0 % (v/v). No external alcohol was added to the growth medium. This suggests that extent of cell growth is not affected across the ethanol concentration range used.

(6) Yeast cooled at higher cooling rates was more susceptible to damage by hydrodynamic stress. In this study the hydrodynamic stress was induced by sudden pressure drop and shear stress on exposure to sub-lytic pressure in a French Press. In the process hydrodynamic stress may be induced by de-pressurising the suspension on leaving the high aspect ratio fermenter, pumping, passage through constrictions, cavitation etc. Cell wall integrity is compromised by an exposure to shear and a decrease in temperature.

(7) The average size of haze particles was found to be in a range of 0.05 to 2 μm, in agreement with the work of Robinson (2001). Haze generation increased with an

increasing flow rate and consistency however, an increase in cooling rate at the same consistency did not bring a significant change in haze generated. The relative contribution of flow rate or cooling rate was not determined separately.

(8) The temperature of the diluent used for particle size analysis by the Malvern particle size analyser was shown to play a major role in the estimation of the cell envelope flexibility. On examining the effect of the difference in the temperatures of the yeast sample and the Malvern diluent on the measured flexibility of yeast, it was shown that it is crucial not to introduce temperature shock when determining the relationship between the yeast sample and cell envelope flexibility. However, for the cooled yeast sample, it was difficult to maintain the diluent temperature at the same temperature as the sample because use of a low temperature within the Malvern particle size analyser resulted in condensation on the lens which reduced the accuracy of data. When carrying out cell envelope flexibility for yeast cooled below 10^{o}C, the temperature shock was reduced to a minimum while avoiding clouding of the lens by keeping the Malvern diluent at 14^{o}C.

(9) The rate and extent of change in size of heated or cooled yeast increased with increasing cooling or heating rate, illustrating increased cell wall flexibility. This suggests potential to pre-stress yeast to induce improved flexibility for yeast handling.

Proper handling of yeast at the brewery is very important to prevent deterioration of yeast quality. Subsequent to cropping SAB 5 at the brewery, the cooling rates obtained will remain the same irrespective of the alcoholic content of the yeast. From observations, the average yeast consistency of cropped yeast at the Newlands brewery is 60 ± 2 %. A linear relationship between the flowrate and cooling rate is expected for yeast consistency range of 30 to 64 % with an alcohol percentage of 8 % (v/v). Yeast of lower consistency will cool faster because of increased suspension velocity, however, cell integrity is compromised due to hydrodynamic stress. High concentrated yeast is preferred at the brewery because it is a sign of healthy fermentation inside the FV, however, more haze was associated with a concentrated yeast suspension. Special attention should be paid to a higher concentrated yeast suspension to prevent further downstream haze problems.

The average cooling rate for the heat exchanger used for cooling yeast from the FV at SAB Miller (Newlands, Cape Town) was 138^{o}C.min^{-1} (2.3^{o}C.s^{-1}) (Nkosi, 2001; Hofmann, 2004). In this study, it was shown that cooling yeast between 0.1 and 2.15^{o}C.s^{-1} did not alter any metabolic activity of brewers yeast. Uncooled yeast gave a better cell growth compared to cooled yeast. In breweries, it is difficult to use freshly cropped yeast for pitching because it does not allow flexibility of eliminating and controlling the microbial contamination in cropped yeast.

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APPENDICES

APPENDIX A: ASSAY REAGENTS AND METHODS

A1. FRAGILITY ANALYSIS

Phosphate buffer saline (PBS)

- $KCl = 0.1342\ g.l^{-1}$
- $NaCl = 8.0647\ g.l^{-1}$
- $KH_2PO_4 = 0.245\ g.l^{-1}$
- $Na_2HPO_4 = 1.496\ g.\ l^{-1}$

A.1.1 Method

- Re-suspend yeast in cold buffer (wash buffer) solution (10 mM Tris and 50 mM NaCl) at a mass ratio of 1:4 i.e. 4 g yeast in 16 ml buffer
- Vortex to mix
- Place on ice
- Assemble piston chamber and fill with yeast
- Place assembled unit onto French press
- Eliminate air from the collection pod
- Pump pressure to 1 MPa, collect zero
- Pump pressure to 6 MPa, collect supernatant
- Take hydraulic pressure up in steps of 10 MPa, then to 16 MPa
- Take samples at each pressure and place them on ice
- Remove yeast from the supernatant by centrifuging for 5 minutes. Use PBS solution to zero spectrophotometer at 280 nm
- Measure absorbance at 280 nm in the supernatant. Note, absorbance should be between zero and 1, so dilute samples down in buffer.
- Plot absorbance versus pressure.

A.2 BIO- RAD PROTEIN ASSAY

BSA standard

- 1 g BSA
- 1000 ml water

A.2.1 Reconstituting the Standard

To reconstitute the lyophilised Bovine Serum albumin, add 20 ml of deionised water and mix until dissolved. If the standard will not be used within 60 days, it should be aliquoted and frozen at -20^{o}C.

A.2.2 Standard Procedure

1. Prepare dye reagent by diluting 1 part Dye reagent Concentrate with 4 parts distilled, deionised (DDI) water. Filter through Whatman # 1 filter (or equivalent) to remove particulates. This diluted reagent may be used for approximately 2 weeks when kept at room temperature.

2. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of the assay for BSA is 0.2 to 0.9 mg.ml^{-1}, whereas with I gG the linear range is 0.2 to 1.5 mg.ml^{-1}.

3. Pipette 100 µL of each standard and sample solution is not a clean, dry test tube. Protein solutions are normally assayed in duplicate or triplicate.

4. Add 5.0 mL of diluted dye reagent to each tube and vortex.

5. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.

6. Measure absorbance at 595 nm.

A.3 HAZE ANALYSIS

Yeast supernatant preparation

Yeast was removed by centrifuging the sample for 5 minutes at 3000 g in a Beckman J-25 Avanti centrifuge. The supernatant was recovered to be analysed for haze using a Malvern Mastersizer. The supernatant consisted mainly of haze material with a trace of yeast still present. Data of volume% as a function of size range was obtained. A size analysis of the haze particles was then conducted.

A.4 ACCELERATED SMALL-SCALE FERMENTATION

Growth Medium or MYPG media

- 3 $g.l^{-1}$ malt extract
- 3 $g.l^{-1}$ yeast extract
- 5 $g.l^{-1}$ peptone
- 10 $g.l^{-1}$ glucose.

The media have to be autoclaved immediately after preparation at 121°C and 15 *psi* for 15 min.

PBS (phosphate buffered saline) solution

EDTA

- 0.83 $g.l^{-1}$ EDTA-tetra sodium salt in distilled water

0.025 % (w/v). Methylene violet staining

- 0.25 $g.l^{-1}$ Methylene violet
- 9 $g.l^{-1}$ NaCl
- 0.42 $g.l^{-1}$ KCL
- 0.48 $g.l^{-1}$ CaCl
- 0.2 $g.l^{-1}$ $NaHCO_3$
- 10 $g. l^{-1}$

A.4.1 Method

Aliquots (300 µl) of yeast were inoculated into 120 ml MYPG media. Each flask was stoppered and fitted with a glass tube containing silica gel to absorb any moisture evaporating from the flask. These flasks are incubated at 30°C in an orbital shaker (at 170 rpm) for 24 hours and sample every two hours for the first 8 hours of the 24 hours period, and thereafter at 24 hours. Mass of the flasks before and after sampling was recorded. Any mass loss observed between sampling was attributed to carbon dioxide evolution (vitality test- predicting the activity of fermentation). Samples taken were analysed for glucose concentration, biomass concentration, growth and methylene blue viability. These things are measured so as to gain more accurate prediction of the effects of cold stress and mechanical handling on yeast physiology.

A.5 DIRECT CELL COUNTING

In order to determine the population size of the yeast culture in the reactor, a 1 ml sample was taken at various times during fermentation. This sample was used to determine the approximate number of yeast cells in the conical flasks. The number of cells was then used to determine the growth phase of the culture in the conical flasks.

In this method, a suitable dilution of cells in PBS is placed in the counting chamber of a haemocytometer, which is covered with a cover slip. This is then viewed under a light microscope at 400x magnification with brightfield optics. The counting chamber is comprised of 25 identical blocks. In each block, there are 16 small squares. Cells falling on the left and top grid perimeter lines are included in the cell count along with all cells enclosed by the perimeter lines. Cells are counted across 25 blocks, counting an average of 200 cells to reduce statistical error. When used in conjunction with methylene blue staining, the cell concentration (cells/ml) and viability may be determined simultaneously.

A.5.1 Analytical method

The solution consists of methylene violet (0.25g.l^{-1}), NaCl (9 g.l^{-1}), KCL (0.42 g.l^{-1}), CaCl (0.48 g.l^{-1}), $NaHCO_3$ (0.2 g.l^{-1}) and glucose (10 g.l^{-1}) dissolved in distilled water giving a final methylene violet concentration of 0.025 % (w/v). Dilute the yeast in PBS (pH 7.4) to give a concentration of 107 cells.ml^{-1}. Add 0.2 ml of yeast suspension to 0.5 ml of methylene blue staining solution and swirl mix for 1 minute. Place a drop of the solution onto a haemocytometer and count the number of blue (N_{blue}) and the total number of cells (N_{total}) under a light microscope with brightfield illumination at 40x magnification.

Viability = $(N_{total} – N_{blue})/N_{total}$ *100 %

Cells.l^{-1} = N_{total} x dilution factor x1000/ [25 x 0.1]

Budding index

N.B: same as microscope cells counting. Budding cells are counted as cells with protrusion.

A.6 RESIDUAL SUBSTRATE / GLUCOSE ANALYSIS

Glucose standard

Dissolve 1 g glucose in a 10 ml volumetric flask with distilled water. For standard curve: Pipette 0, 10, 20, 40, 100, 200, 1000 µl of the above solution into 10ml volumetric flasks. Bring the total volume up to 10 ml with distilled water.

Dinitrosalicyclic Acid Reagent Solution, 1%

Table A.1: Composition of Dinitrosalicyclic acid (DNS) reagent

	Chemical Amount
3,5 Dinitrosalicyclic acid	10 g
NaOH	10 g
Phenol	2 g
Na-metabisulphite or Sodium sulfite	0.5 g
Distilled water	1000 ml

The solution must be pH adjusted as follows. Place a 3 ml sample in a test tube and add a drop of phenolphthalein. Add 0.1 M HCl drop-wise to reach neutrality (pink). If the pH is correct it should take 5 to 6 ml of the 0.1 M HCl solution. If the solution turns pink with the addition of less HCL, add more NaOH to the DNS reagent (2 g NaOH = 1 ml HCl). Repeat the 3 ml test titration until 5-6 ml HCl is required. DNS is light sensitive so wrap storage bottle with tinfoil.

The glucose concentration in samples was determined using the dinitrosalicyclic colorimetric method. This method tests for the presence of free carbonyl group (C=O), also known as "reducing sugar". This method is based on the oxidation of the aldehyde functional group while simultaneously 3,5-dinitrosalicyclic acid (DNS) is reduced to 3-amino, 5-nitrosalicyclic acid under alkaline conditions. These reactions are presented below:

$$\text{Aldehyde } \textit{oxidation} \quad \text{carboxyl group} \longrightarrow$$

$$\text{3,5–dinitrosalicyclic acid} \xrightarrow{\textit{reduction}} \text{3-amino, 5-nitrosalicyclic acid}$$

A.7.1 Preparation glucose standards

Dissolve 1 g glucose in a 10 ml volumetric flask with distilled water. For standard curve: Pipette 0,10, 20, 40, 100, 200, 1000 μl of the above solution into 10 ml volumetric flasks. Bring the total volume up to 10 ml with distilled water.

A.7.2 Procedure

1. Into a test tube pipette 200 µL of the standard glucose solution and 600 µl DNS. Do in triplicate (To avoid loss of liquid due to evaporation, cover the test tube with para-film if a plain test tube is used).

2. Heat (95^{o}C) mixture in a waterbath for exactly 5 minutes to develop the red-brown colour, then cool on ice

3. Add 3200 µl distilled water

4. Measure the absorbance against blank (distilled water) at 510 nm in plastic cuvettes. For unknown samples dilute accordingly and follow steps 1-3

5. Determine a standard glucose curve from the absorbance data of the standard glucose solutions. From this curve, determine the glucose concentrations of the samples.

N.B: Phenol up to 2 g.l^{-1} intensifies the colour density. It changes the slope of the calibration curve of absorbance versus glucose concentration but does not affect the linearity. The above procedure yields an absorbance of 1 for 1 g.l^{-1} of glucose in the original sample in the absence of phenol in the reagent, as opposed to an absorbance of 2.5 for 1 g.l^{-1} of glucose in 2 g.l^{-1} of phenol.

A.8 FLEXIBILITY TEST

A hypersomal buffer and distilled water were used to carry out the flexibility test.

- Hypersormal buffer (650 mM NaCl, 10 mM Tris, pH 7.4)
- Distilled water

N.B: In most cases 4L solution is prepared per experiment

Hypersomal Buffer salt solution

Mass of NaCL = (650/ 1000) x 58.44 x 4 L = 151.94 g

Mass of Tris-HCL = (10/1000) x 121.1 x 4 L = 4.84 g

APPENDIX B: YEAST HISTORY

Yeast was collected from the brewery and the conditions applied to each experiment are labelled C_i

Table B.1: Effect of consistency and diluent

Label	**Source**	**Generation no.**	**Consistency on collection (%)**	**Means of varying consistency**	**Range of Consistency studied (%)**	**Coolant temperature (°C)**
C_1	Black label, 28.08.05	0	58	Dilution with PBS	30, 40 and 60	-1
C_2	Black label, 02.09.05	5	61	Dilution with Beer	30, 40 and 60	-1
C_3	Black label, 09.09.05	3	60	Concentrated by centrifuging, diluted with recovered supernatant (beer)	30, 40 and 60	-1

Table B. 2: Effect of flow rate on cooling rate.

Label	Source	Generation No.	Consistency on collection (%)	Dilution	Pump-setting (% max)	Coolant/ Hot water bath Temperature (^oC)
C_5	Black label, 13.06.05	5	64	none	15, 50, 100	-1
C_6	Black label, 12.07.05	2	61	none	15, 50, 100	-1 and 30^{o}C
C_7	Black label, 10.05.05	1	64	none	15, 25, 50, 75, 100	-1
C_8	Black label, 13.06.05	5	64	none	15, 50, 100	-1 and 35

APPENDIX C: CALCULATIONS

C.1 Cooling rates calculations

Average cooling rate = ΔT_{lm} / Residence Time

Residence time = Hold-up volume of cooling coil / Volumetric flow rate

Similarly, the heating rate was determined as follows:

Average heating rate = ΔT_{lm} / Residence Time

C.2: Converting the cell wall flexibility data from particle side distribution by volume (%) to particle size distribution by number (%)

The conversion of particle size distribution by mass (or volume) to particle size distribution by number is shown in Equation 3-3. The average particle size diameter of 1.95 µm was obtained.

$$f_M(x) = k_3 * x^3 * f_N(x) \qquad \textit{3-3}$$

where f_M (x) denotes the particle size distribution by mass (volume)

k_3 denotes a scaling factor used to make an area under the graph to be 100%.

X denotes the particle size diameter

f_N (x) denotes the particle size distribution by number

C.3: Determine dilution rates and pitching rates for yeast having with the consistency of 30 %, 40 % and 60 %.

Examples of calculations are given for a generation 5 yeast, collected at a 61 % consistency and a 95 % viability. Dilution is carried out with beer.

For 60% consistency:

Preparation of 60 %, 40 % and 30 % yeast consistency by diluting using beer.

$$C_1 V_1 = C_2 V_2$$

$$0.61 * 1L = 0.60 * X$$

$$X = 1.016 \text{ L}$$

Volume of beer to be added = X – 1L

= 0.016 L

Pitching rate = 3.5 * 0.12 L / (0.95*0.6) = 0.74 ml

For 40 % consistency:

$C_1 V_1 = C_2V_2$

0.61* 1L = 0.40 * X

X = 1.525 L

Volume of beer to be added = X – 1L

= 0.525 L

Pitching rate = 3.5* 0.12 / (0.95 * 0.4) = 1. 105 ml

For 30 % consistency :

$C_1 V_1 = C_2V_2$

0.61* 1L = 0.30 * Y

Y = 2.033 L

Volume of beer to be added = Y – 1L

= 1.033 L

Pitching rate = 3.5* 0.12 / (0.95 * 0.3) = 1. 474 ml

C.4: Fragility sample preparation

Yeast to buffer ratio: 1: 4

(i) For the fast cooled yeast pumped at 100 % of the maximum flowrate

Consistency : 60 %

Volume of yeast : 10 ml

Volume of PBS: 40 ml

Total volume of the prepared mixture = 50 ml

Therefore volume wet yeast in the final mixture = 0.6 * 10 ml = 6 ml

N.B: This volume was used to equalize the total amount of yeast used for fragility test in the 40 and the 30 % yeast consistencies

Consistency : 40 %

Volume of yeast : 0.4 x Y = 6 ml

Y = 15 ml

Volume of PBS to be added = 50 ml – 15 ml = 35 ml

Consistency : 30 %

Vol. wet yeast: 0.3 x Z = 6 ml

Y = 20 ml

Vol. of PBS to be added = 50 ml – 20 ml = 30 ml

C.5: Converting the volume size distribution into the % Size change

Table C.3: Data collected from the Malvern machine

Time (s)	**Volume size distribution (**µm)
1	8.78
2	8.39
3	8.47

Size change$_{t1}$ (%) = 100 %

Size change$_{t2}$ (%) = 8.39/8.78 *100 %

= 95.56 %

Size change$_{t3}$ (%) = 8.47/ 8.78 * 100 %

= 96.47 %

The Delta (Δ) Size change$_{t1}$ = 0 %

The Delta (Δ) Size change$_{t2}$ = (95.56- 100) %

= 4.44 %

The Delta (Δ) Size change$_{t3}$ = (96.47-100) %

= 3.53 %

C.5 The percentage alcohol of yeast suspensions diluted with different diluents

Three different yeast suspensions were diluted with three different diluents, namely PBS, beer and a blend of PBS and beer. For each yeast suspension, three different concentrations were prepared: 30 %, 40 % and 60 % consistency. The % alcohol of yeast suspensions ranged from 4 % (v/v) to 8 % (v/v).

An average % of alcohol in cropped yeast is approximately 8 % (v/v). When making the calculations, it was assumed that voidage $\varepsilon = 0.4$

(1) Using beer as the diluent

Yeast suspension 60 %: 8 % alcohol content. The consistency of cropped yeast was 60 % hence it was not necessary to dilute the suspension further to achieve the final yeast consistency of 60 %.

Table C.4: Different alcohol concentration in the three diluents prepared

Yeast consistency (%)	**Beer**	**PBS & Beer**	**PBS**
60 %	8	8	0.36
40 %	8	5.3	1.92
30 %	8	4	~2

APPENDIX D: COOLING RATES AND FERMENTATION DATA

D.1: Raw data used in determining the average yeast consistency

Consistency (%)	*61*	*64*	*58*	*64*	59	64	64

Average yeast consistency: 62 %

Standard deviation: 2.64

D.2 Details of the cooling coil used

Table D.2: Dimensions of the copper cooling coil

Length of copper coil	276	cm
Length of the silicon tubing attached	45	cm
Diameter (i.d)	0.8	cm
Diameter (o.d) 7605914	1.0	cm
Cross-sectional area	0.5024	cm^2
Coil volume	138.66	cm^3
Tube volume	22.61	cm^3
Volume (residence time)	161.27	cm^3

(i) Cooling rates obtained from different yeast consistencies collected on the 9·09.05

(a) Consistency : 60 %

Table D.3: Linear velocities and cooling rates obtained from yeast following dilution with PBS to 60 % yeast consistency

T_i (°C)	T_f (°C)	Pump setting (%)	Duration (s)	Vol flow ($cm^3 . min^{-1}$)	Time residence (min)	Delta T_{lm} (°C)	Cooling rate ($^oC.s^{-1}$)	Cooling Covariance	Linear velocity ($m.s^{-1}$)
14	5.5	50	12	500	0.28	10.16	0.51	0.071	0.17
14	5	50	11.5	521.7	0.27	9.82	0.56		0.17
14.5	7	100	5.85	1025.6	0.14	11.34	0.92	0.066	0.34
14.5	7.5	100	6	1000	0.14	11.65	0.84		0.33

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.5°C.s^{-1}, with the standard deviation of 0.037.

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.17 m.s^{-1}.

Average cooling rate when the pump was set at 100 % of the maximum flowrate: 0.9°C.s^{-1} with the standard deviation of 0.058.

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.34 m.s^{-1}.

(b) Consistency: 40 %

Table D.4: Linear velocities and cooling rates obtained from yeast following dilution with PBS to 40% yeast consistency

T_i (°C)	T_f (°C)	Pump setting (%)	Duration (s)	Vol flow (cm^3 min^{-1})	Time residence (min)	Delta T_{lm} (°C)	Cooling rate (°C.s^{-1})	Cooling Covariance	Linear velocity (m.s^{-1})
14.2	6.5	50	10.28	583	0.237	10.9	0.76	0.00	0.19
14.9	9	100	4.59	1307.2	0.106	12.72	2.00		0.43
15.4	8	100	4.53	1324.5	0.105	12.33	1.96	0.013	0.44

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.8°C.s^{-1}, with the standard deviation of 0.00

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.19 m.s^{-1}

Average cooling rate when the pump was set at 100 % of the maximum flowrate : 1.98°C.s^{-1} with the standard deviation of 0.025

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.44 m.s^{-1}

(c) Consistency : 30 %

Table D.5: Linear velocities and cooling rates obtained from yeast following dilution with PBS to 40 % yeast consistency

T_i (°C)	T_f (°C)	Pump setting (%)	Duration (s)	Vol flow ($cm^3.min^{-1}$)	Time residence (min)	Delta T_{lm} (°C)	Cooling rate (°C.s^{-1})	Cooling Covariance	Linear velocity ($m.s^{-1}$)
14.3	7.7	50	10.63	564.4	0.25	11.69	0.79	0.106	0.187
15.4	7.5	50	9.25	648.6	0.21	11.69	0.91		0.215
15.4	7.2	100	3.97	1511.3	0.09	11.83	2.15	0.00	0.501

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.8°C.s^{-1}, with the standard deviation of 0.084.

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.201m.s^{-1}

Average cooling rate when the pump was set at 100 % of the maximum flowrate : 2.1°C.s^{-1} with the standard deviation of 0.00

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.501m.s^{-1}

(ii) Cooling rates of yeast collected on the 28.08.05

(a) Consistency : 60 %

Table D.6: Linear velocities and cooling rates obtained from yeast following dilution with PBS to 40 % yeast consistency

T_i (°C)	T_f (°C)	Pump set (%)	Duration (s)	Vol flow ($cm^3.min^{-1}$)	Time residence (min)	Delta T_{lm} (°C)	Cooling rate (°C s^{-1})	Cooling rate Covariance	Linear velocity ($m.s^{-1}$)
14.1	6.6	50	10	600	0.23	10.92	0.79	0.0151	0.199
14.5	6	50	10	600	0.23	10.69	0.77		0.199
14.3	6.5	100	6	1000	0.14	10.94	1.31	0.0031	0.332
14.1	6.7		6	1000	0.14	10.99	1.32		0.332

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.8°C.s^{-1}, with the standard deviation of 0.0118.

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.199 m.s^{-1}

Average cooling rate when the pump was set at 100 % of the maximum flowrate: 1.3°C.s^{-1} with the standard deviation of 0.004

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.332 m.s^{-1}

(c) Consistency : 40 %

Table D.7: Linear velocities and cooling rates obtained from yeast following dilution with PBS to 40 % yeast consistency

T_i (°C)	T_f (°C)	Pump setting (%)	Duration (s)	Vol flow (cm^3 min^{-1})	Time $_{residence}$ (min)	Delta T_{lm} (°C)	Cooling rate (°C.s^{-1})	Cooling rate Covariance	Linear velocity (m.$s^{-1)}$
15.4	7	50	9	666.7	0.21	11.7	0.94	0.000	0.221
15.3	9.3	100	5	1200	0.12	13.07	1.89		0.398
15.4	8.8	100	4	1500	0.09	12.82	2.31	0.143	0.498

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.98°C.s^{-1}, with the standard deviation of 0.00.

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.221 m.s^{-1}.

Average cooling rate when the pump was set at 100 % of the maximum flowrate: 2.1°C.s^{-1} with the standard deviation of 0.301.

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.448 m.s^{-1}

(c) Consistency : 30 %

Table D.8: Linear velocities and cooling rates obtained from yeast following dilution with PBS to 40 % yeast consistency

T_i (°C)	T_f (°C)	Pump setting (%)	Duration (s)	Vol flow (cm^3 min^{-1})	Time residence (min)	Delta T_{lm} (°C)	Cooling rate (°C.s^{-1})	Cooling rate Covariance	Linear velocity (m.s^{-1})
15.4	7	50	9	666.7	0.21	11.7	0.94	0.000	0.221
15.4	7.2	100	5	1200	0.12	11.83	1.71		0.398
15.5	9.8	100	4	1500	0.09	13.45	2.42	0.143	0.498

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.90°C.s^{-1}, with the standard deviation of 0.00.

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.221 m.s^{-1}.

Average cooling rate when the pump was set at 100 % of the maximum flowrate: 2.1°C.s^{-1} with the standard deviation of 0.51.

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.448 m.s^{-1}

Table D.9:

Yeast consistency (%)	**Linear velocity**	**Pump setting (%)**	**Cooling rate ($^oC.s^{-1}$)**
60	0.199	50	0.78
40	0.221	50	0.94
30	0.221	50	0.94
60	0.332	100	1.32
40	0.448	100	2.10
30	0.448	100	2.07

Table D.10:

T_i (^oC)	T_f (^oC)	Pump setting (%)	Duration (s)	Vol. flow ($cm^3.min^{-1}$)	Time $_{residence}$ (min)	Delta T_{lm} (^oC)	Cooling rate ($^oC.s^{-1}$)	Linear velocity (m $s^{-1)}$
15.4	7	50	9	666.7	0.21	11.7	0.94	0.221
15.4	7.2	100	5	1200	0.12	13.07	1.89	0.398
15.5	9.8	100	4	1500	0.09	12.82	2.31	0.498

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.9^{o}C.s^{-1} with the standard deviation of 0.00

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.221 m.s^{-1}

Average cooling rate when the pump was set at 100 % of the maximum flowrate: 2.1^{o}C.s^{-1} with the standard deviation of 0.5081

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.448 m

(iii) Cooling rates for yeast collected on the 2·09.05

(a) Consistency : 60 %

Table D.11:

T_i (°C)	T_f (°C)	Pump set (%)	Duration (s)	Vol flow (cm^{3} min^{-1})	Time residence (min)	Delta T_{lm} (°C)	Cooling rate (°C.s^{-1})	Cooling rate Covariance	Linear velocity (m.s$^{-1)}$
15.9	4.8	50	11.93	502.9	0.28	10.38	0.627	0.0179	0.17
15.7	4.8	50	12.15	493.8	0.28	10.31	0.612		0.16
16	6.35	100	5.75	1043.5	0.13	11.51	1.443	0.0465	0.35
15.9	7	100	6.35	944.9	0.15	11.9	1.352		0.31

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.6^{o}C.s^{-1} with the standard deviation of 0.0111

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.17 m.s^{-1}

Average cooling rate when the pump was set at 100 % of the maximum flowrate: 1.4^{o}C.s^{-1} with the standard deviation of 0.065

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.33 m.s^{-1}

(b) Consistency : 40 %

Table D.12:

T_i (°C)	T_f (°C)	Pump set (%)	Duration (s)	Vol flow (cm^3 min^{-1})	Time residence (min)	Delta T_{lm} (°C)	Cooling rate (°C.s^{-1})	Cooling rate Covariance	Linear velocity ($m.s^{-1}$)
14.5	7.2	50	12.63	475.1	0.29	11.47	0.65	0.00	0.158
14.2	7.2	50	11.87	505.5	0.27	11.34	0.69		
14.5	7.9	100	5.53	1085.0	0.13	11.9	1.55	0.1652	0.36
14.1	7	100	6.57	913.2	0.15	11.18	1.23		0.303

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.65°C.s^{-1} with the standard deviation of 0.00

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.158 m.s^{-1}

Average cooling rate when the pump was set at 100 % of the maximum flowrate: 1.389°C.s^{-1} with the standard deviation of 0.229

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.331 m.s^{-1}

(d) Consistency : 30 %

Table D.13:

T_i (^oC)	T_f (^oC)	Pump setting (%)	Duration (s)	Vol flow (cm^3 min^{-1})	Time residence (min)	Delta T_{lm} (^oC)	Cooling rate ($^oC.s^{-1}$)	Cooling rate Covariance	Linear velocity ($m.s^{-1)}$
14.7	4.6	50	10.46	573.6	0.24	9.8	0.68	0.00	0.19
15.3	5.16	100	5.16	1162.8	0.12	10.42	1.46		0.386
15.1	6.5	100	4.9	1224.5	0.11	11.26	1.66	0.0911	0.406

Average cooling rate when the when the pump was set at 50 % of the maximum flowrate: 0.675$^oC.s^{-1}$ with the standard deviation of 0.00

Average linear velocity when the pump was set at 50 % of the maximum flowrate: 0.19 $m.s^{-1}$

Average cooling rate when the pump was set at 100 % of the maximum flowrate: 1.557$^oC.s^{-1}$ with the standard deviation of 0.1418

Average linear velocity when the pump was set at 100 % of the maximum flowrate: 0.396 $m.s^{-1}$

Table D.14:Cooling rates obtained at various yeast consistencies following dilution with a beer

overall table of cooling rates

yeast consistency	**Linear vel**	**pump setting (%)**	**Cooling rate (oC/s)**
60	0.165	50	0.620
40	0.158	50	0.655
30	0.190	50	0.675
60	0.330	100	1.397
40	0.331	100	1.227
30	0.396	100	1.657

D.3: The effect of consistency and linear velocity on cooling rate

50 % pump setting

Table D.15:

Consistency (%)	**Linear velocity ($m.s^{-1}$)**	**Cooling rate ($^{o}C.s^{-1}$)**
30	0.22	0.94
30	0.19	0.68
30	0.20	0.79
40	0.22	0.94
40	0.16	0.65
40	0.19	0.76
58	0.20	0.78
60	0.19	0.62
60	0.17	0.50
64	0.17	0.65
64	0.13	0.57

100 % pump set		
Consistency (%)	**Linear velocity (m.s^{-1})**	**Cooling rate (^{o}C.s^{-1})**
30	0.45	2.07
30	0.40	1.66
30	0.50	2.15
40	0.45	2.10
40	0.33	1.23
40	0.44	1.98
58	0.33	1.32
60	0.33	1.40
60	0.34	0.88
64	0.40	1.66
64	0.24	1.02

D.4: Small-scale fermentation results

(i) **Table D.17**: Cell concentration (cells.ml^{-1}) vs. consistency (control yeast)

		Cell conc data (cells.ml^{-1})	cold stressed yeast (cells.ml^{-1})
	PBS and Beer (a)	Beer (a)	PBS (a)
30 %	1.02E+09	9.76E+08	8.48E+08
40 %	7.20E+08	7.20E+08	7.52E+08
60 %	6.88E+08	6.88E+08	8.00E+08

(ii) Cell Concentration (cells.ml^{-1}) vs. diluent (cold stressed yeast)

Table D.18:

		Cell conc vs. diluents	
		Cell con data (cells.ml^{-1})	cold stressed yeast (cells.ml^{-1})
	30 % (a)	40 % (a)	60 % (a)
PBS+beer (b)	1.02E+09	7.20E+08	6.88E+08
Beer (b)	9.76E+08	7.20E+08	6.88E+08
PBS (b)	8.48E+08	7.52E+08	8.00E+08

(iii) X/X_o for cold stressed yeast

Table D.19:

	30 % (a)	40 % (a)	60 % (a)
X/X_o	5.3	4.7	3.0
X/X_o	6.5	5.6	3.0
X/X_o	4.7	3.9	5.7

(iv) Cell concentration (cells/ml) vs. yeast consistency (control yeast)

Table D.20:

	PBS and Beer (b)	Beer (b)	PBS (b)
30 %	1.01E+09	1.52E+09	8.16E+08
40 %	9.76E+08	8.24E+08	9.12E+08
60 %	9.36E+08	8.72E+08	1.36E+09

(v) **Table D.21**: Cell concentration (cells.ml^{-1}) vs. diluent (control yeast)

	30 % (b)	40 % (b)	60 % (b)
PBS+Beer (b)	1.0E+09	9.76E+08	9.36E+08
Beer (b)	1.52E+09	8.24E+08	8.72E+08
PBS (b)	8.E+08	9.E+08	1.E+09

(vi) **Table D.22**: X/X_o for control yeast

X/X_o	4.10	4.78	3.47
X/X^o	6.18	4.04	4.77
X/X_o	5.91	8.69	6.77

D.5: Yeast consistency data to prepare the following consistencies (30 %, 40 % and 60 %)

(i) Date: 28.08.05

Table D.23:

Eppe #	**Eppe mass (g)**	**Eppe+Y Suspension (g)**	**Eppe+ Pellet (g)**	**Supernatant (g)**	**Pellet (g)**	**Consistency**
1	0.931	1.714	1.384	0.330	0.454	0.58
5	0.943	1.740	1.402	0.338	0.460	0.58
16	0.938	1.781	1.427	0.354	0.488	0.58
					AVE	**58%**
					STDEV	**0.0017**
					CO VAR	**0.0029**

Table D.24:

Eppe #	Eppe mass (g)	Eppe+Y Suspension (g)	Eppe+ Pellet (g)	Supernatant (g)	pellet (g)	Consistency
45	0.935	1.885	1.290	0.595	0.355	0.37
72	0.936	1.936	1.304	0.632	0.369	0.37
71	0.932	1.847	1.279	0.568	0.346	0.38
					AVE	**37 %**
					STDEV	**0.0051**
					CO VAR	**0.0137**

Table D.25:

Eppe #	Eppe mass	Eppe+Y suspension	Eppe+ pellet	Supernatant	pellet	Consistency
24	0.947	1.973	1.237	0.737	0.290	0.28
8	0.938	1.966	1.255	0.712	0.317	0.31
22	0.937	1.869	1.215	0.654	0.278	0.30
					AVE	**30 %**
					STDEV	**0.0128**
					CO VAR	**0.0431**

(ii) Data used to determine the concentration of the yeast sample collected from the brewery on the 09.09.05

Table D.26: **Consistency**

Eppe #	Eppe mass	Eppe+Y suspension	Eppe+ pellet	Supernatant	pellet	Consistency
1	0.941	1.825	1.469	0.356	0.528	0.60
2	0.948	1.767	1.442	0.325	0.494	0.60
3	0.940	1.832	1.479	0.352	0.539	0.60
					AVE	**60 %**
					STDEV	**0.0043**
					CO VAR	**0.0071**

Determination of the pitching rate of yeast having 60 % consistency, 09.09.05

Table D.27:

	yeast uL	EDTA ml		D
Dilution 1:	150	10		67.67
Dilution 2	50	2		41

Single	**Budded**	**Dead**	**Total cell**	**Cell conc**	**Viability**	**Budding index**	**Ave viab**	**stdev**	**co-variance**
74	3	3	80	3.55E+10	96%	0.04	95%	0.012229	0.01282071
67	2	4	73	32404213333	95%	0.03			

Pitching rate (g/L) 0.73 ml critical

D.6: Glucose standard curve

Table D.28:

Ml

Total volume desired 10

Vol Std	**Vol H_2O**	**Final concentration**				
ml	**ml**	**mg/ml**	**Abs 1**	**Abs 2**	**Abs 3**	**Ave abs**
0	10	0.000	0	0	0	0
0.4	9.6	0.040	0.108	0.107	0.117	0.110667
0.8	9.2	0.080	0.29	0.316	0.309	0.305
1	9	0.100	0.401	0.403	0.402	0.402
2	8	0.200	0.908	0.869	0.865	0.880667
4	6	0.400	1.841	1.799	1.768	1.802667
6	4	0.6	2.65	2.575	2.741	2.655333
10	0	1.000	3.427	3.282	3.23	3.313

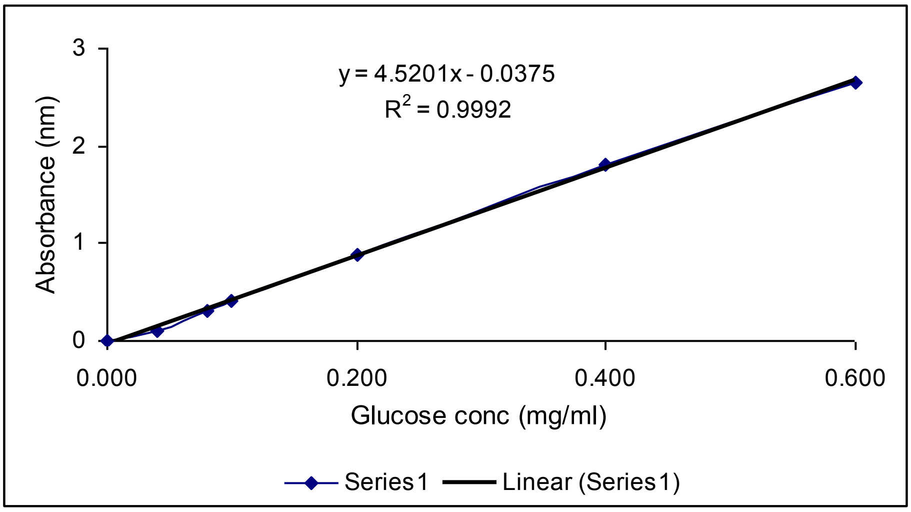


Figure A

(i) Raw data for glucose concentration during the small-scale fermentation (09.09.05)

Dil. Factor 10

Pump setting : 100 %, Consistency = 60

Time	Eppe #	Abs 1	Abs 2	Ave Abs	Stdev	Co-variance	Glu conc (g.l^{-1})	**Final Glucose conc**
0	4	0.896	0.938	0.917	0.0297	0.032	0.21	2.112
2	15	0.581	**0.587**	0.584	0.0042	0.007	0.14	1.375
4	26	0.205	0.212	0.2085	0.0049	0.024	0.05	0.544
6	37	0.086	0.09	0.088	0.0028	0.032	0.03	0.278
8	48	0.074	0.063	0.0685	0.0078	0.114	0.02	0.235
24	59	0.0235	0.0313	0.0274	0.0055	0.201	0.01	0.144

y= 4.5201 x + 0.0375

Pump setting : 100 %, Consistency = 40

Time	Eppe #	Abs 1	Abs 2	Ave Abs	Stdev	Co variance	Glu conc(g/L)	**final Glucose conc**
0	6	0.931	0.935	0.933	0.0028	0.0030	0.215	2.147
2	17	0.477	0.444	0.4605	0.0233	0.0507	0.110	1.102
4	28	0.077	0.075	0.076	0.0014	0.0186	0.025	0.251

6	39	0.086	0.093	0.0895	0.0049	0.0553	0.028	0.281
8	50	0.075	0.07	0.0725	0.0035	0.0488	0.024	0.243
24	**61**	**0.05**	**0.045**	0.0475	0.0035	0.0744	0.019	0.188

Control for 100 % pump setting

Pump setting : 100 %, Consistency = 30

Time	Eppe #	Abs 1	Abs 2	Ave Abs	Stdev	Co variance	Glu conc(g/L)	**Final Glucose conc**
0	8	0.915	0.925	0.92	0.007	0.008	0.21	2.118
2	19	0.572	0.56	0.566	0.008	0.015	0.13	1.335
4	30	0.228	0.181	0.2045	0.033	0.163	0.05	0.535
6	41	0.081	0.09	0.0855	0.006	0.074	0.03	0.272
8	52	0.074	0.068	0.071	0.004	0.060	0.02	0.240
24	63	0.046	0.041	0.0435	0.004	0.081	0.02	0.179

(ii) Raw data of the glucose concentration during the small-scale fermentation (02.09.05)

Dil. Factor 10 02.09.05

Control for cold shock 100 (60%)

Time	Eppe #	Abs 1	Abs 2	Ave Abs	Stdev	Co variance	Glu conc(g/L)	final glucose
0	14	0.791	0.815	0.803	0.016971	0.02113395	0.185947214	1.859472
2	25	0.501	0.52	0.5105	0.013435	0.02631739	0.121236256	1.212363
4	24	0.114	0.112	0.113	0.001414	0.01251516	0.033295724	0.332957
6	45	0.09	0.097	0.0935	0.00495	0.05293848	0.02898166	0.289817
8	56	0.06	0.073	0.0665	0.009192	0.1382314	0.023008341	0.230083
24	67	0.07	0.07	0.07	0	0	0.02378266	0.237827

y= 4.5201 x - 0.0375

306 303

Control for 15% pump setting 100 (40%)

Time	Eppe #	Abs 1	Abs 2	Ave Abs	Stdev	Co variance	Glu conc(g/L)	final glucose
0	16	0.885	0.895	0.89	0.007071	0.00794502	0.205194575	2.051946
2	27	0.729	0.743	0.736	0.009899	0.0134504	0.171124533	1.711245
4	36	0.388	0.356	0.372	0.022627	0.06082639	0.090595341	0.905953
6	47	0.079	0.075	0.077	0.002828	0.03673282	0.025331298	0.253313
8	58	0.059	0.072	0.0655	0.009192	0.1403418	0.022787106	0.227871
24	69	0.049	0.068	0.0585	0.013435	0.22965861	0.021238468	0.212385

Control for 100% pump setting 100(30%)

Time	Eppe #	Abs 1	Abs 2	Ave Abs	Stdev	Co variance	Glu conc(g/L)	final glucose
0	18	0.78	0.781	0.7805	0.000707	0.00090597	0.180969448	1.809694
2	29	0.683	0.696	0.6895	0.009192	0.01333196	0.16083715	1.608371
4	38	0.205	0.199	0.202	0.004243	0.02100317	0.052985553	0.529856
6	49	0.1	0.095	0.0975	0.003536	0.03626189	0.029866596	0.298666
8	60	0.065	0.071	0.068	0.004243	0.06239177	0.023340192	0.233402
24	71	0.061	0.068	0.0645	0.00495	0.07674027	0.022565872	0.225659

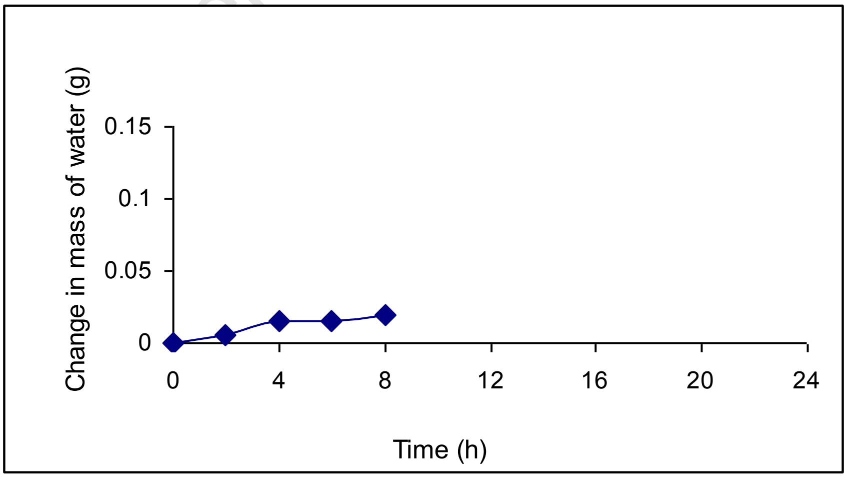
D.7: Evaporation control used during the small-scale fermentation

(i) Date: 09.09.05

Evaporation control for small scale fermentation

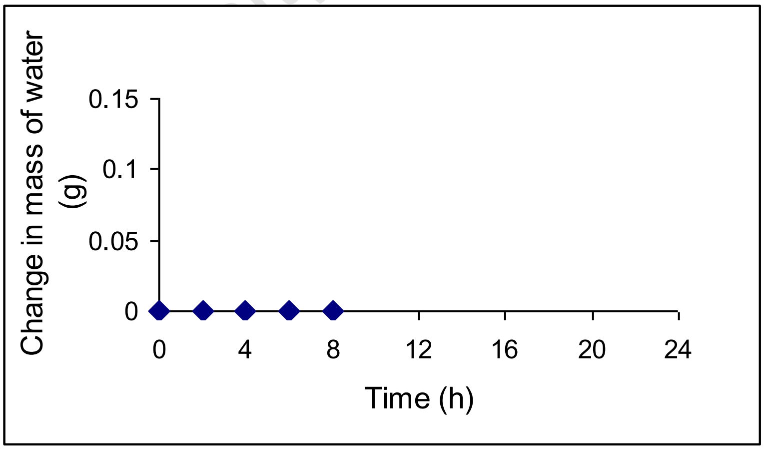
Date: 09.09.05

Time	**F1 mass**	**F2 mass**	**F1 mass**	**F2 mass**	**Ave. mass**	**Cum mass**	**Cum**	**Cum**	
	g	**g**	**loss (g)**	**loss (g)**	**loss (g)**	**loss g**	**F1**	**F2**	stdev
0	323.12	329.21	0	0	0	0	0	0	0
2	323.12	329.2	0	0.01	0.005	0.005	0	0.01	0.007
4	323.11	329.19	0.01	0.01	0.01	0.015	0.01	0.02	0.007071
6	323.11	329.19	0	0	0	0.015	0.01	0.02	0.007071
8	323.1	329.19	0.01	0	0.005	0.02	0.02	0.02	0
24	323.1	329.19							



(ii) Date: 02.09.05

Time	F1 mass g	F2 mass g	F1 mass loss (g)	F2 mass loss (g)	Ave. mass loss (g)	Cum mass loss g	Cum F1	Cum F2	stdev
0	326.42	328.34	0	0	0	0	0	0	0
2	326.42	328.34	0	0	0	0	0	0	0.000
4	326.42	328.34	0	0	0	0	0	0	0
6	326.42	328.34	0	0	0	0	0	0	0
8	326.42	328.34	0	0	0	0	0	0	0
24	326.42	328.34					0	0	



D.8: Vitality tests data

(i) Date : 09.09.05

Consistency : 60 %, pumpsetting = 100%, diluent = PBS

flask 1

undiluted

Time (h)	C1 mass b4 sampling (g)	C1 mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		320	2		0	0
2	319.9	317.86	2	2.04	0.1	0.1
4	317.74	315.66	2	2.08	0.12	0.22
6	315.48	313.46	2	2.02	0.18	0.4
8	313.44	311.38	2	2.06	0.02	0.42
24	311.42		2			0.42

Consistency : 40 %, pumpsetting = 100%, diluent = PBS

flask 1

undiluted

Time (h)	25% mass b4 sampling (g)	25% mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		318.8	2		0	0
2	318.68	316.6	2	2.08	0.12	0.12
4	316.46	314.46	2	2	0.14	0.26
6	314.34	312.26	2	2.08	0.12	0.38
8	312.26	310.18	2	2.08	0	0.38
24	310.18		2		0	0.38

Consistency : 30 %, pumpsetting = 100%, diluent = PBS

flask 1

undiluted

Time (h)	100% mass b4 sampling (g)	100% mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		323.96	2		0	0
2	323.88	321.8	2	2.08	0.08	0.08
4	321.72	319.5	0	2.22	0.08	0.16
6	319.3	317.2	2	2.1	0.2	0.36
8	317.18	315.12	2	2.06	0.02	0.38
24	315.12		2		0	0.38

(ii) Date : 02.09.05

Consistency : 60 %, pumpsetting = 100%, diluent = beer
Initial mass before sampling 2x 1000uL undiluted

Time	C1 mass b4 sampling (g)	C1 mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		330.2	2		0	0
2	330.16	328.12	2	2.04	0.04	0.04
4	328.04	325.98	2	2.06	0.08	0.12
6	325.7	323.62	2	2.08	0.28	0.4
8	323.54	321.48	2	2.06	0.08	0.48
24	321.48		2		0	0.48

flask 1
Consistency : 40 %, pumpsetting = 100%, diluent = PBS & bee undiluted
Vitality test for 25% pump setting

Time	25% mass b4 sampling (g)	25% mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		327.92	2		0	0
2	327.92	325.76	2	2.16	0	0
4	325.7	323.62	2	2.08	0.06	0.06
6	323.36	321.24	2	2.12	0.26	0.32
8	321.2	319.1	2	2.1	0.04	0.36
24	319.19		2			0.27

flask 1
Consistency : 30 %, pumpsetting = 100%, diluent = PBS & bee undiluted
Vitality test for 100% pump setting

Time	100% mass b4 sampling (g)	100% mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		329.2	2		0	0
2	329.14	327	2	2.14	0.06	0.06
4	326.9	324.82	2	2.08	0.1	0.16
6	324.58	322.58	2	2	0.24	0.4
8	322.36	320.36	2	2	0.22	0.62
24	320.36	318.36	2		0	0.62

(iii) Date : 28.08.05

Date : 28.08.05

100,58,pbs flask 1

Consistency : 60 %, pumpsetting = 100%, diluent = PBS & beer

undiluted

Time (h)	C1 mass b4 sampling (g)	C1 mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		327.76	2		0	0
2	327.7	325.5	2	2.2	0.06	0.06
4	325.26	323.1	2	2.16	0.24	0.3
6	323.06	320.84	2	2.22	0.04	0.34
8	320.78	318.72	2	2.06	0.06	0.4
24	318.72		2		0	0.4

Consistency : 40 %, pumpsetting = 100%, diluent = PBS & beer

100,40,pbs flask 1

undiluted

Time (h)	25% mass b4 sampling (g)	25% mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		323.32	2		0	0
2	323.3	321.1	2	2.2	0.02	0.02
4	320.9	318.78	2	2.12	0.2	0.22
6	318.68	316.56	2	2.12	0.1	0.32
8	**316.54**	**314.48**	2	2.06	0.02	0.34
24	314.48		2		0	0.34

Consistency : 30 %, pumpsetting = 100%, diluent = PBS & beer

flask 1

undiluted

Time (h)	100% mass b4 sampling (g)	100% mass after (g)	mass sampled g	Actual mass sampled	Ave. mass loss (g)	Cum mass loss g
0		326.46	2		0	0
2	326.4	324.18	2	2.22	0.06	0.06
4	323.96	321.86	2	2.1	0.22	0.28
6	321.82	319.7	2	2.12	0.04	0.32
8	319.62	317.56	2	2.06	0.08	0.4
24	317.56	318.36	2		0	0.4

D.9: Cell concentration data (28.08.05, 02.09.05 and 09.09.05)

Date : 28.08.05
Pumpsetting : 100 %, consistency : 58

	Average values for flasks								
Time	**single L**	**Budding**	**Dead**	**Total cells**	**cell conc**	**ln X**	**Budding index (%)**	**Viability (%)**	Stdev
0	9.5	27	2	38.5	2.31E+08	19.258	0.70	0.95	2.12E+07
2	13	42.5	5	60.5	3.63E+08	19.710	0.70	0.92	3.82E+07
4	31.5	25	1.5	58	5.22E+08	20.073	0.43	0.97	2.55E+07
6	41.5	14	2.5	58	6.38E+08	20.274	0.24	0.96	4.67E+07
8	45	13	5.5	63.5	6.99E+08	20.364	0.20	0.91	5.44E+07
24	32	6.5	4.5	43	6.88E+08	20.349	0.15	0.90	2.26E+07

Date : 02.09.05
Pumpsetting : 100%, consistency : 61

	Average values for flasks								
Time	single L	Budding	Dead	Total cells	Ln X	cell conc	Budding index (%)	Viability (%)	stdev
0	9.5	27	2.0	38.5	19.26	2.31E+08	0.701	0.948051948	1.34E+08
2	12	38.5	4.0	54.5	19.61	3.27E+08	0.706	0.926605505	8.91E+07
4	36.5	26.5	4.0	67	20.22	6.03E+08	0.396	0.940298507	8.91E+07
6	51.5	14.5	3	69	20.45	7.59E+08	0.210	0.956521739	1.24E+08
8	45	13	5.5	63.5	20.36	6.99E+08	0.205	0.913385827	5.44E+07
24	32	6.5	4.5	43	20.35	6.88E+08	0.151	0.895348837	2.26E+07

Date : 09.09.05
Pumpsetting : 100%, consistency : 60

100 (60%)

	Average values for flasks								
Time	single L	Budding	Dead	Total cells	cell conc	Ln X	Budding index (%)	Viability (%)	stdev
0	3.5	16	4	24	1.41E+08	1.88E+01	0.68	8.30E-01	4.67E+07
2	3.5	36	7.5	47	2.82E+08	1.95E+01	0.77	8.40E-01	1.70E+07
4	16.5	35	4.5	56	5.04E+08	2.00E+01	0.63	9.20E-01	2.55E+07
6	41	28	3.5	73	7.98E+08	2.05E+01	0.39	9.52E-01	5.44E+07
8	49	25	3	77	8.47E+08	2.06E+01	0.32	9.61E-01	9.33E+07
24	36	10	5.5	52	8.00E+08	2.05E+01	0.19	8.93E-01	3.39E+07

Date : 28.08.05
Pumpsetting : 100%, consistency : 40

	Average values for flasks								
Time	**single L**	**Budding**	**Dead**	**Total cells**	**cell conc**	**ln X**	**Budding index (%)**	**Viability (%)**	Stdev
0	4	20.5	1	25.5	1.53E+08	18.846	0.80	0.96	1.27E+07
2	10.5	38.5	0	49	2.94E+08	19.499	0.79	1.00	0.00E+00
4	30.5	29.5	2	62	5.58E+08	20.140	0.48	0.97	8.91E+07
6	38.5	13.5	2	54	5.94E+08	20.202	0.25	0.96	3.11E+07
8	46	18	1.5	65.5	7.21E+08	20.395	0.27	0.98	1.32E+08
24	35	7.5	2.5	45	7.20E+08	20.395	0.17	0.94	1.36E+08

Date : 2.09.05
Pumpsetting : 100 %, consistency : 40

	Average values for flasks								
Time	single L	Budding	Dead	Total cells	Ln X	cell conc	Budding index (%)	Viability (%)	stdev
0	3	17.5	1	21.5	18.68	1.29E+08	0.814	0.95	2.12E+07
2	10.5	38.5	0	49	19.50	2.94E+08	0.786	1.00	0.00E+00
4	30.5	29.5	2	62	20.14	5.58E+08	0.476	0.97	8.91E+07
6	38.5	13.5	2	54	20.20	5.94E+08	0.250	0.96	3.11E+07
8	46	18	1.5	65.5	20.40	7.21E+08	0.275	0.98	1.32E+08
24	35	7.5	2.5	45	20.39	7.20E+08	0.167	0.94	1.36E+08

Date : 9.09.05
Pumpsetting : 100 %, consistency : 40

	Average values for flasks								
Time	single L	Budding	Dead	Total cells	cell conc	Ln X	Budding index (%)	Viability (%)	stdev
0	11.5	20	0.5	32	1.92E+08	19.07	0.63	0.98	2.55E+07
2	5	47	1	53	3.18E+08	19.58	0.89	0.98	0.00E+00
4	19.5	60.5	4.5	84.5	7.61E+08	20.45	0.72	0.95	9.55E+07
6	34	24	2	60	6.60E+08	20.31	0.40	0.97	0.00E+00
8	47.5	24	1	72.5	7.98E+08	20.50	0.33	0.99	3.73E+08
24	35	11	1	47	7.52E+08	20.44	0.23	0.98	2.60E+08

Date : 28.08.05
Pumpsetting : 100 %, consistency : 30

	Average values for flasks								
Time	**single L**	**Budding**	**Dead**	**Total cells**	**cell conc**	**ln X**	**Budding index (%)**	**Viability (%)**	Stdev
0	7	24	1	32	1.92E+08	19.073	0.75	0.97	2.55E+07
2	11.5	50	2	63.5	3.81E+08	19.758	0.79	0.97	4.24E+06
4	30	25.5	3	58.5	5.27E+08	20.082	0.44	0.95	5.73E+07
6	39.5	20.5	4	64	7.04E+08	20.372	0.32	0.94	1.56E+07
8	60.5	14.5	6.5	81.5	8.97E+08	20.614	0.18	0.92	7.00E+07
24	42	19.5	2.5	64	1.02E+09	20.747	0.30	0.96	2.26E+07

Date : 02.09.05
Pumpsetting : 100 %, consistency : 30

	Average values for flasks								
Time	single L	Budding	Dead	Total cells	Ln (X)	cell conc	Budding index (%)	Viability (%)	stdev
0	4.5	18.5	2	25	18.83	1.50E+08	0.740	0.92	3.39E+07
2	11.5	50	2	63.5	19.76	3.81E+08	0.787	0.97	4.24E+06
4	30	25.5	3	58.5	20.08	5.27E+08	0.436	0.95	5.73E+07
6	35	10	0	45	20.02	4.95E+08	0.222	1.00	0.00E+00
8	46	13	2	61	20.32	6.71E+08	0.213	0.97	3.11E+07
24	42	14	5	61		9.76E+08	0.230	0.92	2.26E+07

Date : 09.09.05
Pumpsetting : 100 %, consistency : 30

	Average values for flasks								
Time	single L	Budding	Dead	Total cells	cell conc	Ln X	Budding index (%)	Viability (%)	stdev
0	7.5	20.5	2	30	1.80E+08	19	0.683	0.933	3.39E+07
2	5	47	1	53	3.18E+08	20	0.887	0.981	0.00E+00
4	9	30	2	41	3.69E+08	20	0.732	0.951	6.36E+06
6	38	19.5	3	61	6.66E+08	20	0.322	0.950	3.89E+07
8	54	28	4	86	9.46E+08	21	0.326	0.953	1.79E+08
24	41	11	1	53	8.48E+08	21	0.21	0.98	0.00E+00

(i) Small-scale fermentation data at time zero for all control yeasts (28.08.05, 02.09.05 and 09.09.05)

Cold stressed and cotrol yeast at time zero

pbs **09.09.06**

Time	Flask	Ave. Singl	Ave. Bud	Ave. dead	Total cells	Cell conc	Budding Index (%	viability (%)
0	100,1,58	3.5	16	4	23.5	1.41E+08	0.68	0.83
0	100,1,40	11.5	20	0.5	32	1.92E+08	0.63	0.98
0	100,1,30	7.5	20.5	2	30	1.80E+08	0.68	0.93
0	con,1,58	9	23	1.5	33.5	2.01E+08	0.69	0.96
0	con,1,40	2	13.5	2	17.5	1.05E+08	0.77	0.89
0	con,1,30	3.5	16	3.5	23	1.38E+08	0.70	0.85

02.09.05

Time	Flask	Ave. Singl	Ave. Bud	Ave. dead	Total cells	Cell conc	Budding Index (%	viability (%)
0	100,1,58	9.5	27	2	38.5	2.31E+08	0.70	0.95
0	100,1,40	3	17.5	1	21.5	1.29E+08	0.81	0.95
0	100,1,30	4.5	18.5	2	25	1.50E+08	0.74	0.92
0	con,1,58	10	17.5	3	30.5	1.83E+08	0.57	0.90
0	con,1,40	12	21.5	0.5	34	2.04E+08	0.63	0.99
0	con,1,30	16	24.5	0.5	41	2.46E+08	0.60	0.99

28.08.05

Time	Flask	Ave. Singl	Ave. Bud	Ave. dead	Total cells	Cell conc	Budding Index (%	viability (%)
0	100,1,58	9.5	27	2	38.5	2.31E+08	0.70	0.95
0	100,1,40	4	20.5	1	25.5	1.53E+08	0.80	0.96
0	100,1,30	7	24	1	32	1.92E+08	0.75	0.97
0	con,1,58	15	30	0	45	2.70E+08	0.67	1.00
0	con,1,40	12	21.5	0.5	34	2.04E+08	0.63	0.99
0	con,1,30	16	24.5	0.5	41	2.46E+08	0.60	0.99

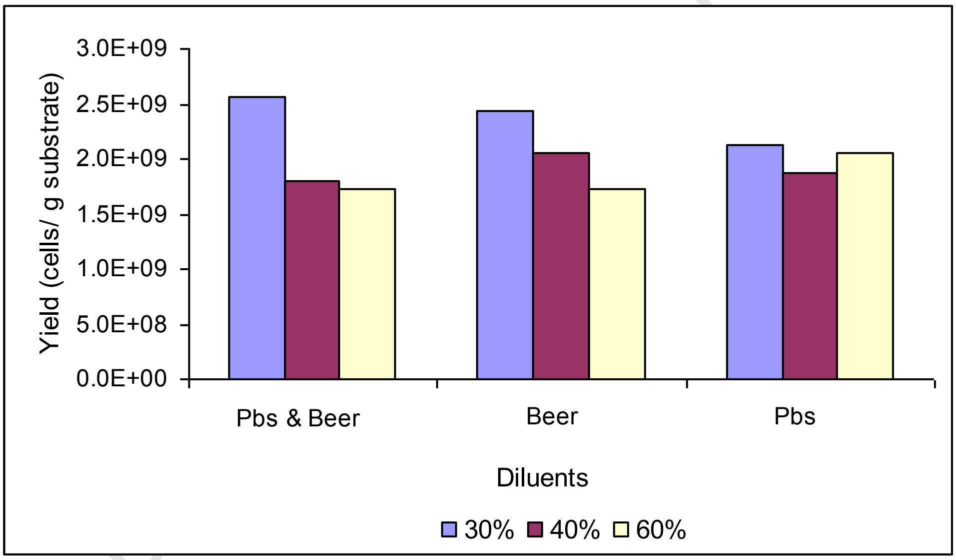
D.10: Specific growth rate for small-scale fermentation: 09.09.05

	PBS & Beer	Beer	PBS
30 %	0.153	0.162	0.295
40 %	0.320	0.319	0.218
60 %	0.136	0.198	0.289

D.11: Biomass yield

Date: 09.09.05

	30 %	40 %	60 %
PBS & beer	2.56E+09	1.80E+09	1.72E+09
Beer	2.44E+09	2.06E+09	1.72E+09
Pbs	2.12E+09	1.88E+09	2.06E+09



APPENDIX E: CELL ENVELOPE FLEXIBILITY

Date: 09.09.05

Cell wall flexibility of control yeast injected into water

run 1

0909uco60w				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	10.23	100.00	0.00
3	5	9.65	94.33	5.67
4	10	9.25	90.42	9.58
5	15	8.99	87.88	12.12
6	20	8.81	86.12	13.88
7	25	8.65	84.56	15.44
8	30	8.52	83.28	16.72
9	35	8.42	82.31	17.69
10	40	8.34	81.52	18.48
11	45	8.30	81.13	18.87
12	50	8.24	80.55	19.45
13	55	8.15	79.67	20.33
14	65	8.13	79.47	20.53
15	75	8.11	79.28	20.72
16	85	8.10	79.18	20.82

run 1

0909uco40w				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	8.94	100.00	0.00
3	5	8.64	96.64	3.36
4	10	8.42	94.18	5.82
5	15	8.30	92.84	7.16
6	20	8.20	91.72	8.28
7	25	8.16	91.28	8.72
8	30	8.11	90.72	9.28
9	35	8.06	90.16	9.84
10	40	8.02	89.71	10.29
11	45	7.91	88.48	11.52
12	50	7.89	88.26	11.74
13	55	7.98	89.26	10.74
14	65	7.87	88.03	11.97
15	75	7.86	87.92	12.08
16	85	7.86	87.92	12.08

run 1

0909uco30w				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	8.81	100.00	0.00
3	5	8.57	97.28	2.72
4	10	8.36	94.89	5.11
5	15	8.25	93.64	6.36
6	20	8.15	92.51	7.49
7	25	8.11	92.05	7.95
8	30	7.96	90.35	9.65
9	35	7.97	90.47	9.53
10	40	7.98	90.58	9.42
11	45	7.95	90.24	9.76
12	50	7.94	90.12	9.88
13	55	7.93	90.01	9.99
14	65	7.92	89.90	10.10
15	75	7.90	89.67	10.33
16	85	7.90	89.67	10.33

run 1

09096050w				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	10.90	100.00	0.00
3	5	10.12	92.84	7.16
4	10	9.72	89.17	10.83
5	15	9.39	86.15	13.85
6	20	9.15	83.94	16.06
7	25	9.03	82.84	17.16
8	30	8.92	81.83	18.17
9	35	8.83	81.01	18.99
10	40	8.73	80.09	19.91
11	45	8.69	79.72	20.28
12	50	8.61	78.99	21.01
13	55	8.53	78.26	21.74
14	65	8.51	78.07	21.93
15	75	8.43	77.34	22.66
16	85	8.41	77.16	22.84

0909u60100hy				
No	**t (s)**	**D (µm)**	**Change (%)**	**Delta size (%)**
2	0	9.60	100.00	0.00
3	5	8.47	88.23	-11.77
4	10	8.11	84.48	-15.52
5	15	7.92	82.50	-17.50
6	20	7.78	81.04	-18.96
7	25	7.75	80.73	-19.27
8	30	7.65	79.69	-20.31
9	35	7.55	78.65	-21.35
10	40	7.51	78.23	-21.77
11	45	7.49	78.02	-21.98
12	50	7.47	77.81	-22.19
13	55	7.41	77.19	-22.81
14	65	7.42	77.29	-22.71
15	75	7.38	76.88	-23.13
16	85	7.38	76.88	-23.13
		7.34	76.46	-23.54

090940100w1				
No	**t (s)**	**D (µm)**	**Change (%)**	**Delta size (%)**
2	0	10.13	100.00	0.00
3	5	9.62	94.97	5.03
4	10	9.29	91.71	8.29
5	15	9.07	89.54	10.46
6	20	8.94	88.25	11.75
7	25	8.83	87.17	12.83
8	30	8.69	85.78	14.22
9	35	8.64	85.29	14.71
10	40	8.51	84.01	15.99
11	45	8.38	82.72	17.28
12	50	8.34	82.33	17.67
13	55	8.31	82.03	17.97
14	65	8.2	80.95	19.05
15	75	8.19	80.85	19.15
16	85	8.25	81.44	18.56

090910040hy				
No	**t (s)**	**D (µm)**	**Change (%)**	**Delta size(%)**
2	0	7.80	100.00	0.00
3	5	7.55	96.79	-3.21
4	10	7.43	95.26	-4.74
5	15	7.32	93.85	-6.15
6	20	7.23	92.69	-7.31
7	25	7.15	91.67	-8.33
8	30	7.10	91.03	-8.97
9	35	6.99	89.62	-10.38
10	40	6.96	89.23	-10.77
11	45	6.95	89.10	-10.90
12	50	6.91	88.59	-11.41
13	55	6.93	88.85	-11.15
15	65	6.89	88.33	-11.67
17	75	6.90	88.46	-11.54
19	85	6.88	88.21	-11.79

090910030w				
No	**t (s)**	**D (µm)**	**Change (%)**	**Delta size (%)**
2	0	9.94	100.00	0.00
3	5	9.34	93.96	6.04
4	10	9.08	91.35	8.65
5	15	8.87	89.24	10.76
6	20	8.69	87.42	12.58
7	25	8.53	85.81	14.19
8	30	8.37	84.21	15.79
9	35	8.30	83.50	16.50
10	40	8.23	82.80	17.20
11	45	8.14	81.89	18.11
12	50	8.10	81.49	18.51
13	55	8.1	81.49	18.51
14	65	8.04	80.89	19.11
15	75	7.99	80.38	19.62
16	85	7.95	79.98	20.02

Date: 28.08.05

run 1

280858coW				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	10.33	100.00	0.00
3	5	9.61	93.03	6.97
4	10	9.22	89.25	10.75
5	15	8.94	86.54	13.46
6	20	8.78	85.00	15.00
7	25	7.57	73.28	26.72
8	30	8.68	84.03	15.97
9	35	8.41	81.41	18.59
10	40	8.28	80.15	19.85
11	45	8.20	79.38	20.62
12	50	8.15	78.90	21.10
13	55	8.09	78.32	21.68
14	65	8.03	77.73	22.27
15	75	7.99	77.35	22.65
16	85	7.96	77.06	22.94

run 1

280840coW				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	9.65	100.00	0.00
3	5	9.61	99.59	0.41
4	10	8.53	88.39	11.61
5	15	8.31	86.11	13.89
6	20	8.20	84.97	15.03
7	25	8.13	84.25	15.75
8	30	8.05	83.42	16.58
9	35	7.95	82.38	17.62
10	40	7.91	81.97	18.03
11	45	7.88	81.66	18.34
12	50	7.85	81.35	18.65
13	55	7.87	81.55	18.45
14	65	7.85	81.35	18.65
15	75	7.83	81.14	18.86
16	85	7.84	81.24	18.76

run 1

280830coW				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	8.78	100.00	0.00
3	5	8.39	95.56	4.44
4	10	8.47	96.47	3.53
5	15	8.29	94.42	5.58
6	20	8.19	93.28	6.72
7	25	8.11	92.37	7.63
8	30	8.05	91.69	8.31
9	35	8.00	91.12	8.88
10	40	7.93	90.32	9.68
11	45	7.90	89.98	10.02
12	50	7.86	89.52	10.48
13	55	7.86	89.52	10.48
14	65	7.82	89.07	10.93
15	75	7.83	89.18	10.82
16	85	7.76	88.38	11.62

run 2

280858coW2				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	11.75	100.00	0.00
3	5	10.54	102.03	2.03
4	10	9.87	95.55	4.45
5	15	9.48	91.77	8.23
6	20	9.13	88.38	11.62
7	25	8.92	86.35	13.65
8	30	8.82	85.38	14.62
9	35	8.59	83.16	16.84
10	40	8.48	82.09	17.91
11	45	8.39	81.22	18.78
12	50	8.29	80.25	19.75
13	55	8.2	79.38	20.62
14	65	8.11	78.51	21.49
15	75	8.08	78.22	21.78
16	85	8.02	77.64	22.36

run 1

280840cohy				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	8.72	100.00	0.00
3	5	8.27	85.70	14.30
4	10	7.96	82.49	17.51
5	15	7.74	80.21	19.79
6	20	7.77	80.52	19.48
7	25	7.95	82.38	17.62
8	30	7.82	81.04	18.96
9	35	7.75	80.31	19.69
10	40	7.67	79.48	20.52
11	45	7.69	79.69	20.31
12	50	7.59	78.65	21.35
13	55	7.63	79.07	20.93
14	65	7.58	78.55	21.45
15	75	7.50	77.72	22.28
16	85	7.49	77.62	22.38

run 1

280830cohy				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	8.52	100.00	0.00
3	5	8.17	95.89	-4.11
4	10	7.85	92.14	-7.86
5	15	7.59	89.08	-10.92
6	20	7.46	87.56	-12.44
7	25	7.32	85.92	-14.08
8	30	7.23	84.86	-15.14
9	35	7.16	84.04	-15.96
10	40	7.26	85.21	-14.79
11	45	7.15	83.92	-16.08
12	50	7.12	83.57	-16.43
13	55	7.08	83.10	-16.90
14	65	7.04	82.63	-17.37
15	75	7.02	82.39	-17.61
16	85	7.05	82.75	-17.25

run 1

280858com				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	12.64	100.00	0.00
3	5	11.98	94.78	-5.22
4	10	11.60	91.77	-8.23
5	15	11.28	89.24	-10.76
6	20	11.07	87.58	-12.42
7	25	10.76	85.13	-14.87
8	30	10.70	84.65	-15.35
9	35	10.57	83.62	-16.38
10	40	10.26	81.17	-18.83
11	45	10.75	85.05	-14.95
12	50	10.08	79.75	-20.25
13	55	10.00	79.11	-20.89
14	65	9.92	78.48	-21.52
15	75	9.82	77.69	-22.31
16	85	9.76	77.22	-22.78

run 1

280840com				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	11.11	100.00	0.00
3	5	10.55	94.96	-5.04
4	10	10.13	91.18	-8.82
5	15	9.86	88.75	-11.25
6	20	9.73	87.58	-12.42
7	25	9.55	85.96	-14.04
8	30	9.43	84.88	-15.12
9	35	9.23	83.08	-16.92
10	40	9.23	83.08	-16.92
11	45	9.08	81.73	-18.27
12	50	9.03	81.28	-18.72
13	55	8.90	80.11	-19.89
14	65	8.80	79.21	-20.79
15	75	8.76	78.85	-21.15
16	85	8.71	78.40	-21.60

run 1

280830com				
No	t (s)	D (um)	change(%)	delta size(%)
2	0	10.77	100.00	0.00
3	5	10.24	95.08	-4.92
4	10	9.92	92.11	-7.89
5	15	9.57	88.86	-11.14
6	20	9.43	87.56	-12.44
7	25	9.28	86.17	-13.83
8	30	9.13	84.77	-15.23
9	35	9.07	84.22	-15.78
10	40	8.84	82.08	-17.92
11	45	8.77	81.43	-18.57
12	50	8.71	80.87	-19.13
13	55	8.66	80.41	-19.59
14	65	8.63	80.13	-19.87
15	75	8.60	79.85	-20.15
16	85	8.53	79.20	-20.80

Consistency: 60 %

run 1

28085850w				
No	**t (s)**	**D (um)**	**change(%)**	**delta size(%)**
2	0	12.58	100.00	0.00
3	5	10.59	88.18	11.82
4	10	9.67	80.52	19.48
5	15	9.38	78.10	21.90
6	20	8.92	74.27	25.73
7	25	8.78	73.11	26.89
8	30	8.55	71.19	28.81
9	35	8.42	70.11	29.89
10	40	8.29	69.03	30.97
11	45	8.21	68.36	31.64
12	50	8.07	67.19	32.81
13	55	8.03	66.86	33.14
14	65	7.98	66.44	33.56
15	75	7.92	65.95	34.05
16	85	7.89	65.70	34.30

run 1

280858100w				
No	**t (s)**	**D (um)**	**change(%)**	**delta size(%)**
2	0	12.01	100.00	0.00
3	5	10.65	88.68	11.32
4	10	9.94	82.76	17.24
5	15	9.56	79.60	20.40
6	20	9.24	76.94	23.06
7	25	8.99	74.85	25.15
8	30	8.83	73.52	26.48
9	35	8.66	72.11	27.89
10	40	8.53	71.02	28.98
11	45	8.44	70.27	29.73
12	50	8.33	69.36	30.64
13	55	8.26	68.78	31.22
14	65	8.20	68.28	31.72
15	75	8.11	67.53	32.47
16	85	8.05	67.03	32.97

run 1

28085850m				
No	**t (s)**	**D (um)**	**change(%)**	**delta size(%)**
2	0	13.33	100.00	0.00
3	5	12.35	92.65	-7.35
4	10	11.77	88.30	-11.70
5	15	11.31	84.85	-15.15
6	20	11.02	82.67	-17.33
7	25	10.75	80.65	-19.35
8	30	10.48	78.62	-21.38
9	35	10.30	77.27	-22.73
10	40	10.13	75.99	-24.01
11	45	10.01	75.09	-24.91
12	50	9.88	74.12	-25.88
13	55	9.7	72.77	-27.23
14	65	9.67	72.54	-27.46
15	75	9.56	71.72	-28.28
16	85	9.52	71.42	-28.58

run 1

280858100m				
No	**t (s)**	**D (um)**	**change(%)**	**delta size(%)**
2	0	12.64	100.00	0.00
3	5	11.93	94.38	-5.62
4	10	11.42	90.35	-9.65
5	15	11.01	87.10	-12.90
6	20	10.84	85.76	-14.24
7	25	10.60	83.86	-16.14
8	30	10.46	82.75	-17.25
9	35	10.25	81.09	-18.91
10	40	10.05	79.51	-20.49
11	45	9.84	77.85	-22.15
12	50	9.79	77.45	-22.55
13	55	9.66	76.42	-23.58
14	65	9.63	76.19	-23.81
15	75	9.57	75.71	-24.29
16	85	9.46	74.84	-25.16

run 1

28085850m				
No	**t (s)**	**D (um)**	**change(%)**	**delta size(%)**
2	0	13.33	100.00	0.00
3	5	12.35	92.65	-7.35
4	10	11.77	88.30	-11.70
5	15	11.31	84.85	-15.15
6	20	11.02	82.67	-17.33
7	25	10.75	80.65	-19.35
8	30	10.48	78.62	-21.38
9	35	10.30	77.27	-22.73
10	40	10.13	75.99	-24.01
11	45	10.01	75.09	-24.91
12	50	9.88	74.12	-25.88
13	55	9.7	72.77	-27.23
14	65	9.67	72.54	-27.46
15	75	9.56	71.72	-28.28
16	85	9.52	71.42	-28.58

run 1

280858100hy				
No	**t (s)**	**D (um)**	**change(%)**	**delta size(%)**
2	0	10.58	100.00	0.00
3	5	9.75	77.14	-22.86
4	10	9.20	72.78	-27.22
5	15	9.52	75.32	-24.68
6	20	9.27	73.34	-26.66
7	25	9.09	71.91	-28.09
8	30	8.93	70.65	-29.35
9	35	8.79	69.54	-30.46
10	40	8.84	69.94	-30.06
11	45	8.56	67.72	-32.28
12	50	8.41	66.53	-33.47
13	55	8.31	65.74	-34.26
14	65	8.33	65.90	-34.10
15	75	8.21	64.95	-35.05
16	85	8.18	64.72	-35.28

Consistency: 40 %

run 1

280840100w				
No	**t (s)**	**D (um)**	**change(%)**	**delta size(%)**
2	0	8.83	100.00	0.00
3	5	8.28	93.77	6.23
4	10	8.06	91.28	8.72
5	15	7.93	89.81	10.19
6	20	7.89	89.35	10.65
7	25	7.87	89.13	10.87
8	30	7.81	88.45	11.55
9	35	7.89	89.35	10.65
10	40	7.86	89.01	10.99
11	45	7.86	89.01	10.99
12	50	7.81	88.45	11.55
13	55	7.83	88.67	11.33
14	65	7.83	88.67	11.33
15	75	7.79	88.22	11.78
16	85	7.77	88.00	12.00

run 1

280840100m				
No	**t (s)**	**D (um)**	**change(%)**	**lta size(**
2	0	9.55	100.00	0.00
3	5	9.30	105.32	5.32
4	10	9.10	103.06	3.06
5	15	8.89	100.68	0.68
6	20	8.78	99.43	0.57
7	25	8.70	98.53	1.47
8	30	8.56	96.94	3.06
9	35	8.47	95.92	4.08
10	40	8.48	96.04	3.96
11	45	8.40	95.13	4.87
12	50	8.38	94.90	5.10
13	55	8.29	93.88	6.12
14	65	8.27	93.66	6.34
15	75	8.25	93.43	6.57
16	85	8.22	93.09	6.91

run 1

280840100w				
No	**t (s)**	**D (µm)**	**Change (%)**	**Delta size(%)**
2	0	8.83	100.00	0.00
3	5	8.28	93.77	6.23
4	10	8.06	91.28	8.72
5	15	7.93	89.81	10.19
6	20	7.89	89.35	10.65
7	25	7.87	89.13	10.87
8	30	7.81	88.45	11.55
9	35	7.89	89.35	10.65
10	40	7.86	89.01	10.99
11	45	7.86	89.01	10.99
12	50	7.81	88.45	11.55
13	55	7.83	88.67	11.33
14	65	7.83	88.67	11.33
15	75	7.79	88.22	11.78
16	85	7.77	88.00	12.00

Consistency: 30 %

Table : Yeast with 30 % yeast consistency

		28.08, 30, 100, w		
No	Time(s)	D(um)	Size change (%)	Delta size (%)
2	0	11.03	100	0.00
3	5	9.9	89.76	10.24
4	10	9.3	84.32	15.68
5	15	8.92	80.87	19.13
6	20	8.67	78.6	21.4
7	25	8.53	77.33	22.67
8	30	8.42	76.34	23.66
9	35	8.3	75.25	24.75
10	40	8.15	73.89	26.11
11	45	8.13	73.71	26.29
12	50	8.03	72.8	27.2
13	55	7.98	72.35	27.65
14	65	7.95	72.08	27.92
15	75	7.91	71.71	28.29

16	85	8.01	72.62	27.38

		28.08, 30, 100, w		
No	Time(s)	D(um)	Size change (%)	Delta size (%)
2	0	11.03	100	0.00
3	5	9.9	89.76	10.24
4	10	9.3	84.32	15.68
5	15	8.92	80.87	19.13
6	20	8.67	78.6	21.4
7	25	8.53	77.33	22.67
8	30	8.42	76.34	23.66
9	35	8.3	75.25	24.75
10	40	8.15	73.89	26.11
11	45	8.13	73.71	26.29
12	50	8.03	72.8	27.2
13	55	7.98	72.35	27.65

14	65	7.95	72.08	27.92
15	75	7.91	71.71	28.29
16	85	8.01	72.62	27.38

run 1 w

280830100w

No	t (s)	D (um)	change(%)	delta size(%)
2	0	11.03	100.00	0.00
3	5	9.90	89.76	10.24
4	10	9.30	84.32	15.68
5	15	8.92	80.87	19.13
6	20	8.67	78.60	21.40
7	25	8.53	77.33	22.67
8	30	8.42	76.34	23.66
9	35	8.30	75.25	24.75
10	40	8.15	73.89	26.11
11	45	8.13	73.71	26.29
12	50	8.03	72.80	27.20
13	55	7.98	72.35	27.65
14	65	7.95	72.08	27.92
15	75	7.91	71.71	28.29
16	85	8.01	72.62	27.38

run 1 w

280830100m

No	t (s)	D (um)	change(%)	delta size(%)
2	0	11.69	100.00	0.00
3	5	10.95	99.27	0.73
4	10	10.67	96.74	3.26
5	15	10.21	92.57	7.43
6	20	10.14	91.93	8.07
7	25	9.82	89.03	10.97
8	30	9.71	88.03	11.97
9	35	9.44	85.58	14.42
10	40	9.51	86.22	13.78
11	45	9.28	84.13	15.87
12	50	9.35	84.77	15.23
13	55	9.3	84.32	15.68
14	65	9.21	83.50	16.50
15	75	9.07	82.23	17.77
16	85	9.22	83.59	16.41

APPENDIX F: HAZE

control data		18.07.05					02.09.05			haze						
15%		100%			30% consistency beer		40%		60%	CoNTROL		25% 10.05.05		100% 10.05.05		control
Sizes	fx/x3 scld	Sizes	fx/x3 scld	Sizes	fx/x3 scld	Sizes	fx/x3 scld	Sizes	fx/x3 scld		Sizes	fx/x3 scld	Sizes	fx/x3 scld	Sizes	fx/x3 scld
0.0582	0		0		0		0		0	0		0		11.79536		11.11555
0.0679	0		1.077007		0		0		0	0		0		14.62719		12.86522
0.0791	0		2.258641		0		0		0	0		0		13.50989		12.15257
0.0921	0		3.367252		0		0		0	0		0		12.75308		12.6642
0.1073	0		4.504791		0		0		0	0		0		11.84622		12.70639
0.125	0		5.723442		0		0		0	0		0		8.38728		9.395186
0.1456	0		6.988016		0		0		0	0		0		6.423446		7.378323
0.1697	0		8.149908		0		0		0	0		0		5.304793		6.175305
0.1977	0		9.028001		0		0		0	0		3.675732		4.587334		5.184843
0.2303	0		9.526965		0		0		0	0		11.28679		3.47542		3.705183
0.2683	0		9.561546		0		0		0	0		15.832		2.700626		2.664613
0.3125	0		9.049716		0		0		0	0		16.20891		1.819202		1.694185
0.3641	0		7.952399		0		0		10.71797	0		15.15464		1.201952		1.04162
0.4242	0		6.435317		3.284176851		0		16.38116	0		12.38606		0.717816		0.585685
0.4941	0		4.903615		8.312926829		8.411985		17.14333	0		9.343709		0.412234		0.312566
0.5757	0		3.633874		13.13862266		15.95422		15.34543	0		6.600408		0.225583		0.161404
0.6707	0		2.605575		15.78727631		18.16151		12.44536	0		4.041631		0.109034		0.07596
0.7813	25.3368		1.765911		15.24344237		15.74423		9.395044	0		2.461785		0.052524		0.041578
0.9103	19.74455		1.164562		12.96132818		12.10687		6.228416	0		1.28117		0.022637		0.026351
1.0604	14.47034		0.754051		10.09184585		8.510085		4.05436	0		0.647039		0.008944		0.01598
1.2354	10.70534		0.491716		6.780877727		5.274075		2.579817	3.396958		0.328522		0.00336		0.009722
1.4393	8.295704		0.326106		4.119842676		3.062891		1.627644	4.296418		0.179961		0.000331		0.00583
1.6767	6.220103		0.22421		1.48912264		1.894347		1.053082	8.150731		0.11491		0.002257		0.003567
1.9534	4.108294		0.158148		0.739924928		1.470257		0.72648	11.1706		0.08125		0.001732		0.002247
2.2757	2.616876		0.109201		0.787037134		1.394803		0.544247	12.49838		0.061699		0.00142		0.001936
2.6512	1.852108		0.074142		0.914786965		1.383086		0.434319	12.37248		0.050281		0.001173		0.001876
3.0887	1.386663		0.053044		1.029429748		1.356788		0.355639	11.30252		0.047873		0.0012		0.002041
3.5983	1.134245		0.038356		1.081539733		1.267576		0.288477	9.347189		0.045295		0.001264		0.002056
4.192	0.931434		0.027679		1.051557888		1.107478		0.22549	7.128876		0.041932		0.00135		0.001966
4.8837	0.791344		0.018831		0.942683065		0.907745		0.167343	5.498389		0.036252		0.0013		0.001719
5.6895	0.647923		0.012269		0.774512603		0.694211		0.116772	3.998989		0.029628		0.001184		0.001403
6.6283	0.523959		0.007413		0.579358153		0.492704		0.07592	2.793063		0.022481		0.000981		0.001051
7.7219	0.403463		0.004225		0.386022167		0.323076		0.045581	1.947246		0.015966		0.000744		0.000734
8.996	0.292344		0.002269		0.23208661		0.198196		0.024911	1.442682		0.010519		0.00052		0.000477
10.4804	0.199267		0.001069		0.13022853		0.117412		0.0122	1.079326		0.006399		0.000338		0.00029
12.2096	0.13076		0.000475		0.069554558		0.068572		0.005255	0.855012		0.003629		0.000172		0.000169
14.2242	0.083655		0.000188		0.035626921		0.040194		0.001965	0.683177		0.001911		7.54E-05		9.57E-05
16.5712	0.052617		5.94E-05		0.017573867		0.023771		0.000683	0.548884		0.000934		2.83E-05		5.28E-05
19.3055	0.032582		9.26E-06		0.008431614		0.014103		0.000339	0.432587		0.000417		7.73E-06		2.86E-05
22.4909	0.018977		0		0.004054486		0.008705		0.000346	0.331567		0.000166		5.27E-07		1.54E-05
26.2019	0.010521		0		0.002034666		0.005235		0.000426	0.244944		5.45E-05		0		8.31E-06
30.5252	0.005491		0		0.000995959		0.002997		0.000468	0.174053		1.34E-05		0		4.61E-06
35.5618	0.002737		0		0.000824991		0.001597		0.000442	0.119762		0		0		2.73E-06
41.4295	0.001266		0		0.000599319		0.000782		0.000363	0.080877		3.21E-06		0		1.76E-06
48.2654	0.000506		0		0.000465991		0.00035		0.000264	0.049043		7.11E-06		0		1.22E-06
56.2292	0.000134		0		0.000365218		0.00013		0.000174	0.027919		7.81E-06		0		8.81E-07
65.507	0		0		0.000294299		2.45E-05		0.000106	0.014832		5.99E-06		0		6.38E-07
76.3157	0		0		0.000218277		0		6.17E-05	0.007608		3.4E-06		0		4.55E-07
88.9077	0		0		0.000148036		0		3.64E-05	0.003691		1.5E-06		0		3.17E-07
103.5775	0		0		9.02407E-05		0		2.25E-05	0.001625		6.42E-07		0		1.85E-07
120.6678	0		0		4.90821E-05		0		1.44E-05	0.00058		4.33E-07		0		9.67E-08
140.578	0		0		2.42739E-05		0		1.17E-05	0		4.15E-07		0		4.48E-08
163.7733	0		0		1.11858E-05		0		9.43E-06	0		3.6E-07		0		1.77E-08
190.7959	0		0		4.98093E-06		0		6.39E-06	0		2.32E-07		0		4.53E-09
222.2773	0		0		1.91749E-06		0		3.47E-06	0		1.07E-07		0		0
258.953	0		0		5.05294E-07		0		1.53E-06	0		3.4E-08		0		0
301.6802	0		0		0		0		5.48E-07	0		3.47E-10		0		0
351.4575	0		0		0		0		8.08E-08	0		0		0		0
409.4479	0		0		0		0		0	0		0		0		0
477.0068	0		0		0		0		0	0		0		0		0
555.713	0		0		0		0		0	0		0		0		0
647.4056	0		0		0		0		0	0		0		0		0
754.2275	0		0		0		0		0	0		0		0		0
878.675	0		0						0	0		0		0		0
	25.3368		9.561546		15.78727631		18.16151		17.14333	0				0		0
										12.49838		16.20891		14.62719		12.86522