THE EFFECT OF COOLING ON BREWERS' YEAST QUALITY

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SYNOPSIS

The primary concern of a brewer is to produce beer of a desired flavour and quality, in an economic and efficient manner. In large scale brewing operations, process efficiency and beer quality rely on consistent fermentations. Stewart (1977) reports that improper handling of yeast in the brewing process may result in beer of sub-standard quality. Therefore, the maintenance of yeast quality during yeast handling is imperative. A typical brewery yeast handling regiment consists of (Basson, 1996):

- The transfer of yeast from propagation vessels into aerated wort for fermentation.
- Pumping the settled yeast from the bottom of a cylindroconical fermentation vessel.
- The flow of yeast through pipes of specified diameter and length.
- Cooling to ± 3 ° C by passage through a heat exchanger.
- Holding in an agitated storage tank.
- Possible re-circulation through a heat exchanger to maintain temperature.

Study of the current yeast handling system at South African Breweries (SAB)-Newlands by Robinson (2001) and work conducted by Fargher and Smith (1995) highlighted cold injury sensitivity in brewing yeast. Literature studies indicate that response of yeast to low positive temperatures is governed by:

i) yeast cell condition,
ii) cell growth phase,
iii) growth temperature,
iv) temperature range over which cooling is performed,
v) cooling rates employed, and
vi) level of low molecular weight protectants in the yeast.

An alternative process for cooling yeast has been reported by Wittl and Maier (1980). This involves the slow cooling of diluted yeast slurries in a jacketed agitated vessel. Possible stresses on yeast include osmotic stress on dilution, shear stress on agitation and cold stress from cooling.

The objectives of this research project were to investigate the effects of cooling, in terms of both extent of cooling and cooling rates, on stationary phase yeast. Systems examined included the cooling of diluted yeast slurries in an agitated jacketed vessel and in a heat

exchanger. Analytical techniques for the identification and quantification of a loss in yeast quality were selected and validated, and the cooling rates (critical) above which cold injury will impinge on yeast performance were identified. Because laboratory studies were limited in their ability to simulate the brewery circuit, on-line trials were conducted at SAB-Newlands to assess the potential for a loss of yeast quality during cooling under routine brewery operations.

The following was demonstrated:

- The osmotic stress encountered by cropped yeast on dilution had little or no effect on yeast cell integrity, growth and fermentative ability. Prolonged storage of yeast at 14 ^{0}C in a nutrient depleted media may however lead to a decrease in internal glycogen content of the yeast and the release of extracellular protease.
- The agitation employed in the jacketed agitated vessel, in the absence of cooling, had no effect on cell envelope (wall and membrane) integrity. Yeast growth and the ability of the yeast to adapt to the fermentation media were also not affected by the agitation.
- Over the range of final cooling temperatures investigated (14 to 4 ^{0}C) on cooling diluted yeast slurries in a jacketed agitated vessel, there was an increase in the level of expression of membrane stress compounds (trehalose and hsp) in the yeast with decreasing final cooling temperature. Cellular and membrane desiccation (indicated by reduction in cell size and expression of hsp 12) also increased with decreasing final cooling temperature. The exposure of yeast to temperature stress at 8-4 ^{0}C led to cell membrane permeabilisation and a decrease in cell wall strength. Damage to the cell envelope was dependent on the intensity of the cold stress. There was also a decrease in biomass yield as indicated by small-scale fermentations with decreasing final cooling temperature.
- Over the range of cooling rates that could be achieved in the heat exchange systems in the laboratory and on-line at SAB-Newlands (1.03 to 4.03 ^{0}C/sec), the amount of haze generated and protease released increased with increasing cooling rate (flowrate employed). Yeast growth and metabolic activity were similarly affected by increasing cooling rate. In laboratory and brewery experiments, yeast growth rate, biomass and CO_2 yield decreased with increasing cooling rate. Overall, cooling rates above 2 ^{0}C/sec (in the brewery, cropping flowrates in excess of 60 Kg/min, where the exit temperature of yeast from the heat exchanger is 2.5 ^{0}C) aggravated damage to the cell

envelope and showed the most detrimental effect on subsequent small-scale fermentation performance of the yeast.

- In general, the exposure of yeast to a 4 ^{0}C cold stress led to:
    - Increased levels of stress compounds such as trehalose and heat shock proteins (hsp 12) in yeast.
    - A decrease in yeast cell size due to cellular and membrane desiccation.
    - Loss of cell membrane integrity.
    - A weakening of the cell wall which resulted in haze formation and, on exposure extreme hydrodynamic stress in a French Press, increased cell disruption.
    - A decrease in biomass yield during subsequent small-scale fermentations.

From this study, it was shown that cold stress increases the susceptibility of the cell envelope to damage. Further work should be carried out to investigate the effects of agitation rates and duration of agitation on cooled yeast slurries. As the investigation was limited to conditions representative of brewery yeast handling circuits, work should also be conducted to identify rates below which cold injury will not occur in yeast. Protease release and haze formation were shown to be assay sensitive to the detection of cold stress generated in the heat exchanger. It is therefore valuable to conduct regular haze and protease analyses after the heat exchanger during routine brewery operations.

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NOMECLATURE

DEFINITIONS

Attenuation	The conversion of wort sugars to ethanol, CO_2 and other fermentation products leading to a drop in the specific gravity of fermentation media
Attenuation rate	The rate of change in specific gravity of the fermentation medium as a result of the conversion of wort sugars to fermentation products
Cavitation	Formation of vapour cavities in a fluid, due to a significant reduction in pressure, which oscillate through collapse and rebound cycles until they are destroyed by pressure recovery
Consistency	The biomass concentration of yeast suspension expressed as the percentage wet mass
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 inoculated into a polysaccharide rich medium (wort) convert sugars to carbon dioxide, ethanol, glycerol and biomass
Flocculation	The agglomeration of yeast during fermentation
Generation number	Indicator of the number of times a batch of yeast has been cropped and re-used
Gravity	Specific gravity of wort at 20 ^{0}C (expressed in ^{0}Plato) and used as a measure of sugar concentration
Integrity of the cell envelope	The intactness of the cell envelope
Physiological state	Yeast condition in terms of structural and functional integrity
Pitching	Inoculation of fermentation
Propagation	The step-wise generation of biomass from laboratory to plant scale to obtain sufficient biomass for production purpose
Re-circulation	The re-use of yeast during brewery fermentation involving harvesting, storage, batching and re-inoculation
Replicative competence	The ability to reproduce

Replicative deactivation	Loss of ability to reproduce
Sedimentation	The settling of yeast during fermentation
Viability	A term 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 within the brewing industry to describe the metabolic activity of yeast; in some cases, overall fermentative capacity may be implied
Wort	The cereal-based extract containing a range of carbohydrates which are converted to ethanol, CO_2 and other organoleptic products during the production of beer
Yeast quality	The physiological condition of the yeast in terms of its fermentative capacity and the quality of the beer produced

SYMBOLS

ln	Natural log
C_x	Biomass concentration (cells/mL)
C_s	Substrate concentration (g/L)
μ	Growth rate (/hr)
t	Time (hr)

ABBREVIATIONS

CIP	Cleaning in place
FV	Fermentation vessel
GOD	Glucose oxidase enzymes
MB	Methylene Blue stain
MYPG	Malt, yeast, peptone and glucose media
NIR	Near infrared spectroscopy
PBS	Phosphate buffered saline solution
POD	Peroxidase enzyme
SAB	South African Breweries
UCT	University of Cape Town
YCV	Yeast collection vessel
YPV	Yeast pitching vessel

CHAPTER

1

INTRODUCTION

This chapter serves to provide the reader with a background to the work presented in this thesis. The objectives of the research project are given and the importance of optimizing post-fermentation handling of yeast discussed.

1.1 BACKGROUND AND OBJECTIVES OF STUDY

Yeasts are of major economic, social and health significance in human culture. Their diverse and dynamic activities impinge on many areas of science, technology and medicine (Walker, 1998a). Taxonomists have identified yeasts of the genus *Saccharomyces* as having crucial roles in the production of foods, beverages and pharmaceuticals. The species *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* represent the foundation on which the world's largest and oldest biotechnology industry, beer brewing, is built (Oliver, 1991). In beer brewing, *Saccharomyces* convert sugars in cereal based extracts to ethanol, carbon dioxide and organoleptic compounds (Basson, 1996).

The production of beer is both lucrative and competitive. Annually, close to 10^{11} liters of beer are consumed worldwide (Oliver, 1991). As a result, maintenance of a product consistent in quality and taste is imperative (McCaig and Bendiak, 1985). Stewart (1977) reports that the improper handling of yeast during the brewing process may result in beer of sub-standard quality. A close examination of the brewery yeast handling procedure is therefore required in order to identify areas in which conditions may be detrimental to yeast.

In this regard, the Department of Chemical Engineering at the University of Cape Town has conducted studies into the effects of mechanical handling on yeast quality. Mechanical handling of yeast in a brewery involves the physical process of transfer, separation and agitation of yeast slurries (Robinson, 2001). Basson (1996) reports that a typical yeast handling regiment consists of:

- The transfer of yeast from propagation vessels into aerated wort for fermentation.
- Pumping the settled yeast from the bottom of a cylindroconical fermentation vessel.
- The flow of yeast through pipes of specified diameter and length.
- Cooling to ± 3 ° C by passage through a heat exchanger.
- Holding in an agitated storage tank.
- Possible re-circulation through a heat exchanger to maintain temperature.

Deterioration of yeast quality during handling may occur as a result of exposure to several stresses. These stresses may be categorized as mechanical and physiological. Mechanical stresses are exacted on yeast upon agitation, flow through pumps and flow restrictions such as pipe reductions and valves. Physiological stresses may stem from exposure of yeast to low temperatures, variations in water potential and the depletion of yeast cellular reserve molecules in storage. It is against this background that this study investigates the effects of low temperature on yeast and its possible implications on quality and resilience to other stresses.

Much evidence has shown that exposure of yeast to low positive temperatures may cause cell membrane fatty acids to undergo a phase transition from fluid to 'gel' states, compromising cell integrity. Furthermore, sterol synthesis is reduced, weakening membranes and thereby

increasing chances of cell disruption. Damage of this sort may cause the release of intracellular compounds affecting flavor and foam stability of beer (SAB, 1993).

The objectives of this research thesis are to investigate the effects of cooling, in terms of both extent of cooling and cooling rates, on stationary phase yeast. The investigation explores the hypothesis that exposure of yeast to cold stress may have adverse effects on quality and on the ability of the yeast to withstand subsequent mechanical handling. Systems to be examined include the cooling of diluted yeast slurries in an agitated jacketed vessel and in a heat exchanger. Analytical techniques for the identification and quantification of a loss in yeast quality are selected and validated, and the cooling rates (critical) above which cold injury will impinge on yeast performance are identified.

1.2 STRUCTURE OF THESIS

An overview of the brewing process is presented in Chapter 2, together with a comprehensive review of the yeast handling circuit. The term 'yeast quality' is defined and a literature survey on the potential for loss of yeast quality during mechanical handling of yeast in the brewery is given.

Chapter 3 deals with the potential for loss of yeast quality during cooling. The chapter is introduced by discussing yeast cooling systems used by brewers. Thereafter, a review of the effects of cooling, cooling rates and osmotic stress during dilution on yeast viability, vitality and integrity is presented. Factors affecting yeast tolerance to low temperatures are also discussed and further work required is detailed.

In Chapter 4, methods selected for the identification, characterization and quantification of a loss of yeast quality are presented. This includes a discussion of the literature on each method and the rationale for the choice of the method. The relationship between yeast quality and small-scale fermentation performance is investigated and a scheme for the loss of yeast quality in response to cold stress is proposed.

Chapter 5 gives a detailed account of the experiments performed to assess the potential for a loss of yeast quality as a consequence of cooling diluted yeast slurries in a jacketed agitated vessel and in a heat exchanger. These experiments were carried out in order to ascertain the effects of osmotic shock, agitation, final cooling temperature and cooling rate on the physiological state of yeast and its ability to withstand subsequent mechanical handling. Cooling rate experiments were carried out in the laboratory, using laboratory scale copper coils, and on-line at SAB-Newlands brewery during routine brewery operations.

In Chapter 6, results obtained from the experiments are presented and discussed. Efforts are also made to establish the critical cooling rates above which extensive cold injury will occur in yeast. Based on these findings, conclusions are made and recommendations put forward in Chapter 7.

There are two appendices to the text. Appendix A provides the detailed assay methods employed in the study. In Appendix B, calculations used to estimate brewery heat exchanger cooling rates are outlined. Calibration curves for the Marlow S40 pump used in laboratory scale heat exchanger experiments are also given.

CHAPTER

2

BEER BREWING AND MECHANICAL HANDLING OF YEAST IN A BREWERY

In this Chapter, an overview of the brewing process is presented, together with a comprehensive review of the yeast handling circuit. The term 'yeast quality' is defined, and the possible implications of mechanical handling on quality are given. Chapter 3 goes on to consider physicochemical handling of yeast, with specific reference to cold shock.

2.1 INTRODUCTION

Fermentation is the anaerobic process by which yeast cells inoculated into a polysaccharide rich medium (wort), convert sugars to carbon dioxide, ethanol, glycerol and biomass (Daude and Searle, 1986). The course of a fermentation is influenced by the wort composition and the type of yeast pitched (Stewart, 1977). At South African Breweries, beer is brewed in the so-called bottom or lager fermentation process (SAB, 1993). On depletion of sugars, the yeast *Saccharomyces carlsbergensis* aggregates and settles to the bottom of the fermentation vessel from where it is removed. Oliver (1991) reports that beers and ales traditionally drunk in the United Kingdom are produced using brewing strains of the yeast *Saccharomyces cerevisiae*. These microorganisms rise to the top of the vessel during fermentation and are skimmed off when the process is complete (Phaff *et al.*, 1966). Work detailed in this chapter is limited to bottom fermenting yeasts.

2.2 OVERVIEW OF THE BREWING PROCESS

The primary concern of a brewer is to produce beer of a desired flavor and quality, in an economic and efficient manner. As a result, modern breweries are designed to optimize beneficial biological and chemical transformations occurring throughout processing (Daude and Searle, 1986). Operations inside a brewery are generally in the following sequence: wort preparation, fermentation, and post fermentation treatment. A schematic summary of these steps is given in Figure 2.1.

2.2.1 WORT PREPARATION

Barley is the major raw material in beer production. It serves as the principal source of fermentable sugars in brewing (Oliver, 1991). Cereals such as sorghum and wheat are used in conventional African beers and are termed adjuncts (SAB, 1993). In wort preparation, several varieties of barley are allowed to undergo limited germination in a process known as malting. This results in the development of enzymes that convert starch and proteins in barley to sugars and amino acids, which may later be utilized by yeast. The malted barley (malt) is then kilned (dried) to arrest growth and remove moisture. The temperature employed on drying determines the principal characteristics of the malt. If moderate temperatures are used, the malt will be pale in color and high in enzyme activity. Kilning at high temperature produces dark malt suitable for stouts.

The next stage in wort preparation involves milling the malt into coarse flour, which is steeped in water and incubated at approximately 65 ^{0}C, continuing the hydrolysis of starch and proteins. The aqueous extract formed, wort, is separated from spent grain and other solids. Spent-grain residue is a useful by-product, sold as animal feed.

Hops and other process sundries are added to clarified wort in a brew kettle. The wort is boiled to eliminate contaminants, remove undesirable flavor compounds and stop enzymatic reactions. After two hours, the hot media is clarified by passing it through a whirlpool tank. The resulting solution is cooled in a heat exchanger, and an oxygen/air mixture is injected. Yeast is now pitched into the aerated wort and pumped to a fermentation vessel where fermentation commences.

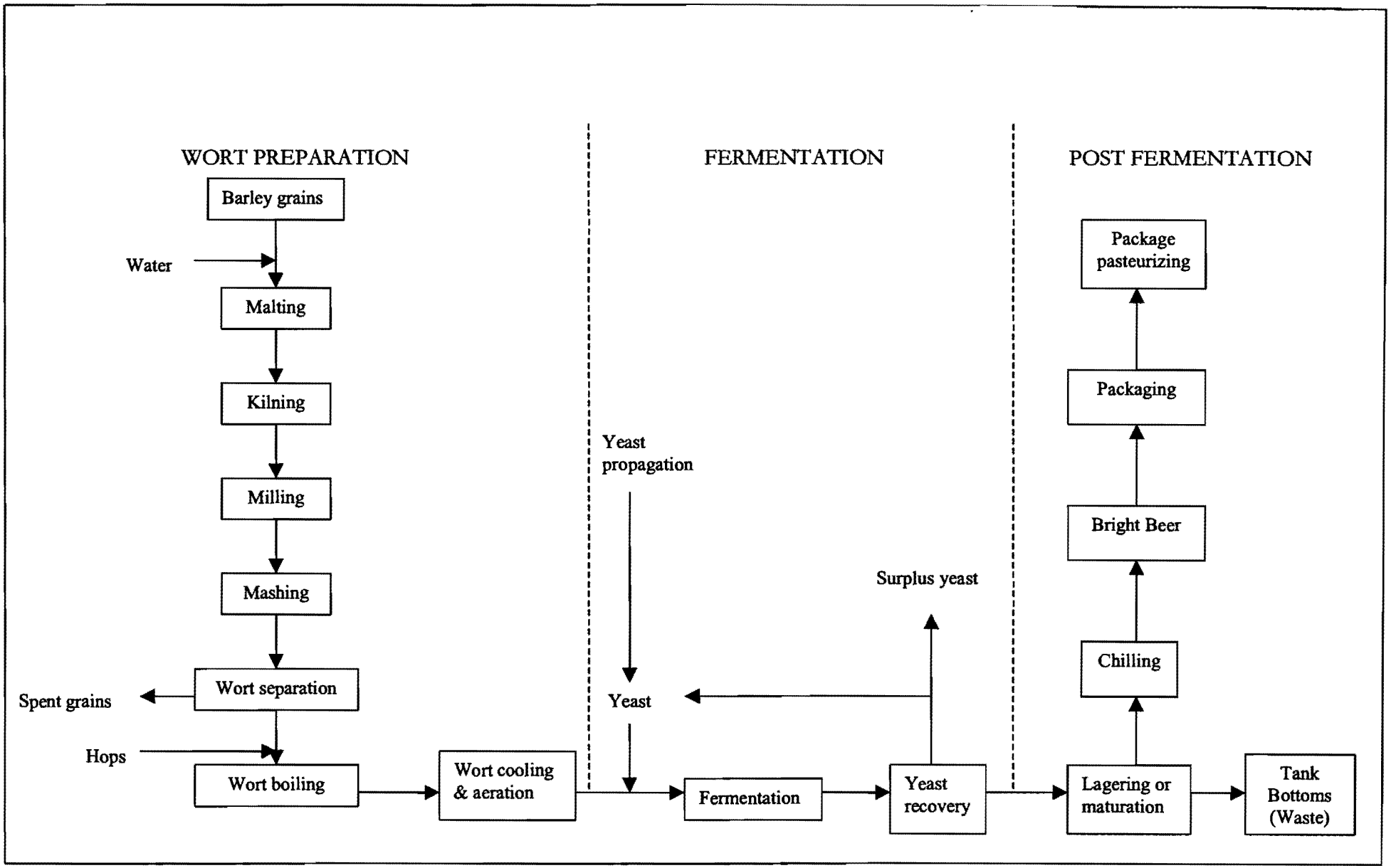


Figure 2.1 Outline of the brewing process. Adapted from Walker (1998a).

2.2.2 FERMENTATION

The fermentation process results in the conversion of wort components to beer and yeast. The amount of yeast pitched into a fermentation vessel depends on the starting sugar concentration (gravity) of the wort. A rule of thumb is the use of 1 million cells per milliliter per degree Plato of wort (O'Connor-Cox, 1995). This initial carbohydrate concentration determines the ultimate ethanol content of beer.

During the early phases of fermentation, yeasts use oxygen to convert their internal carbohydrate stores to carbon dioxide and energy. As the dissolved oxygen concentration decreases, the yeast begin to respire anaerobically, converting wort sugars to ethanol, carbon dioxide and a number of higher alcohols. These may further react to form esters and other flavor compounds. The following equation provides a simple representation of the fermentation of wort to beer (Hough, 1985):

$$\text{Maltose} + \text{Amino acids} \longrightarrow CO_2 + \text{Ethanol} + \text{Yeast} + \text{Heat} \qquad (2.1)$$

The conversion of wort sugars to ethanol leads to a drop in specific gravity of the medium. This process is called attenuation. The sugar concentration at which all fermentable carbohydrates are utilized is called the attenuation limit of the wort. Once the desired attenuation of the wort is reached, normally after 7-10 days at 10-15 ^{0}C, it is cooled to stop metabolism and to induce yeast sedimentation into the cone shaped base of the fermentation vessel. From here, the yeast is either transferred to storage (cropped) or spent yeast vessels (scrapped) through the yeast handling circuit.

2.2.3 POST-FERMENTATION TREATMENT OF BEER

Racking is the movement of beer from the fermenting tank to storage. In storage, the young beer is held at 14-18 ^{0}C for two days to allow the volatilization of undesirable products of fermentation. These compounds are purged in a very slow secondary fermentation between residual yeast in suspension and residual fermentable matter. The beer may then be chilled and carbonated. Any haze formed on chilling and all other solids in the beer are removed by filtration. This is normally achieved by passing beer through an inert filter aid called kieselguhr. The mature beer is now bottled, ready for consumption (SAB, 1993).

2.3 THE YEAST HANDLING CIRCUIT

Cropping refers to the process of removing yeast from a fermentation vessel (FV) at the end of fermentation. On average, there is a three-fold increase in yeast biomass during fermentation (SAB, 1993). Avis (1990) reports that handling can be kept to a minimum by pitching yeast directly from one FV to another, possibly under hydrostatic pressure. However, this is seldom done in large breweries. The handling of yeast via a collection vessel before re-pitching is important because (SAB, 1993):

- It allows a hold-up time for the collected yeast so that its quality can be determined.
- It gives the brewer the opportunity to calculate the amount of yeast to be pitched for a subsequent fermentation.
- It eases the scheduling time between cropping and wort availability.
- It allows the brewer to eliminate and control microbial infections that may plague yeast.

A typical yeast handling circuit employed in modern breweries is depicted schematically in Figure 2.2. Yeast handling circuits may differ depending on vessel geometry and on the type of fermenting yeast used. During handling, yeast is exposed to a variety of mechanical equipment including pumps, pipes, valves, bends, fittings and agitators. Firstly, flocculated yeast is pumped using positive displacement pumps from the cone of the FV to a paraflow plate and frame heat exchanger. Here, the yeast is cooled from 14 ^{0}C to temperatures close to 0 ^{0}C. It is then transferred to an uninsulated storage tank where it is held for up to 24 hours. The slurry is agitated to maintain its homogeneity and to avoid compaction of yeast (Lewis and Poerwantaro 1991). The amount of yeast required for a subsequent fermentation is then determined based upon its quality, concentration and the sugar content of wort. In instances where bacterial contamination is to be minimized, acid washing is used. The infected yeast is mixed with 1 % phosphoric acid solution prior to pitching and the resulting low pH kills any contaminants present. Infections in yeast affect beer flavor and the rate of fermentation (Roesler, 1968). Yeast is typically reused 6 to 8 times to minimize poor performance resulting from infection, mutation or a deteriorated physiological state (Knudsen, 1985). The stresses experienced by yeast during mechanical handling and their possible implications on quality are discussed in Section 2.5.

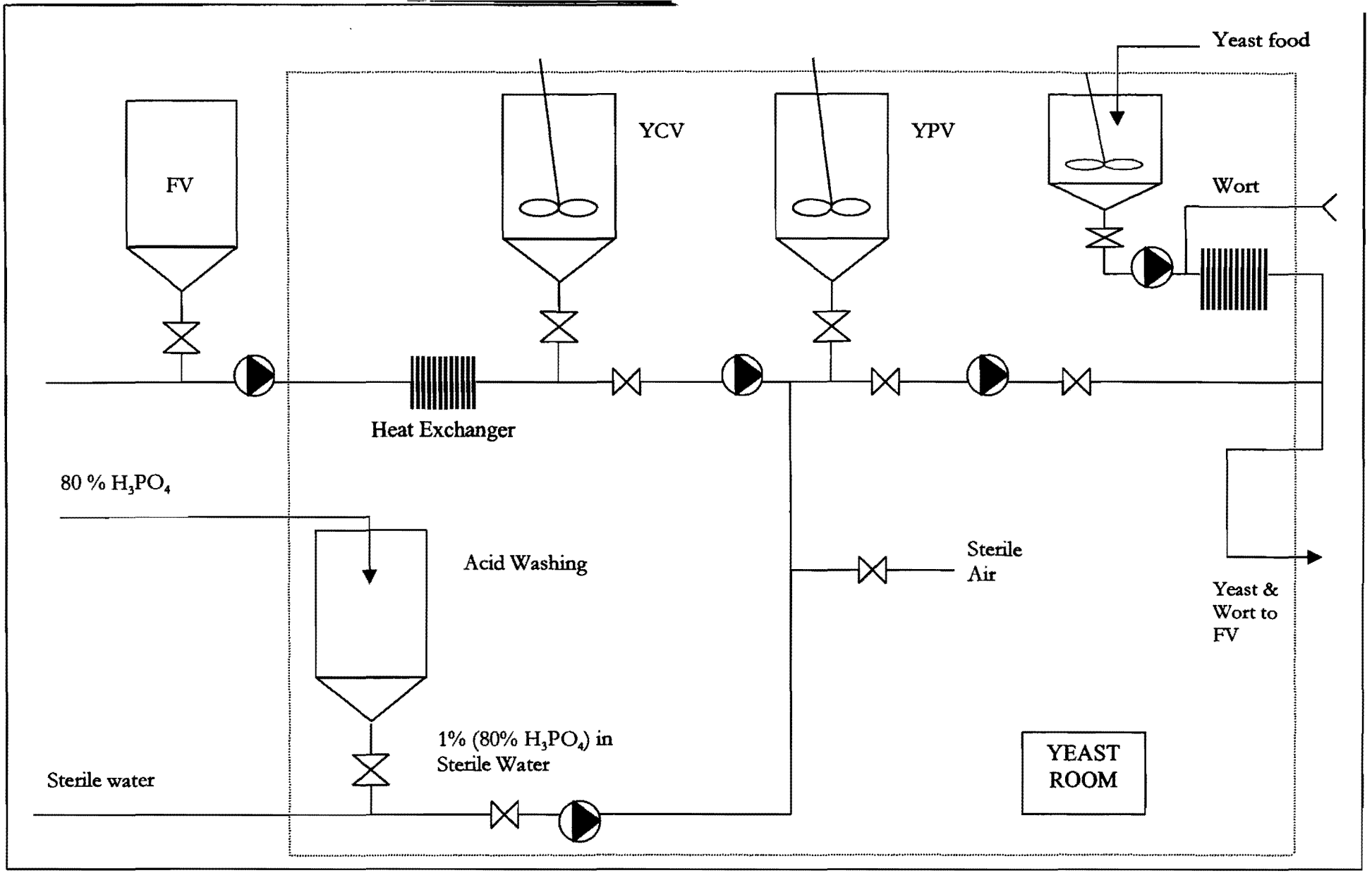


Figure 2.2 Schematic of the yeast handling circuit (FV, fermentation vessel; YCV, yeast collection vessel; YPV, Yeast pitching vessel). Oliver (1991).

2.4 YEAST QUALITY

The selection of yeast for re-pitching is a crucial process in brewing. The age or generation number of the yeast and its previous fermentation performance are two important factors to consider (SAB, 1993). Basson (1996) reports that yeast quality is related to fermentation performance and the quality of beer produced. The chief parameters used to ascertain yeast quality are (Avis, 1990):

i) stability,

ii) purity,

iii) viability,

iv) vitality and

v) integrity.

Stability refers to the occurrence of mutants in yeast. Genetic mutations in yeast are not discussed here, as it is not within the scope of this study.

2.4.1 PURITY

Yeast purity implies the absence of contamination. Contamination of yeast by bacteria and wild yeasts is very common. A wild yeast is defined as one that does not produce the same desirable product as the brewers strain (Lieberman, 1980). However, unless the infection is severe, acid washing can eliminate most bacterial contaminants effectively. Infection can cause a host of problems, all of which normally result in off flavors and cloudiness in packaged beer.

2.4.2 VIABILITY

Cell death and the inability of cells to grow and reproduce are said to constitute a loss of viability (Lentini, 1993). Viability is an important aspect of yeast quality as sufficient biomass growth is required after inoculation to affect an acceptable rate of attenuation. Furthermore, replicative deactivation is a precursor to cell death. Dead or autolysed cells result in the introduction of intracellular substances into beer leading to haze formation and flavor variations (SAB, 1993).

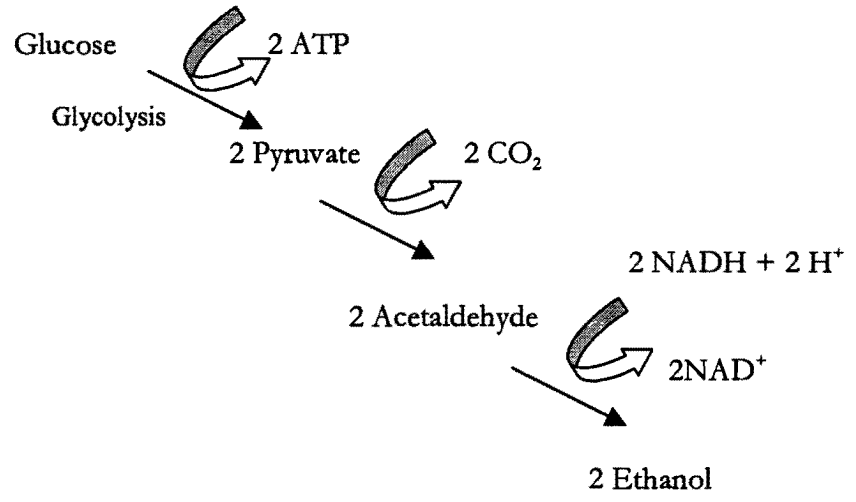
2.4.3 VITALITY

Detrimental changes in the physiological condition of yeast may be described as a loss of "vitality". These changes may manifest themselves as:

- Changes in partitioning between metabolic pathways influencing flavor compounds; or
- Changes in metabolic rate and biomass growth.

The implications of the above factors on yeast quality are now examined.

2.4.3.1 METABOLIC PATHWAYS



(2.2)

The sequence of enzyme catalyzed reactions that oxidatively convert sugars to pyruvic acid and finally ethanol and carbon dioxide is outlined in Equation 2.2 (Walker, 1998a). In order to utilize this Embden-Meyerhof-Parnas pathway (EMP), yeast enzymes degrade complex wort sugars such as sucrose and maltose to their monosaccharide units. Although ethanol and carbon dioxide are the principal products of the EMP pathway, a variety of other compounds, including alcohols, sterols, esters, diacetyl and sulphur containing compounds are produced by yeast metabolism and by interactions between metabolic products and wort constituents (Rose, 1977). These substances contribute to the distinctive flavor of beer. Deviations from characteristic flavour or the production of undesirable flavours in final beer constitute a loss in yeast quality. Pickerell *et al.* (1991) report that excess amounts of

acetaldehyde and sulphur dioxide are undesirable and that their levels at the end of fermentation may reflect stress in yeast.

Levels of diacetyl in wort may also be indicative of a loss in yeast quality. Yeast produces diacetyl from amino acid metabolism. This compound imparts a butterscotch flavor in beer and should be present in concentrations less than 0.1 ppm. During the early stages of fermentation, up to 0.5 ppm of diacetyl are produced. As fermentation progresses yeast assimilates the compound. Warm fermentation temperatures, prolonged storage, the amount of yeast cells in suspension and bacterial infections in yeast affect diacetyl metabolism. Aspects of yeast quality such as purity, viability and integrity (Section 2.4.4) may therefore influence diacetyl levels in the final product (Siebel Institute of Technology, 1978).

2.4.3.2 METABOLIC RATES AND BIOMASS GROWTH

The metabolic rate of yeast determines the rate at which wort sugars are utilized and hence the rate of attenuation. Yeast metabolic rates vary according to oxygen and nutrient availability, as well as product inhibition (ethanol). Ormrod *et al.* (1991) report that a cell's condition, in terms of structural and functional integrity, may also affect the metabolic rate. Changes in metabolic rate can be measured by monitoring the oxygen utilization and carbon dioxide evolution of yeast. During the lag phase of fermentation, yeasts convert their internal stores of glycogen, in the presence of oxygen, to carbon dioxide, sterols, unsaturated fatty acids and energy. This energy, acquired by aerobic respiration, is used for growth and maintenance. Sterols and unsaturated fatty acids are integral components of the cell membrane, and their concentration determines biomass growth. Two factors may limit the rate and extent of yeast growth: the amount of glycogen in yeast and the dissolved oxygen present in wort (Pickerell *et al.*, 1991). Low levels of glycogen and oxygen on pitching correlate with a reduced rate of attenuation and extent of biomass growth (Quain *et al.*, 1981).

2.4.4 INTEGRITY

Shear forces acting at the interface between the cell and its fluid environment may cause changes in cell integrity. This may result in a rearrangement or loss of surface molecules from the cell wall, or in extreme cases, may cause damage to the cell membrane, releasing intracellular compounds into beer. Loss in wall and membrane integrity may thus result in loss of beer quality, altered flocculation and sedimentation, and replicative deactivation and cell death (Dunlop *et al.*, 1994).

2.4.4.1 LOSS OF BEER QUALITY

Partial removal of cell wall glycoproteins represents the least extreme degree of damage to yeast. This may contribute to the occurrence of haze in beer (Lewis and Poerwantaro, 1991), compromising beer quality. Moreover, the presence of haze material may lead to filtration difficulties due to increased fine particle concentration (Siebert *et al.*, 1987). Damage to the cell membrane may result in "leaky" cells. The subsequent release of intracellular material into beer may affect flavor profiles, and in the case of protease enzymes, may cause foam instability (Ormrod *et al.*, 1991).

2.4.4.2 CHANGES IN FLOCCULATION AND SEDIMENTATION

Flocculation is a phenomenon of particular importance in brewing. Flocculation refers to the asexual cellular aggregation that occurs when yeasts adhere to one another reversibly and sediment out of suspension. In general, the flocculation of brewing yeast is associated with the onset of stationary phase. If flocculation occurs too early in the brewing process, fermentation will cease leaving residual sugars in the wort. Conversely, if flocculation is delayed causing the yeast cells to settle out of the fermentation slowly, downstream processing problems may be encountered (Walker, 1998). Yeast flocculation is genetically determined, but wort composition and the physiological state of the yeast control the expression of the phenomena (Hohmann and Mager, 1997). In 1982, Miki *et al.* proposed the widely accepted lectin hypothesis for yeast flocculation (Figure 2.3).

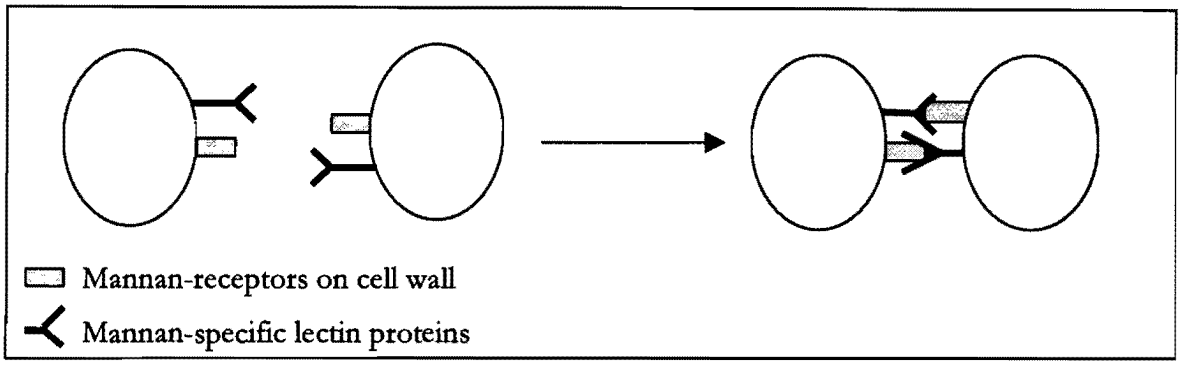


Figure 2.3 Lectin hypothesis for flocculation of yeast. Adapted from Miki *et al.* (1982).

The hypothesis claims that flocculation occurs when surface proteins with lectin properties specifically bind to mannose residues in the walls of neighboring yeast cells (Walker, 1998). Changes in yeast cell surface topography can therefore be related to loss in flocculation ability. Smart *et al.* (1995) report that physiological stresses such as starvation may affect surface properties of yeast. Smart *et al.* (1995) found that starved yeast cells were less flocculent in beer than those that were not starved. Robinson and Harrison (2001) observed a change in extent of flocculation of laboratory propagated yeast following French Press treatment. Damage to the cell surface was influenced by the magnitude of shear force and the growth conditions of the yeast (Figure 2.4).

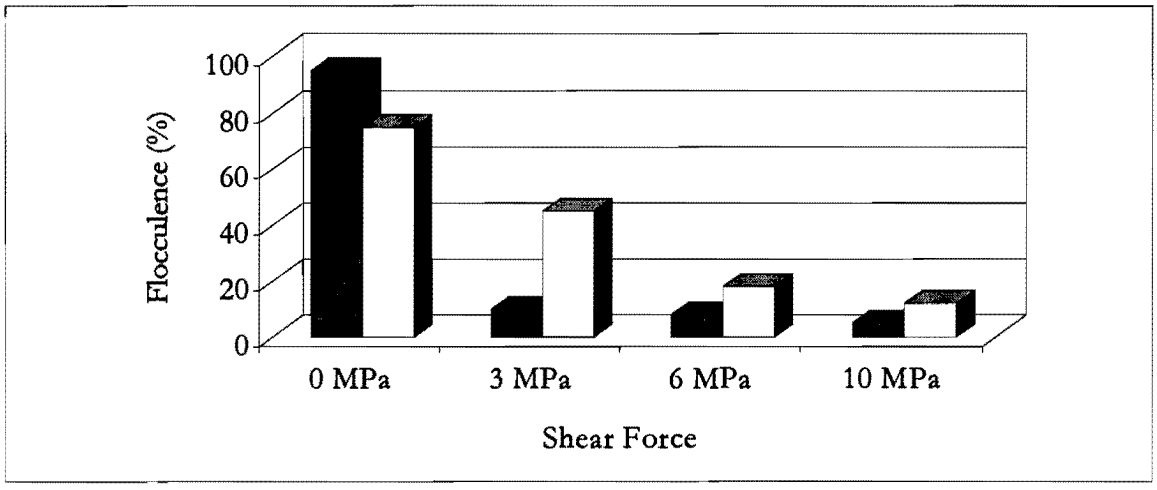


Figure 2.4 Flocculation of laboratory propagated yeast following French Press treatment. (■ Aerobic propagation □ anaerobic propagation). Adapted from Robinson and Harrison (2001).

Aerobically propagated yeasts were more flocculent than the anaerobic equivalent (0 MPa value in Figure 2.4). However, on subjection to sublytic shear forces in the French Press, the aerobic yeast lost its flocculence more rapidly than the anaerobic yeast. Robinson and Harrison (2001) report that surface components most likely affected by shear forces are phosphomannan and lectin proteins, both required for flocculation.

2.4.4.3 REPLICATIVE DEACTIVATION AND CELL DEATH

Replicatively deactivated cells are unable to reproduce. Jones (1987) reports that cell reproduction is dependent on the presence of a functional cell membrane. While this condition is a precursor to cell death (loss of viability), it may be reversible permitting the cells to return to a replicating state after a period of adaptation. It is not clear to what extent the cell membrane needs to be damaged for replicative deactivation to occur (Basson, 1996).

2.5 EFFECT OF MECHANICAL HANDLING ON YEAST QUALITY

Structural and functional deterioration of yeast may occur in the yeast handling circuit. The origin of the degeneration is most likely to be found by examining the stresses encountered by yeast during mechanical handling. The potential sources of these stresses will now be examined and their consequences on yeast quality discussed.

2.5.1 HYDRODYNAMIC SHEAR

Pipe fittings, constrictions and expansions as well as other pieces of process equipment (pumps, heat exchangers, agitated vessels and centrifuges) which cause changes in the flow pattern of yeast slurries generate hydrodynamic shear. Thomas (1993) reports that 'shear' refers to the action of a force on, and parallel to, the interface between a cell and its fluid environment. Here, it may cause the rearrangement of or loss of surface molecules from the cell wall. In addition to these surface effects, the effect of shear could be propagated through the cell wall and affect the cell membrane. At this level, the energy dissipated may result in a loss of membrane integrity (Basson, 1996). Further, a loss of cell viability and vitality may result. Indeed, Engler (1981) states that cell disruption equipment such as high-pressure homogenisers, beadmills and sonication devices use severe shear forces to rupture cells. The degree of disruption achieved is dependent on the physiological state of the cell, cell growth

phase and magnitude of the force applied. Figure 2.5 shows a proposed scheme for loss of yeast quality due to hydrodynamic shear stress (Robinson, 2001).

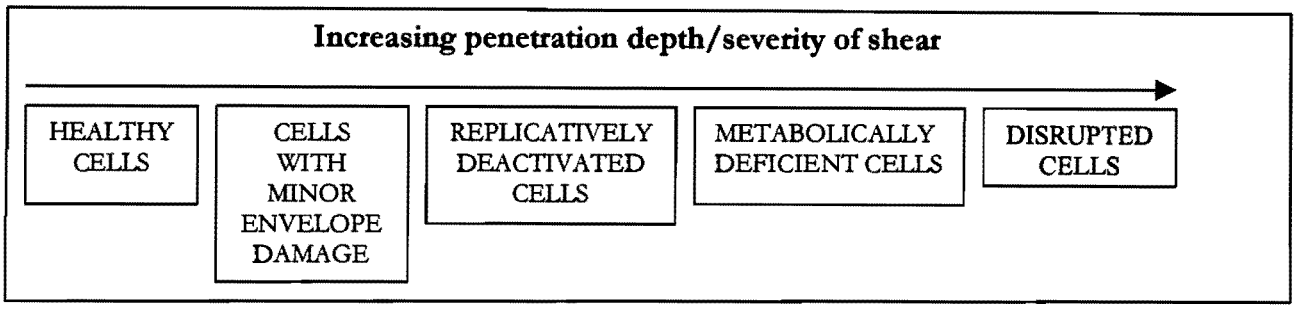


Figure 2.5 Loss of yeast quality due to hydrodynamic shear. Adapted from Robinson (2001).

Schur (1990) conducted pilot scale studies to determine the effects of shear forces during cropping and pitching on yeast and beer quality. Yeast slurries were throttled through a centrifugal pump and the filterability, foam stability and concentration of intracellular compounds in the suspension were measured.

With freshly cropped yeast in young beer, Schur (1990) observed no effects. This suggests that shear forces were unable to cause cell disruption or damage the cell wall. At low and high biomass concentrations (0.2×10^6 cells/ml beer and 10^7 cells/ml beer respectively), stored yeast suspended in mature beer showed a decrease in filterability but there was no change in beer quality. This decrease in filterability was more significant at the higher concentration, indicating the increased presence of haze material and therefore wall damaged cells. Within large-scale breweries, cell concentrations range from 25×10^6 to 500×10^6 cells/ml beer during pitching to 1000×10^6 cells/ml beer during cropping. This apparent dependence of yeast damage on biomass concentration permits postulation that cell wall damage may occur in high shear environments in the brewery.

Lewis and Poerwantaro (1991) subjected brewers' yeast to mechanical agitation and observed the release of a haze-forming colloidal material. Upon analysis, the presence of mannan, protein and cell wall enzymes was demonstrated. It was also observed that cells in the stationary phase are more susceptible to cell wall damage. The storage of yeast with agitation

is frequently used as a holding phase between cropping and subsequent batching of yeast in the brewery. Cropped yeast (stationary phase) is constantly stirred to maintain slurry homogeneity in terms of consistency and temperature (McCaig and Bendiak, 1985). This agitation causes cell-cell and cell-obstacle interactions which may lead to cell wall damage. Cell wall damage results in increased concentrations of haze material in the beer and loss of yeast quality.

In a study to evaluate the effects of mechanical handling on yeast, Basson (1996) reports that the transfer of yeast from the base of the fermentation vessel to the heat exchanger outlet in routine brewery operations did not affect yeast quality (indicated by membrane integrity, vitality and viability analyses). Flow of yeast slurries through several pump types (peristaltic, lobe, sine, gear and centrifugal pumps), bends and valves in the brewery, over a range of flow rates from 17 to 110 L/min, also had no effect on yeast quality. Robinson (2001) investigated the effects of hydrodynamic shear stress during centrifugation of 'green' or immature beer and on flow of yeast slurries through the cropping circuit. Centrifuges aid the partial removal of suspended yeast at the end of primary fermentation, before lagering begins. The hydrodynamic shear generated within the brewery disk stack centrifuge caused:

- Release of cell wall polymers resulting in haze formation.
- Release of cell surface polymers resulting in altered cell surface properties (hydrophobocity and flocculation).
- Loss of membrane integrity and viability.
- Release of protease through permeabilised membrane.

On-line flow trials conducted by Robinson (2001) confirmed no significant effect on yeast quality (measured as changes in viability, vitality, and flocculence), although there was increased protease release along the handling circuit and a decrease in surface charge after flow through the heat exchanger. In these experiments, higher flow rates (61, 163 and 172 L min^{-1}) than typically used in the brewery (48 L min^{-1}) were employed to generate higher shear rates. Based on these results and laboratory French Press analysis, Robinson (2001) postulated that the extent of cell damage due to hydrodynamic stress is determined by the magnitude of shear stress and exposure time to the stress. To minimize cell damage in

the brewery, Robinson (2001) recommends either operating at flow rates corresponding to low shear rates in the laminar flow regime (below 61 L min^{-1}) or under complete turbulence to ensure a reduced exposure time of yeast to wall shear stress.

2.5.2 HYDRODYNAMIC CAVITATION

Vapour cavities form in a fluid when a significant reduction in pressure causes the fluid vapor pressure to be approached. The cavities oscillate through collapse and rebound cycles until they are destroyed by pressure recovery. This occurrence is known as cavitation and is cited frequently for the damage it causes to pumps and pipe work (Coulson and Richardson, 1990). Pressure fluctuations of the order 1000 MPa are associated with cavitation and may be sufficient to compromise cell integrity. In the yeast handling circuit, local increase in linear velocity and decreases in pressure may induce hydrodynamic cavitation. This may occur during pumping or upon flow through restrictions such as bends, valves and pumps.

In experiments conducted by Harrison and Pandit (1992) on stationary phase *Alcaligenes eutrophus* and *Saccharomyces cerevisiae* (Bakers yeast), hydrodynamic cavitation generated on flow through a constriction resulted in cell disruption. Operating parameters influenced the disruption attained. Increasing the pressure drop across and number of passes through the constriction increased extent of disruption. An increase in biomass concentration from 19 to 140 kg/m^3 decreased the extent of disruption. The authors suggest that the higher viscosity and shear-thinning behavior of the suspension at high cell concentrations reduced cavitation due to the damping of energy fluctuations. Hence, there is a decrease in cell disruption. It should be noted however that the assessment of yeast damage was limited to cell disruption. Save *et al.* (1994) report that growing cells, or the cells which are in the exponential phase cell cycle, are more susceptible to disruption by cavitation than cells which are already in the stationary phase.

In a brewery, the dry biomass concentration of yeast on cropping is approximately 160 kg/m^3 and is expected to be about 80 kg/m^3 when re-pitching. Furthermore, cropped yeast is in the stationary phase. Basson (1996) postulates that cell disruption due to hydrodynamic cavitation is not expected under these conditions. Save *et al.* (1994) however report that temperature has a coupled effect on cell breakage. On decreasing the

temperature, the severity of the cavitation increases due to a more violent collapse of the vapour cavity as the vapour pressure is reduced at low temperature. On the contrary, an increase in the temperature weakens the cell wall and the cell becomes more susceptible to breakage, but the cavitation damage is reduced because of the increased vapour pressure of water. Hence, cell damage due to hydrodynamic cavitation may occur on passage through the brewery heat exchanger.

2.6 CHAPTER SUMMARY

The brewing process involves wort preparation, yeast inoculation, and fermentation of wort by yeast. Fermentation performance of yeast largely depends on the structural and metabolic state of yeast (Oliver, 1991). Avis (1990) reports that yeast quality can be described in terms of viability, vitality, stability, purity and integrity. After cropping, yeast is pumped, cooled and stored as inoculum for a subsequent fermentation. As a result, yeast is exposed to a variety of mechanical equipment including positive displacement pumps, pipes, valves, bends, fittings and agitators, which may affect the structural and functional state of yeast.

Work conducted by Basson (1996) indicates that pump design and operation under brewery conditions does not affect yeast quality. Flow of yeast slurries through bends, valves and constrictions in the handling circuit also had no significant effects on fermentation performance. However, exposure to more extreme hydrodynamic conditions generated in the centrifuge and French Press led to cell wall and membrane damage. Further study by Robinson (2001) identified the heat exchanger as a critical unit in maintaining yeast quality. Save *et al.* (1994) report that cell breakage due to hydrodynamic cavitation increases at low temperature. Furthermore, Fargher and Smith (1995) report that low temperatures weaken the cell membrane, making the cell more susceptible to damage. Hence, a direct study on the effect of low temperature stress on brewers yeast quality is required.

CHAPTER

3

POTENTIAL FOR LOSS OF YEAST QUALITY DURING COOLING

This chapter is introduced by discussing yeast cooling systems used by brewers. Thereafter, a review of the effects of cooling, cooling rates and osmotic stress during dilution on yeast viability, vitality and integrity is presented. Factors affecting yeast tolerance to low temperatures are also discussed and further work required is detailed.

3.1 INTRODUCTION

To maintain yeast quality at the end of fermentation, it is common practice to cool cropped yeast from approximately 14 °C to a storage temperature approaching 0 °C in order to reduce yeast metabolic activity. However, the cooling processes may have an adverse effect on quality. Pitching poor quality yeast leads to slow or incomplete fermentation and the production of beer with uncharacteristic flavors. Experimental evidence has shown that exposure of yeast to low positive temperatures may have the following effects on cell physiology (Walker, 1998a):

- Cell membrane fatty acids may undergo a phase transition, compromising cell membrane integrity.
- Sterol synthesis is reduced, weakening membranes and thus increasing potential for cell disruption.

- Yeast cells shrink uniformly, inhibiting budding.
- Vacuolar membrane damage may occur leading to changes in metabolic activity.

The most common method of cooling yeast slurries during the handling process is by flow through a plate and frame heat exchanger. The operating conditions utilized are based on tradition and experience of brewers rather than rigorous scientific study (Table 3.1). The paraflow plate heat exchanger supplied by APV can, for example, achieve cooling rates in excess of 2 ^{0}C/sec. It has been postulated that this rapid decrease in temperature may cause the yeast to become more susceptible to mechanical and physiological damage.

Following the trend set by modern breweries in Europe and America (Witttl and Maier, 1980), an alternative process for cooling yeast has been proposed (Figure 3.1). In this process, cropped yeast is diluted into 12 ^{0}C deaerated, non-carbonated water. The water (40 % of the final estimated volume) is added to the YCV before cropping commences. The ensuing mixture is agitated to ensure homogeneity. Yeast required for pitching is batched to a YPV in which it is cooled via cooling jackets to 2-4 ^{0}C at approximately 2 ^{0}C/hr. Dilution of yeast slurries in water has the added advantage of lowering ethanol stress on cropped yeast as well as reducing suspension viscosity.

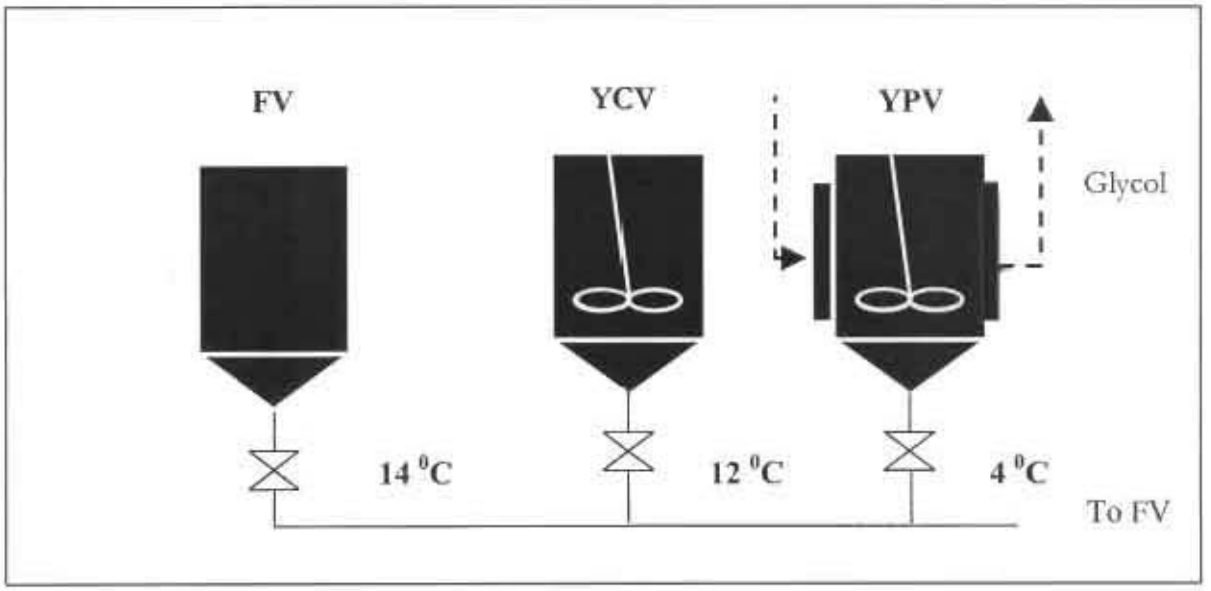


Figure 3.1 Diagram of proposed yeast cooling system.

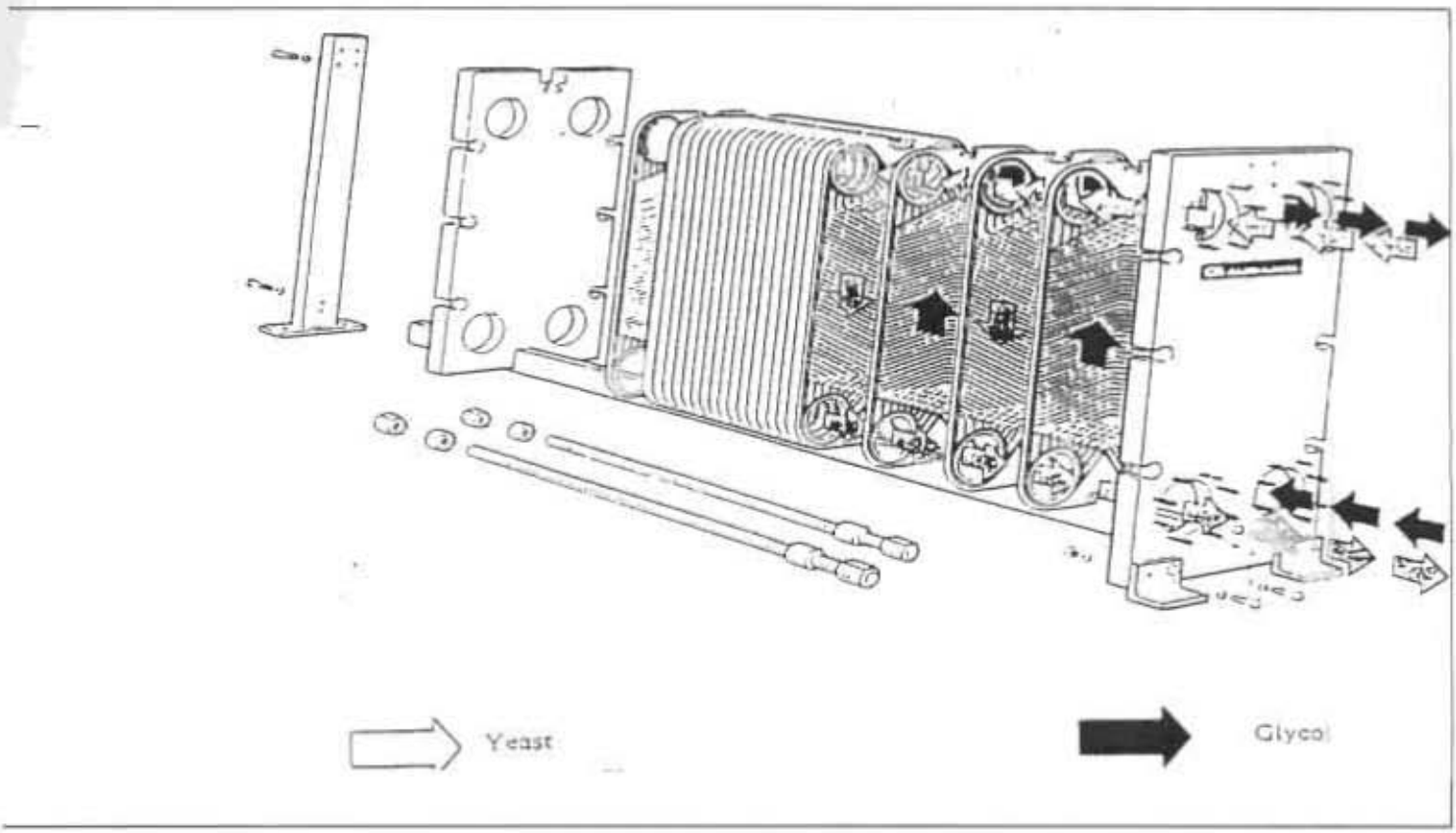


Figure 3.2 Schematic of a paraflow heat exchanger. Adapted from Coulson and Richardson (1990).

Table 3.1 Paraflow heat exchanger specifications typical for yeast handling circuits.

Type	Paraflow plate heat exchanger
Manufacturer	APV
Material of construction	316 stainless steel
Series	SR 20
Serial number	SA 1076
Number of plates	127
Space between plates (mm)	2.51
Heat exchange area (m^2)	21.6
Inlet diameter (mm)	80
Coolant	Glycol
Temp. Glycol in (^{0}C)	-3
Temp. Glycol out (^{0}C)	14
Glycol flowrate (kg/hr)	6300
Substance being cooled	Yeast
Temp. yeast in (^{0}C)	15
Temp. yeast out (^{0}C)	2.8
Yeast flowrate (m/s)	0.1-0.3
Range of yeast flowrates used(hl/hr)	33-35
Yeast density (g/cm^3)	1.09

3.2 LOSS OF YEAST QUALITY DURING COOLING

The term cold shock is used to describe damage often seen on cooling micro-organisms (Williams, 1990). Sensitivity of cells to cold shock can be both lethal (changes in viability and integrity) and sub-lethal (changes in vitality). Lethal injury results from the loss of proteins, ATP and other cellular components through leaks in the cell (plasma) membrane. Sub-lethal injury results in physiological changes such as inhibition of bud development and vacuolar rearrangement (Fargher and Smith, 1995).

3.2.1 LETHAL INJURY: LOSS OF MEMBRANE INTEGRITY

The structural organization of plasma membranes is largely determined by weak interactions in the form of Van der Waals forces and hydrogen bonds, existing between membrane components and between these components and their aqueous environment. The stability of these electrostatic interactions is particularly sensitive to temperature and hydration (Williams, 1990). Russsel (1990) reports that exposure to low temperatures (below 5-10 ^{0}C) leads to changes in membrane composition and fluidity, and to the weakening of membrane lipid-integral protein bonds. In this state, membranes are prone to damage. A detailed discussion of the function, composition and structure of the yeast cell membrane is given below to enable cold shock effects to be better understood and predicted in yeast handling.

3.2.1.1 THE CELL MEMBRANE

All cells are surrounded by a membrane which separates the functional cell from its environment. Furthermore, many internal cellular functions are performed within compartments bounded by membranes. Plasma membranes, as well as those enclosing cytoplasmic organelles, are primarily lipid structures with embedded protein (Quinn, 1980). The majority of membranous lipids are phospholipids of which 75-85% are typically phosphoglycerides. The remaining 15-25 % of the phospholipids are mostly phosphatidylethanolamines (Williams, 1990). Other lipids associated with cellular membranes include glycolipids and sterols. The hydrophobic character of the plasma membrane is due to the lipid component. Owing to their combined hydrophobic and polar constituents, phospholipids form a noncovalent bi-layer in aqueous solutions, as illustrated in Figure 3.3.

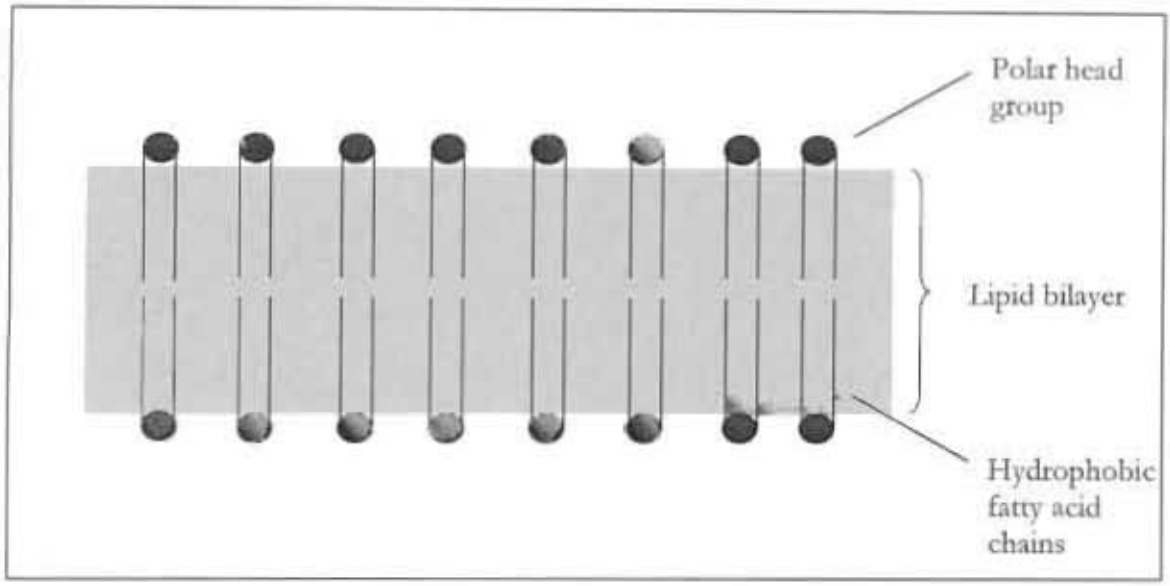


Figure 3.3 Lipid-bilayer of plasma membranes. Adapted from Prescott *et al.* (1996).

The lipid-bilayer is a selectively permeable barrier. It restricts the type of molecules that will diffuse readily from one side of the membrane to the other. Substrates carrying a charge, for example sugars and inorganic ions, will not diffuse at a significant rate because of their attraction to water and exclusion by the hydrophobic portion of the lipid membrane. Proteins, held in the membrane by electrostatic interactions with phosphatidylethanolamines, facilitate the movement of these molecules either by forming diffusion channels or providing an active transport mechanism (Prescott *et al.*, 1996). These proteins are classified on the basis of the energetics used in transporting species (Table 3.2). Further functions of membrane proteins include catalytic activity and ligand binding.

Table 3.2 Classification of membrane protein translocation system (Prescott *et al.*, 1996).

Type of protein	Class	Substance transported
Channel	Voltage regulated	Na^+ ions
Transporter	Passive mediated	Glucose

Stryer (1988) reports that the two sides of the bilayer are different, both in lipid composition and in placement and orientation of proteins (Figure 3.4). As a result of this structural asymmetry, the outer and inner surfaces of all biological membranes have different functions and enzymatic activities. Glycolipids and glycoproteins on the outer surface of the cell

membrane constitute important cell-surface antigens which act as specific receptors for external stimuli (Quinn, 1980). These control the flow of information between the cell and its environment.

3.2.1.2 MEMBRANE FLUIDITY

The arrangement of lipids and proteins in membranes was originally conceived as a static array of metabolically inert molecules (Quinn, 1980). However, with the advent of the electron microscope and improvements in biochemical understanding, membranes are now thought to consist of highly mobile molecules interacting with each other and with those in the surrounding environment. This is referred to as the 'fluid mosaic' model to indicate the movement of both lipids and proteins in the membrane (Figure 3.4). Such movement provides membrane properties such as flexibility and fluidity, which permit cells to change shape and form (Prescott *et al.*, 1996). Furthermore, the fluidity of cell membranes has been correlated with numerous biological processes including growth, membrane transport and enzyme activities.

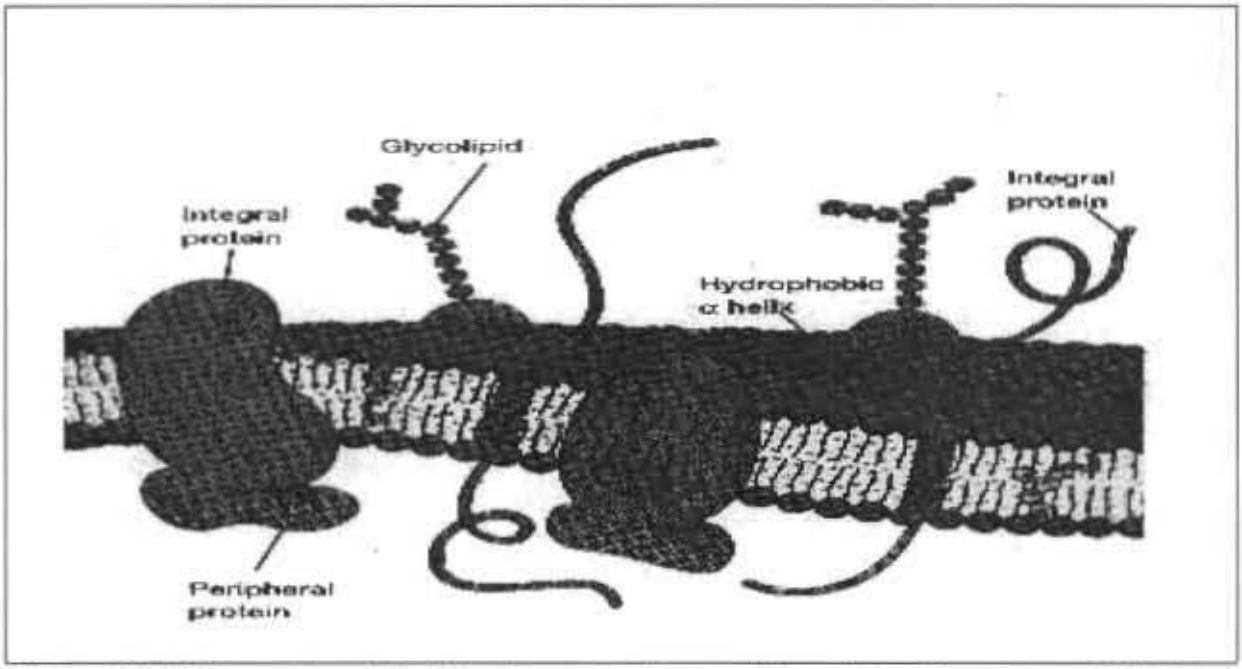


Figure 3.4 Fluid mosaic model of the plasma membranes. Adapted from Prescott *et al.* (1996).

The interactions among different lipids and between lipids and proteins in membranes are complex and dynamic. Physical studies of phospholipid dispersions have shown that lipid molecules readily diffuse in a lateral direction parallel to the plane of the bilayer (Quinn, 1980).

The hydrocarbon chains of the lipids have a motion, which produces fluidity in the hydrophobic core. This central area of the bilayer is occupied by the ends of fatty acid chains and is more fluid than areas closer to the two surfaces, where there are more constraints due to stiffer parts of the hydrocarbon chains. It is assumed that pockets of space between individual chains are filled with water molecules and small ions, contributing to the overall fluidity (Prescott *et al.*, 1996). Thiering *et al.* (1998) demonstrated lateral movement of membrane proteins in the bilayer and found it to be slower than that of lipids, possibly due to restrictions by cellular structural elements such as microtubules. Biological membranes are thus in a constantly changing state, with not only proteins and lipids moving but with molecules passing into and out of the membrane. In yeast, sterols, particularly ergosterol, are a key regulator of membrane fluidity. Sterols contain a bulky steroid nucleus with a hydroxyl group at one end and a flexible hydrocarbon tail at the other end (Figure 3.5). Sterols inserts into the bilayer with their long axis perpendicular to the plane of the membrane. High concentrations of sterols may sterically block large motions of fatty acid chains, making membranes less fluid.

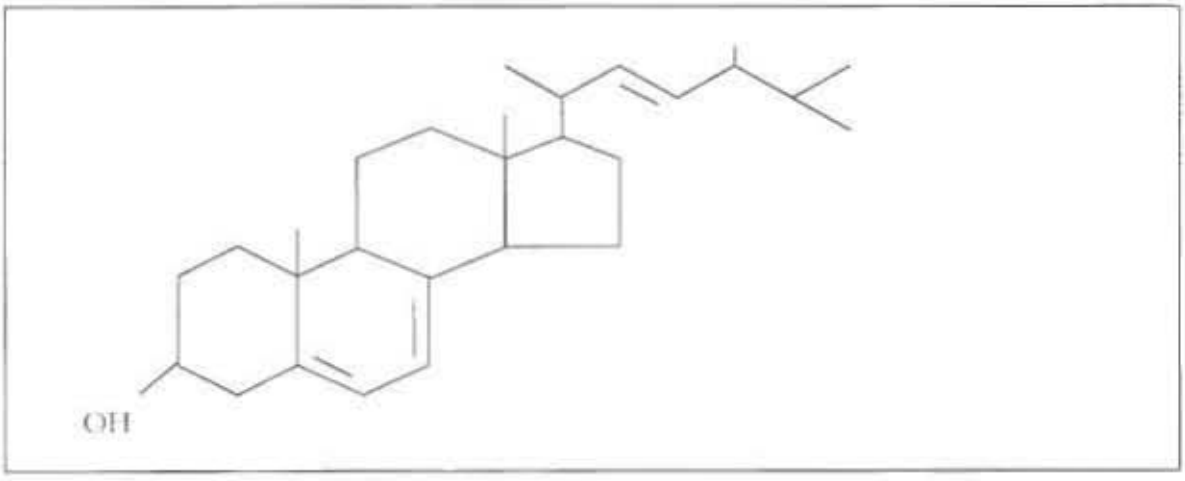


Figure 3.5 Schematic of the compound ergosterol. Adapted from Stryer (1988).

The degree of fluidity of a membrane is dependent on both temperature and composition of the membrane. Farghet and Smith (1995) report that, under all growth conditions, cells attempt to preserve plasma membrane fluidity in order to maintain functionality. Hence, it may be expected that the cell will, for example, tailor its membrane composition to handle changes in temperature.

3.2.1.3 EFFECT OF COOLING ON MEMBRANE FLUIDITY AND COMPOSITION

The requirement of membrane fluidity to maintain functionality with respect to cell growth, reproduction and activity of membrane bound enzymes is reported by Thiering *et al.* (1998), Prescott *et al.* (1996), Fargher and Smith (1995), and Williams (1990). At optimum growth temperature, biological membranes are in a liquid crystalline phase, allowing lateral diffusion of lipids and proteins (Quinn, 1980). The response of the membrane to any change in temperature is a function of its composition.

On exposure to a decrease in temperature, membrane lipids in a liquid crystalline state cool and undergo a transition to a gel phase state (Thiering *et al.*, 1998). The temperature at which this loss of fluidity occurs is called the transition temperature (T_m). T_m for a particular membrane lipid depends on the length of its fatty acid chains, their degree of saturation, and the specific content of water in the membrane (Stryer, 1988). Long saturated hydrocarbon chains have higher transition temperatures than do short ones. Phospholipids with unsaturated fatty acids have greater flexibility than those containing saturated fatty acids. Thus, to maintain some movement within an increasingly viscous membrane, cells alter the level of saturation and shorten the length of phospholipid hydrocarbon chains. Additional methods of preserving fluidity at low temperatures involve the reduced synthesis of sterols and the manufacture of low molecular weight protectants such as trehalose, glycerol and heat shock proteins. Presumably these compounds form hydrogen bonds with proteins and glycolipids in the membrane, preventing membrane fusion and providing a hydrophilic net to reduce water loss from the membrane at low temperature (Sales *et al.*, 2000).

Generally, cells regulate their membrane composition so as to keep T_m below their environmental temperature. Most membrane lipids form a rigid gel phase at temperatures well below 10 °C. Figure 3.6 shows the typical phase behavior of membrane lipid extracts. The different regions in the diagram refer to regions of phase-separation rather than uniform phases and the boundaries indicate the onset of such phase separations rather than the formation of discrete phases. The low temperature (below 5-10 °C) gel-lamella region corresponds to a region in which lipids with high T_m tend to separate out as gel-phase patches. The gel-liquid interfaces formed are susceptible to leakage (Williams, 1990). At temperatures close to and below 0 °C, a phenomenon known as freeze dehydration is observed (Quinn, 1980). In a suspension of cells,

ice tends to form preferentially in the extracellular medium. The osmotic strength of water in the cell membrane and inside the cell imposes a gradient of water potential on the cell. Loss of cellular water in response to this gradient leads to membrane dehydration and cell shrinkage. Subsequent increases in electrolyte concentration weaken electrostatic interactions between lipid groups leaving the cell membrane vulnerable to fracture (Williams, 1990). Walker (1998a) reports that ice formation within the cell may also occur, causing cell lysis due to the expansion of water upon freezing.

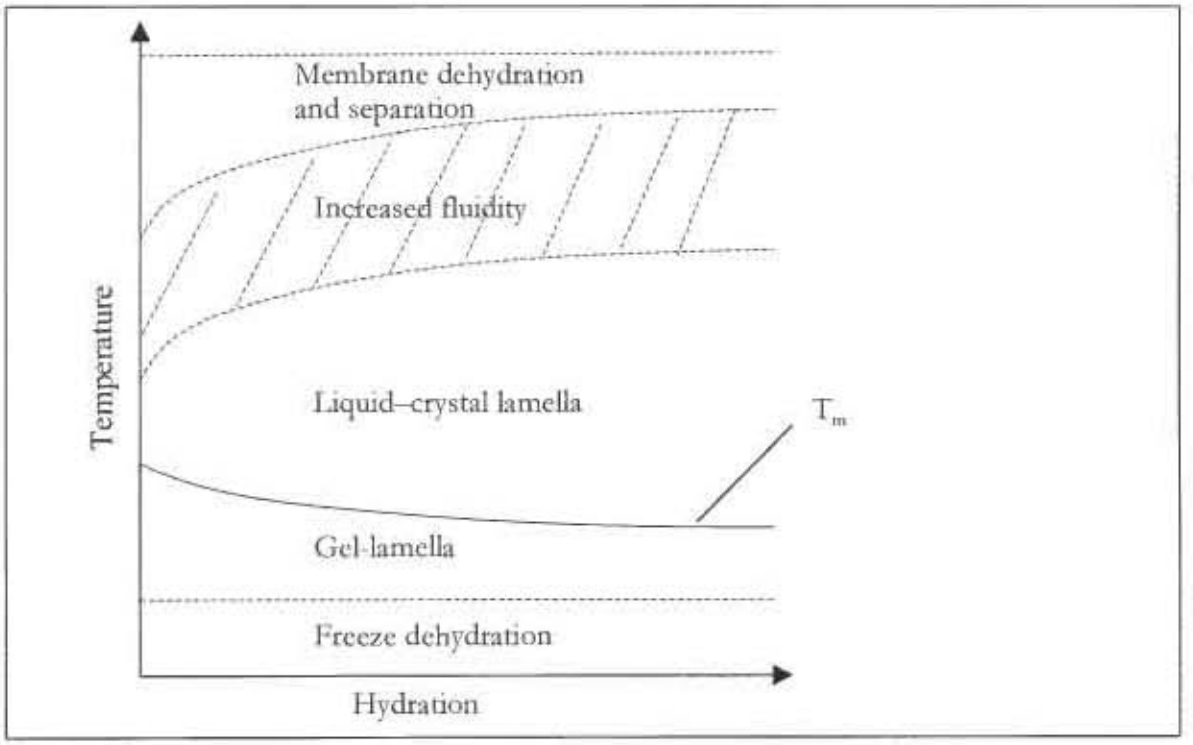


Figure 3.6 Diagram illustrating the types of phase transitions that might be anticipated on subjecting membranes of differing levels of hydration to temperature stress (Williams, 1990).

If a cell is to acclimatize successfully to moderate low temperature stresses, it must have sufficient time to make the necessary changes to its membrane composition. This requires that the rate at which heat is extracted from the cell be such that it allows all necessary membrane and cellular adaptations to take place.

3.2.1.4 EFFECT OF COOLING RATE ON MEMBRANE INTEGRITY

Following a rapid decrease in temperature, plasma membranes lose heat (Fargher and Smith, 1995). If the rate of cooling is greater than the rate at which the cells can modify their membrane composition, then the cell membrane will pass through a temperature zone in which some membrane lipids may undergo a phase transition to gel-crystalline state before others. The membrane is then fixed in a random disorganized form, compromising integrity. Several explanations have been put forward to explain this phase separation of otherwise miscible components. Williams and Quinn (1987) postulate that saturated minor lipid components found in cell membranes have a relatively high gel transition temperature, allowing limited precipitation of bulk lipid components at temperatures well above T_m of most membrane lipids. On rapid cooling, cells simply do not have enough time to unsaturate or shorten these compounds. Williams and Quinn (1987) report that leaks in the cell membrane may also occur due to the inability of phosphadylethanolamines to seal membrane proteins at low temperature causing leakage at protein-lipid interfaces.

3.2.2 SUB-LETHAL INJURY: YEAST VITALITY

Lipid liquid-crystal to gel-phase transitions observed on cooling cells are not unique to the plasma membrane. Williams (1990) observed phase separation in total lipid extracts of chloroplast membranes at low temperature. Thus, it can be postulated that membrane bound cytoplasmic organelles that play important roles in yeast physiology will, to some extent, be affected by cold stress. Stryer (1988) reports that the internal membranes of mitochondria, which are involved in energy transduction, have the highest content of protein (over 75 % by mass) of all biological membranes. These proteins are sealed in the membrane by phosphadylethanolamines. Williams and Quinn (1987) report that protein-lipid interfaces are prone to leaks at low temperature. Such damage may have a detrimental effect on cell metabolism. Work conducted by Fargher and Smith (1995) and Kaul *et al.* (1992) on the effects of cold shock on yeast further highlights cold injury to cellular structures, and its impact on vitality.

Fargher and Smith (1995) subjected ale yeast grown at 25 °C to cold shock by decreasing its temperature to 4 °C at a cooling rate of 8 °C /min. The yeast showed a prolonged lag phase and gave a growth yield 10 % lower than that of control yeast (uncooled) on inoculation into growth media. This apparent loss in vitality is explained by the inhibition of the formation of actin filaments essential for budding and growth by cold shock. This growth arrest may only be temporary as the actin cytoskeleton is reformed within two hours of returning the yeast to suitable conditions (Fargher and Smith, 1995). The extended lag phase corresponds to this period of recovery. Exponential phase yeast is more prone to this type of injury than yeast in the stationary phase. The ability of yeast to grow and reproduce is dependent on the presence of a functional cell membrane (Lentini, 1993). Irreversible alterations of the functional properties of the cell membrane during cooling may have caused the lowered biomass yields.

In work conducted by Kaul *et al.* (1992) and Fargher and Smith (1995), exposure of yeast to conditions causing a cold shock response resulted in vacuolar rearrangement. In yeast, the vacuole is a key organelle involved in the intracellular trafficking of proteins. It is primarily responsible for non-specific proteolysis and contains a wide variety of proteases. Vacuoles also act as storage compartments for basic amino acids and cations, and are involved in regulating cellular pH (Walker, 1998a). Vacuoles have a dynamic structure and are bounded by a single membrane called a tonoplast. This membrane has a different phospholipid, unsaturated fatty acid and sterol content compared to plasma membranes. Changes in vacuole structure may reflect changes in intracellular transport or osmology of the cell, both of which affect metabolism. Kaul *et al.* (1992) viewed vacuolar damage in more than 85 % of a yeast population that received cold shock (direct immersion in liquid nitrogen). The researchers proposed that phase separations associated with plasma membranes of rapidly cooled yeasts may occur in vacuolar tonoplasts. This scenario may cause vacuoles to split into a series of small compartments. Regions of the tonoplast in which phase transitions take place may be liable to leaks. Release of proteases from vacuoles into the cytoplasm may result in the destruction of important enzymes, varying metabolic pathways, and in extreme cases, autolysis (Kaul *et al.*, 1992).

3.2.3 FACTORS INFLUENCING COLD INJURY IN YEAST

From experiments conducted by Fargher and Smith (1995), it is evident that the severity of injury to yeast cells is dependent upon stage of growth and culture conditions prior to cold shock treatment (Figure 3.7). Fargher and Smith (1995) cooled lager yeast at various growth phases from 20 °C to 4 °C at a cooling rate of 8 °C/min. Loss of viability was more prominent in budding and decline phase cells (3 % and 6 % decrease in viability respectively) than in stationary phase cells. Fargher and Smith (1995) also claim that the extent of cold injury to yeast is dependent upon growth temperature, cooling rate and the temperature ranges over which cooling is applied. However, no data is given to support this claim.

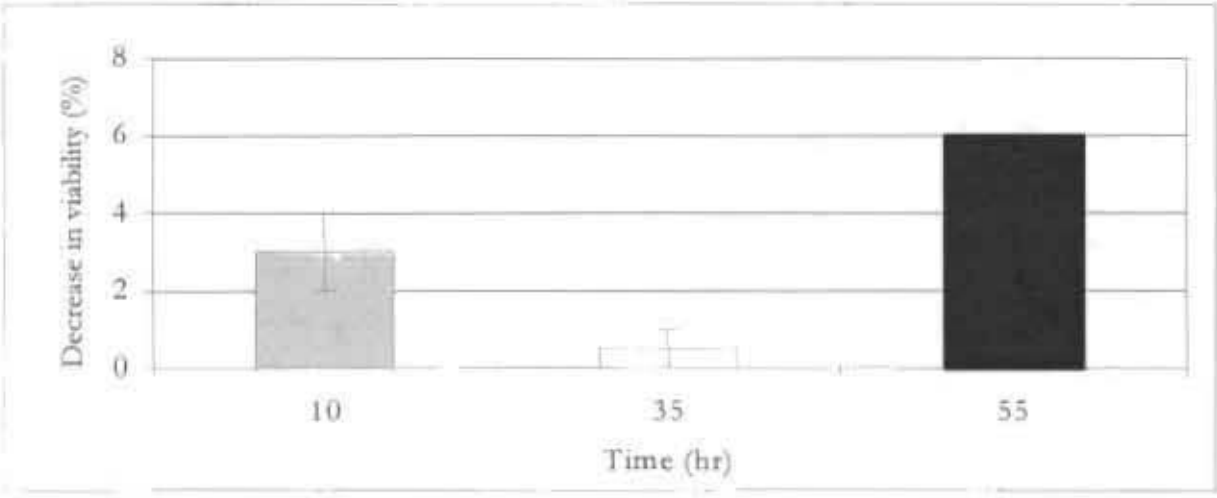


Figure 3.7 Loss in viability of lager yeast grown in a shake flask culture, sampled and cold-shocked at times indicated (□ exponential, □ stationary and■ decline phase). Fargher and Smith (1995).

Komatsu *et al.* (1990) report that damage to yeast subjected to low temperature stress is affected by the level of cryoprotectants present in the cell. Cryoprotectants are a diverse group of compounds which help in preventing cell damage under various conditions of cold stress (Lewis *et al.*, 1994). The role of heat shock proteins, ethanol, and trehalose in the ability of yeast to survive near-freezing temperatures is discussed below.

3.2.3.1 COLD INJURY PROTECTION PROVIDED BY HEAT SHOCK PROTEINS

Obuchi *et al.* (1991) observed that the viability of *S. cerevisiae* decreased by 99 % when rapidly cooled to sub-zero temperatures in liquid nitrogen. However, a prior heat shock (two hours at

43 °C) increased cell survival 20 to 30 fold. They correlated the increased survival to the increased inducibility of heat shock proteins (hsp) in the cell. Differential scanning calorimetric analysis of the cells revealed that the presence of heat shock proteins (hsp 90, 85, 70) decreased the denaturation of total cellular protein. Komatsu *et al.* (1990) also observed that prior heat treatment of yeast significantly improved cell viability during subsequent rapid cooling. After exposure to 43 °C heat shock (normal growth temperature being 30 °C), yeast cells (*S. cerevisiae*) were frozen by plunging them directly into liquid nitrogen. Cells frozen without exposure to prior heat shock showed a loss of viability on freezing approaching 100 %. Heat exposure prior to freezing significantly increased the cell viability to 12 %. Komatsu *et al.* (1990) associate this increase in cell viability with the higher initial hsp content of heat shocked cells (hsp 70 and 90). It is postulated that these hsp act by strengthening the bonds within macromolecules thus reducing denaturation, and by increasing the hydrophobic interactions within the cells, preventing ice crystal formation, freeze dehydration and membrane fusion. Time course studies by Obuchi *et al.* (1991) indicate that hsp are induced within 10 minutes of heat shock and attain their maximum amount within two hours of treatment.

3.2.3.2 REDUCTION OF COLD SHOCK SENSITIVITY OF YEAST BY ETHANOL

Lewis *et al.* (1994) studied the cryoprotective effects of ethanol on brewing strains of *S. cerevisiae* under various freezing conditions. They observed that under conditions of slow cooling (cooling rate 3 °C/min), ethanol (0.1 to 2 M) acted as a cryosensitizer, severely decreasing the viable cell count compared to control cells (yeast cooled at 3 °C/min in growth media). However, under conditions of rapid freezing (cooling rates above 25 °C/min), ethanol greatly improved cryotolerance of the yeast. At a cooling rate of 200 °C/min, yeast population submerged in 2 M (92 g/L) ethanol showed 61 % survival compared with 1 % survival of yeast in growth media. Lewis *et al.* (1994) suggest that the mechanism of protection may be associated with the increased permeability that ethanol is able to induce in the plasma membrane, leading to the rapid efflux of water during freezing, and therefore prevention of intracellular ice crystal formation. The ethanol concentration of cropped yeast from a typical lager fermentation ranges from approximately 1.1 M (52 g/L) to 1.4 M (67 g/L) (Lodolo, 1995). Brewery heat exchangers are operated at cooling rates in excess of 25 °C/min. Hence, during rapid cooling of yeast in the brewery, ethanol is postulated to have a protective effect.

3.2.3.3 COLD INJURY PROTECTION PROVIDED BY TREHALOSE

Survival of yeast at low temperatures has been related to trehalose content (Walker, 1998a). Trehalose is known to protect yeast membranes and decrease cold shock sensitivity of yeast. Yeasts with substantial levels of trehalose have an added advantage at low temperatures. Walker (1998a) reports that bakers' yeast with a trehalose concentration of 4-5 weight % was protected from injury on rapid cooling and was successfully used in the production of frozen bread dough. The synthesis of trehalose is enhanced by exposing yeast to temperatures slightly greater than optimum for growth (Iwahashi, 1995). Sales *et al.* (2000) report that trehalose aids in maintaining membrane integrity upon changes in lipid bi-layer fluidity. In experiments to determine the effects of trehalose on maintaining integrity of desiccated membranes during rehydration, it was found that in the presence of trehalose, up to 70 % of liposomal membrane integrity was maintained. In the absence of trehalose, rehydration led to total loss of membrane integrity. As with hsp, trehalose is thought to prevent fusion between adjacent membrane phospholipids due to osmotic or cold stress induced membrane desiccation (Figure 3.8). This results in improved fluidity, therefore stabilizing the bilayer.

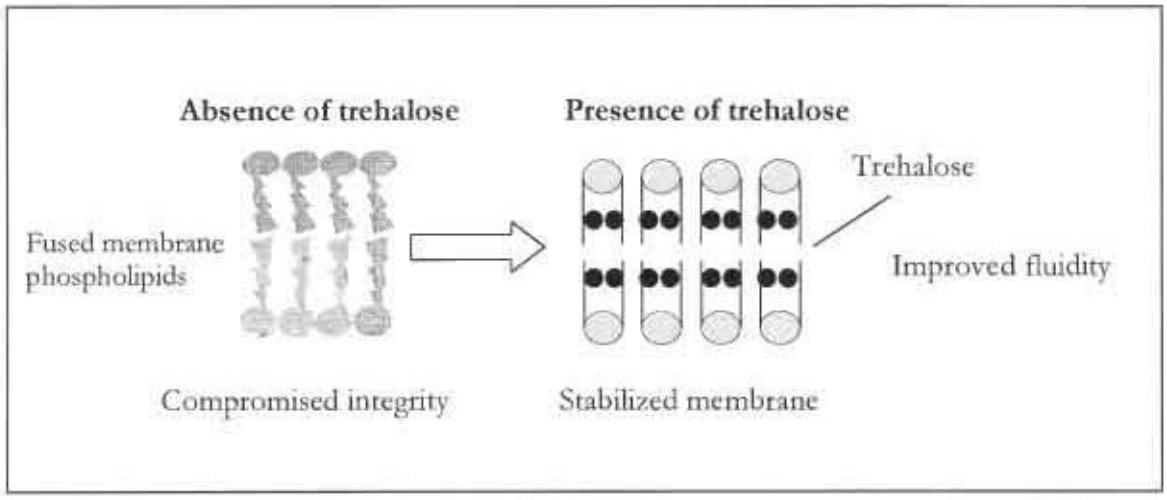


Figure 3.8 Effect of trehalose on membrane fluidity at low temperature. Based on Sales *et al* (2000).

3.3 EFFECT OF OSMOTIC STRESS ON YEAST

Ethanol is a major metabolic product of fermentation. Its accumulation inside and outside the cell during the brewing process creates a potent chemical stress on yeast. Hallsworth and Nomura (1998) report that ethanol reduces the water potential of aqueous phases. The term water potential (ψ_w, expressed in MPa) refers to the potential energy of water and is used to quantitate the availability of water in the presence of dissolved solutes. Pure water has a ψ_w of zero, while impure water has a lower or negative value. Yeasts able to withstand conditions of low water potential are referred to as osmotolerant. Walker (1998a) reports that the yeast *S. cerevisiae* is non-osmotolerant. Figure 3.9 shows the cardinal water potential range of growth for *S. cerevisiae*.

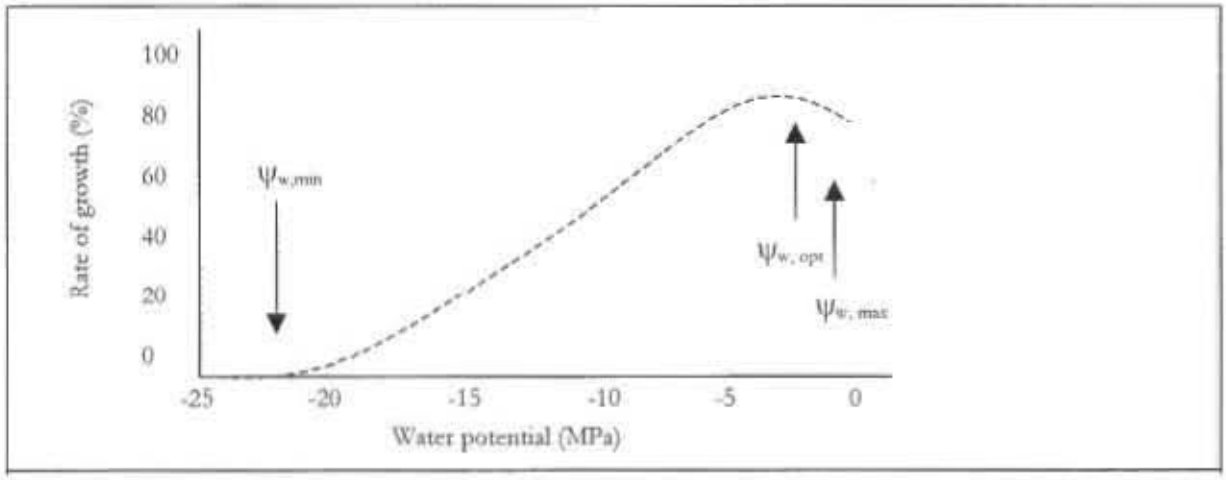


Figure 3.9 Generalized graph of the relative rate of yeast growth in relation to ψ_w of growth medium. Adapted from Walker (1998a).

In a study to investigate the influence of thermal and osmotic stress on the viability of *S. cerevisiae*, Beney *et al.* (2000) found that survival of yeast at low water potential was dependent on both the temperature and intensity of osmotic stress. They observed that temperatures equal to or below 11 °C allowed the preservation of viability after an osmotic stress whereas temperatures above 11 °C did not preserve yeast survival (Figure 3.10). Beney and co workers (2000) also showed that the extent of damage to yeast due to osmotic shock was determined by the rate of change of water potential. Yeast cell viability (> 90 %) was maintained at low water potential when the extracellular osmotic pressure was decreased slowly (by addition of glycerol) from -1.38 MPa to -100 MPa at -1.6 MPa /min. On the other hand, cell viability decreased

significantly (to <30 %) when supernatant–free yeast cells were resuspended (-600 MPa/ min) in a hyperosmotic glycerol solution (–100 MPa).

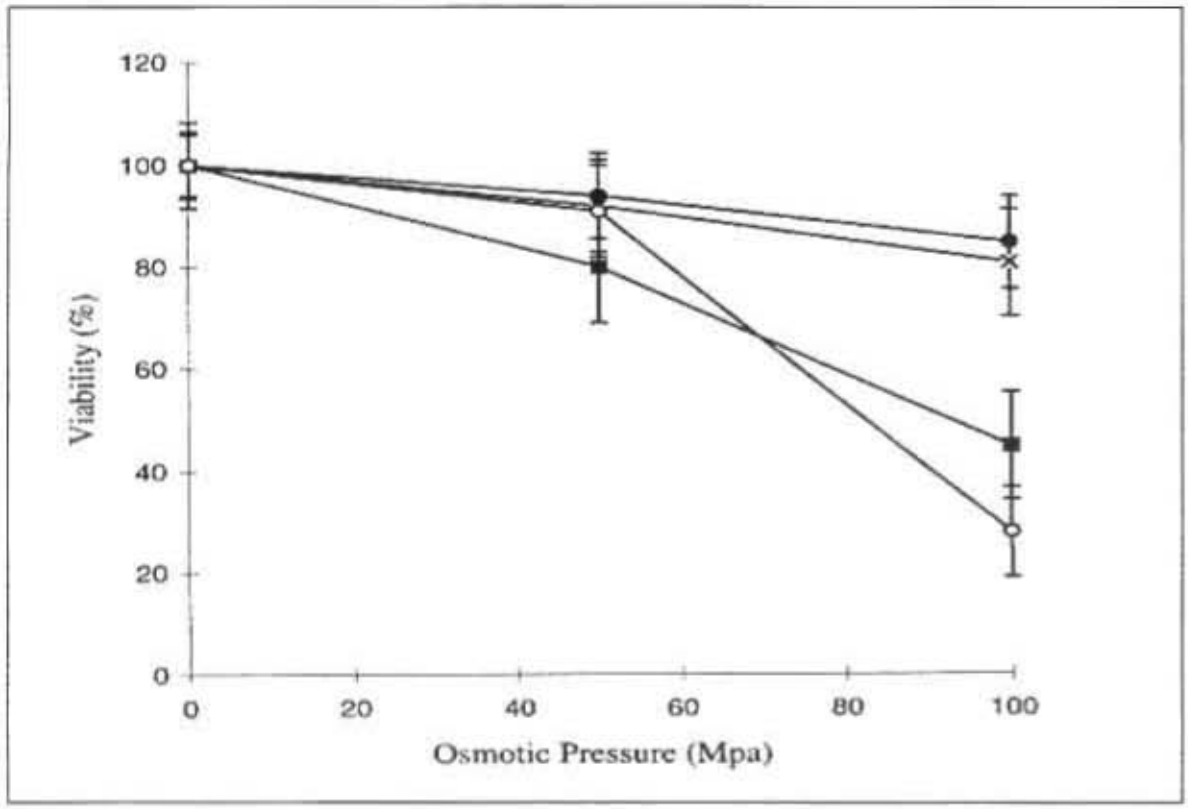


Figure 3.10 Effect of hyperosmotic stress on viability of *S. cerevisiae*. Hyperosmotic shock at ○ 22 °C, ■ 15 °C, ● 11°C and x 5 °C. Adapted from Beney *et al.* 2000.

The ethanol rich environment (1.1 M to 1.4 M) of flocculated yeast (14 °C) at the end of fermentation has an osmotic pressure ranging from approximately -2.7 to -3 MPa (Sienko and Plane, 1974). In experiments investigating the use of magnesium as a stress protectant for industrial strains of *S. cerevisiae*, Walker (1998b) observed that exposure of yeast to ethanol concentrations exceeding 1 M at 30 °C (an osmotic pressure of -2.2 MPa) led to a 12 % decrease in cell viability. Walker (1998b) postulates that ethanol alters the fatty acid and sterol composition of the cell membrane, inducing lypolysis of phospholipids (leading to loss of membrane integrity) and inhibiting intracellular proteins and glycolitic enzymes. Diluting the yeast in water (see Section 3.1) would therefore reduce extracellular ethanol concentration, alleviating the chemical stress. However, the transfer of cells from a medium with low ψ_w to one with higher osmotic potential may induce hypoosmotic shock in the cells.

In a higher osmotic potential medium, the osmotically dehydrated cells quickly increase in volume due to high water permeability of the plasma membrane. The cells increase their internal osmotic potential by the efflux of ethanol, glycerol and K^+ ions from the cell through specific protein channels in the cell membrane (Stanley and Pamment, 1993). Hohmann and Mager (1997) term this expulsion of solutes as 'shock excretion'. The response of yeast to hypoosmotic shock is thus highly dependent on a functional cell membrane. Damage to the membrane due to handling may affect the operation of channels that transport water, anions and cations (Prescott *et al.*, 1996). Inability of cells to respond to changes in osmolarity may lead to cell death and complete cell disruption (Stryer, 1988). Furthermore, the loss of glycerol (cryoprotectant) from cells under hypoosmotic stress may have a detrimental effect on the ability of the cell membrane to withstand subsequent low temperature stress.

3.4 CHAPTER SUMMARY

Exposure of yeast to a rapid decrease in temperature may result in a loss of cell viability, vitality and integrity. The extent of cold injury to yeast is governed by:

i) yeast cell condition,
ii) cell growth phase,
iii) growth temperature,
iv) temperature range over which cooling is performed,
v) cooling rates employed, and
vi) level of low molecular weight protectants in the yeast.

Thiering *et al.* (1998), Prescott *et al.* (1996), Fargher and Smith (1995), and Williams (1990) report that membrane fluidity is required to maintain functionality with respect to cell growth, reproduction and activity of membrane bound enzymes. On exposure to low temperatures, membrane lipids undergo a transition to a gel phase state. The temperature and extent to which this occurs is dependent on the composition of the cell membrane, rate of cooling and the presence of low molecular weight protectants such as hsp and trehalose. Membranes in which both gel and liquid states exist are prone to fracture. Pitching such yeast may decrease fermentation performance and quality of beer produced.

The most common method of cooling yeast slurries in the brewery is by flow through a plate and frame heat exchanger. However, an alternative technique has been proposed. This involves diluting cropped yeast in water and slowly cooling the resultant slurry in a jacketed agitated vessel. In order to gain a clearer understanding of the effects of cold shock on yeast, work should be carried out to assess the potential for a loss of yeast quality as a result of flow through a heat exchanger. Here both the rate of cooling as well as the subsequent or concomitant ability of the yeast to withstand mechanical handling is of importance. Critical cooling rates above which cold shock injury will affect brewers' yeast performance should also be quantified. An evaluation of the effects of dilution, agitation as well as final cooling temperature on cooling diluted yeast slurries in a jacketed agitated vessel should also be carried out.

CHAPTER

4

IDENTIFICATION AND CHARACTERISATION OF A LOSS IN YEAST QUALITY

In this chapter, the assays selected to assess yeast quality are reviewed. The review includes a discussion of the literature on each method and the rationale for the choice of the method. The relationship between yeast physiological condition and small-scale fermentation performance is also investigated. Detailed descriptions of the analytical procedures performed and their detection limits are given in Appendix A.

4.1 INTRODUCTION

This project aims to establish the effect of cold stress on the quality and subsequent fermentation performance of pitching yeast. For this study, several assays have been selected to assess yeast quality. It is imperative that these assays are sensitive to differences in yeast quality brought about by yeast handling, that the mechanism of the effect is identified, and that the results of the assays can be used to predict fermentation performance accurately. To characterize a loss of yeast quality, knowledge of the nature of cell damage is required. Cell death and the inability of cells to grow and reproduce constitute a loss of "viability". Detrimental changes in the physiological condition of yeast are said to describe a loss in "vitality", and injury to the cell envelope resulting in the release of cell material into beer illustrates a loss in "integrity".

4.2 POSSIBLE ROUTES LEADING TO THE LOSS OF YEAST QUALITY

Work conducted by Basson (1996) on the effects of mechanical handling on yeast quality highlighted the need for a precise method to describe changes in cell condition in response to stress. The terms 'viability' and 'vitality' commonly used in the brewing industry were found too broad to accurately characterize yeast quality. Basson (1996) proposed a scheme for the progression of yeast between various physiological states (Figure 4.1). Cells in each of these physiological states can be identified by appropriate yeast quality assays. The physiological states are defined as follows:

- **Healthy cells:** These cells are alive and able to withstand stress conditions. When inoculated into fresh media, they have the ability to grow and reproduce. In addition, these cells are sufficiently intact so that intracellular or wall-associated materials are not released into the product.
- **Physiologically stressed cells:** They are intact, able to grow and reproduce, but show signs of reduced metabolic rates. Fermentation is slower and the flavor profile may be different. The cells may also be characterized by altered levels of intracellular stress resistance compounds, such as glycogen, trehalose and heat shock proteins (hsp).
- **Replicatively deactivated cells:** 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 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 envelope damage appear intact and are not necessarily poorer in terms of fermentation performance. However, the presence of these cells may negatively affect beer because of haze release. 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 will go through autolysis.
- **Disrupted cells:** These cells are partially (still visible as cellular entities under the microscope) or completely (visible only as fragments) disrupted. This represents the most extreme damage to yeast.

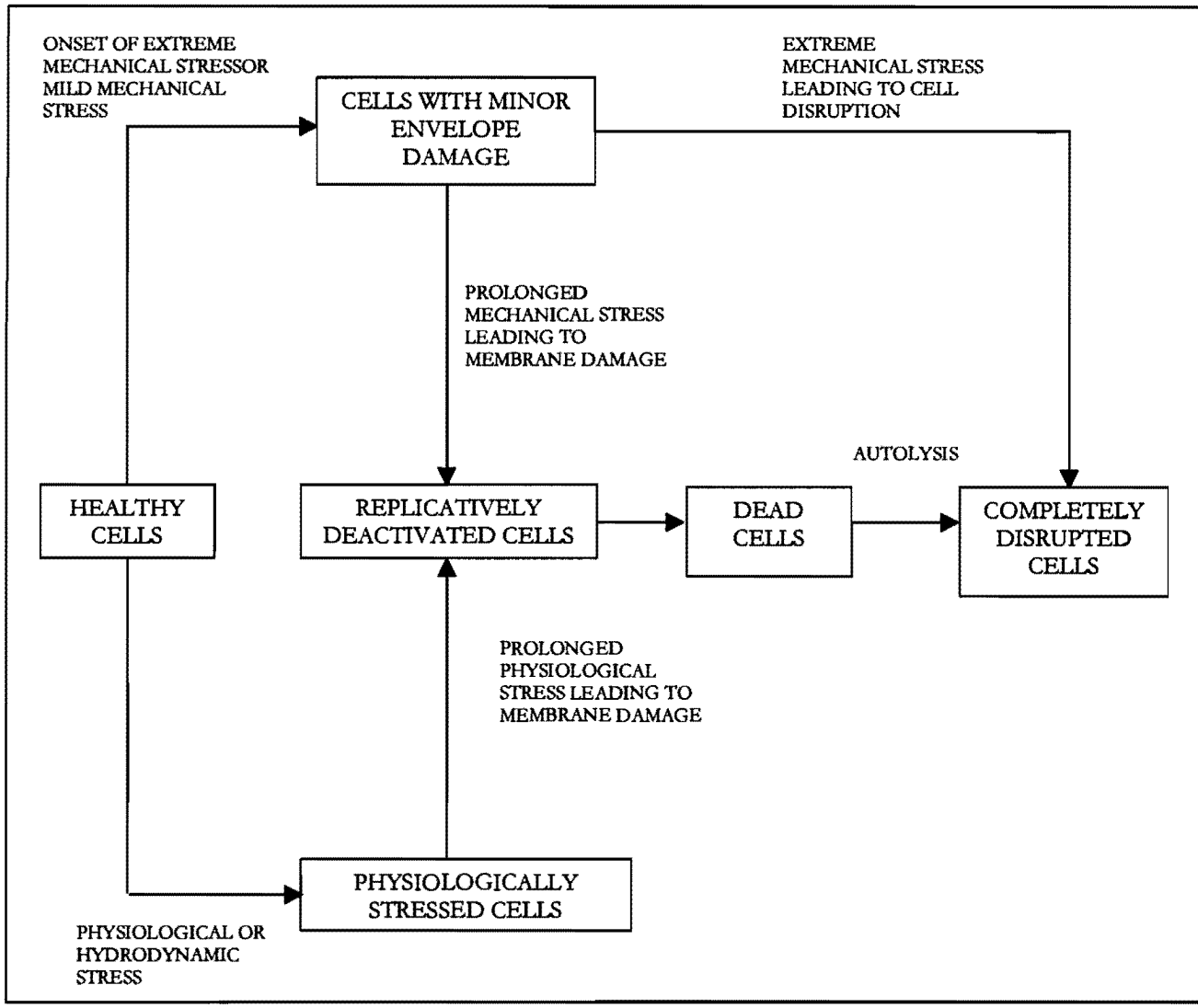


Figure 4.1 Scheme for the loss of yeast quality due applied physiological and hydrodynamic stresses. Adapted from Basson (1996).

This approach is useful as it illustrates the dependency of yeast damage on nature, duration and intensity of stress conditions. In this thesis, the focus is placed on the loss of yeast quality following exposure to low temperature stress. The review of the literature on the potential for a loss of yeast quality during cooling (Chapter 3) indicated that under conditions of cold stress, cell membrane integrity may be compromised, allowing the release of intracellular compounds. Jones (1987) reports that compromised membrane functionality hampers the ability of the cell to reproduce, and may be a precursor to cell death. It was also established that cold injury to yeast may affect its metabolic activity (Fargher and Smith, 1995). Furthermore, cell death due to freeze dehydration and ice crystal formation has been observed at sub-zero temperatures

(Williams, 1990). A proposed scheme for the loss of yeast quality as a result of cold stress is presented in Figure 4.2.

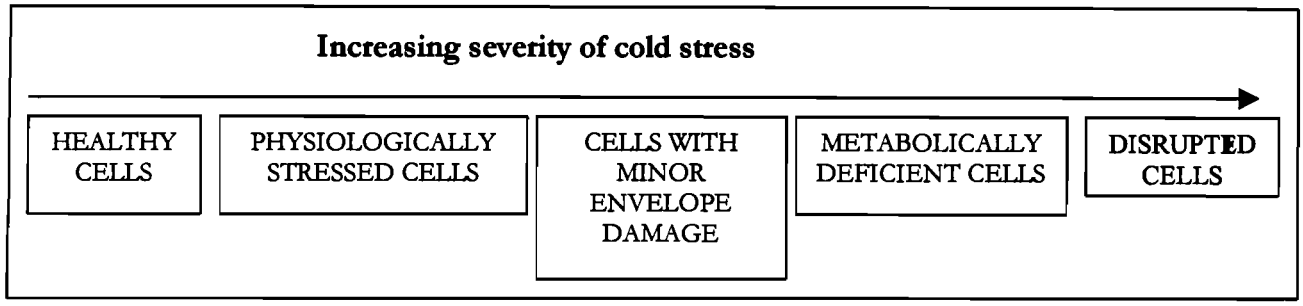


Figure 4.2 Proposed scheme for loss of yeast quality due to low temperature stress.

4.3 ASSAYS FOR IDENTIFYING YEAST QUALITY

The yeast quality indicators as well as assays selected to detect and quantify the loss of yeast quality are summarized in Table 4.1. A more detailed description of each of the indicators and assays is presented in the subsequent sections.

Table 4.1 Analytical methods selected to identify yeast quality.

Yeast Quality Indicator	Selected Assay
Minor envelope damage (membrane)	Protease assay
Cell reproduction and cell death	Methylene blue viability stain Direct cell counting
Minor envelope damage (cell wall)	Haze - particle size distribution Fragility - French press analysis
Metabolic activity indicators	Small-scale fermentation - Carbon dioxide (CO_2) evolution - Cell growth rate - Biomass yield - Substrate utilization Intracellular reserve compounds - Glycogen
Physiological stress indicators	Cold shock - Morphology - Trehalose content - Heat shock protein (hsp) 12

4.3.1 MINOR ENVELOPE DAMAGE (MEMBRANE)

Under conditions leading to cell lysis or the loss of membrane integrity, intracellular compounds may escape from the cell. Components released through a permeabilised cell membrane include amino acids, RNA and proteins. The intactness of the cell membrane has been assessed by the measuring the release of intracellular compounds. The Lowry assay (Lowry, 1951) is a well known test for the release of soluble protein. However this assay is of little value for the assessment of cell integrity in protein rich beer (Basson, 1996). An alternative assay, the protease assay, may provide a more sensitive method for assessing cell integrity.

4.3.1.1 PROTEASE ASSAY

Yeast possess an extensive range of proteases. The main function of these enzymes is the degradation of proteins within the cell and in the surrounding media (Oliver, 1991). Partial or complete disruption of the yeast cell membrane results in the release of proteases into wort. Mochaba *et al.* (1993) showed good correlation between yeast cell membrane damage and protease activity. Under conditions of nitrogen limitation, however, cells tend to release protease without loss of membrane integrity (Slaughter and Nomura, 1992). The released protease aids the extracellular breakdown of proteins to alleviate the limitation. Therefore protease excretion can only be used as a relative indicator of extent of membrane integrity by inclusion of appropriate control samples. The presence of protease in beer is undesirable as it leads to foam instability (Mochaba *et al*, 1993).

Several protease assays are proposed in literature. Ormrod *et al.* (1991) report the use of synthetic substrates dimethylcasein and azocasein to quantify protease activity. The method used in this study is based on the work of Mochaba *et al.* (1993). With this method, resorufin-labelled casein is broken up into resorufin-labelled peptides by proteases in the supernatant of a centrifuged yeast sample. Unhydrolysed protein is precipitated by trichloroacetic acid, while the resorufin-labelled peptides remain in solution. Measured spectrophotometrically, this concentration gives an indication of proteolytic activity in the supernatant (Mochaba *et al.*, 1993). Protease release, measured as such, can be found in the absence of cell disruption, indicating its ability to quantify membrane integrity (leaky membranes). The method and the reproducibility study are

presented in Appendix A.1. The coefficient of variance (percentage error) of replicate samples was calculated to be 3.1 %.

4.3.2 CELL REPRODUCTION AND CELL DEATH

Cell death or the inability of cells to reproduce both constitute a loss in cell viability (Jones, 1987). In the brewing industry, staining techniques are commonly used to monitor cell viabilities. There are two possible mechanisms for the action of viability stains. The first relies on the ability of cell membranes of viable cells to prevent dyes from entering the cytoplasm. The other is based on dyes entering the cell being reduced enzymatically to a colorless form by viable cells (Smart *et al.*, 1999). Hence, the condition of the cell membrane and the presence of a functional enzyme system are assessed. Examples of frequently used stains include methylene blue, aniline blue and eosin yellow (Lentini, 1993). Of these, methylene blue remains an industry standard (Smart *et al.*, 1999). The use of methylene blue as a viability indicator is discussed.

4.3.2.1 METHYLENE BLUE STAINING

Methylene blue is reduced to a colourless compound in the cytoplasm of viable cells while dead cells remain stained (blue). Methylene blue results are generally most accurate and reproducible above viabilities of 90 % (King *et al.* 1981, McCaig 1990). In populations of low viability (<50%) or when yeast has been rendered non-replicative by prolonged storage, methylene blue overestimates the proportion of viable cells (Parkinnen *et al.*, 1976). McCaig (1990) reports that at 0 % true viability, methylene blue can show a viability of about 30 %. Such errors may be due to impurities in the dye, which have the propensity to form variable intensities (from pale to dark blue) in dead cells (Smart *et al.*, 1999). From this study, the use of a more stable stain (methylene violet 3 RAX) is proposed to counter problems associated with methylene blue. Nonetheless, methylene blue stain developed by Lee *et al.* (1981) was chosen as the viability indicator in this work because of expected high viabilities of yeast (>90%). Details of the procedure for this method are given in Appendix A.2

As fermentation performance depends largely on the concentration of cells inoculated, and their ability to grow and reproduce (SAB, 1993), knowledge of the number of cells in a population and their replicative competence is necessary in selecting yeast for re-pitching. To simultaneously determine cell viability and concentration (cells/mL),

methylene blue staining was used in conjunction with direct cell counting. The direct cell counting method used in this project is outlined in Appendix A.3. The standard deviation of replicate cell count viability measurements was calculated to be 0.80 %.

4.3.3 MINOR ENVELOPE DAMAGE (CELL WALL)

Prokop and Bajpai (1992) report that the cell wall and plasma membrane act as a first barrier of defense against shear. Differences in response of cells to hydrodynamic shear can be related to the condition cell envelope. The cell wall is a relatively thick lipid (3-9 % of the wall by mass), protein (4 % of the wall by mass) and polysaccharide (80-90 % of the wall by mass) structure (Oliver, 1991). Despite the apparent strength offered to cells by the cell wall, loss of viability and activity under conditions of hydrodynamic shear well below the mechanical strength of cells have been observed (Dunlop *et al.*, 1994). It is postulated that low temperature stress may affect the interactions between the different components of yeast cell walls. Deterioration in yeast cell wall resistance to hydrodynamic stress was measured by haze and fragility (ease of disruption of the cell envelope) analyses.

4.3.3.1 HAZE ANALYSIS

The presence of sub-micron particles in the yeast slurry can contribute to the occurrence of haze in beer, resulting in compromised beer quality. Lewis and Poerwantaro (1991) demonstrated the release of haze material from cell walls of yeast on agitation. Siebert *et al.* (1987) found reduced filtration performance after yeast removal by centrifugation owing to haze formation. It is therefore reasonable to hypothesize the release of sub-micron particles from the yeast surface on mechanical handling. To investigate this, the size distribution of particles over a broad range must be determined. In this study, the size of haze particles in mechanically handled cold stressed yeast was examined using a Malvern Mastersizer (long bed version 2), based on laser light scattering. Smaller particles scatter the light at a greater angle while larger particles allow light to pass through almost undeflected. By detecting the angle and intensity of the scattered light, the size and concentration of particles in the size range from 0.05 to 850 μm is given. This range is suitable for the detection of yeast and smaller haze material (Robinson, 2001). Data was collected as number percent of particles falling within defined size ranges. The sample preparation method for haze analysis is given in Appendix A.4.

4.3.3.2 FRAGILITY

Mechanical stress was exacted on cold shocked yeast in a French press. The French press is a laboratory scale high-pressure homogenization device (HPH) which is conventionally used to cause microbial cell disruption. Depicted schematically in Figure 4.3, the French Press (40 mL cell, model number FA-073) uses a flow control valve and a hand driven pump to vary the hydraulic pressure within the press. A positive displacement piston forces the pressurized suspension through a restricted discharge valve. The disruption of cells is a function of the operating temperature, pressure and initial cell concentration (Harrison, 1990). In this study, the hydraulic pressure was varied at 5 MPa increments from 1(sublytic) to 30 MPa (lytic). The extent of cell disruption of cold stressed yeast at specific pressures was measured spectrophotometrically by soluble protein release through absorbance at 280 nm. The experimental procedure for the assay is given in Appendix A.5. The standard deviation for replicate samples was calculated to be 1.60 %.

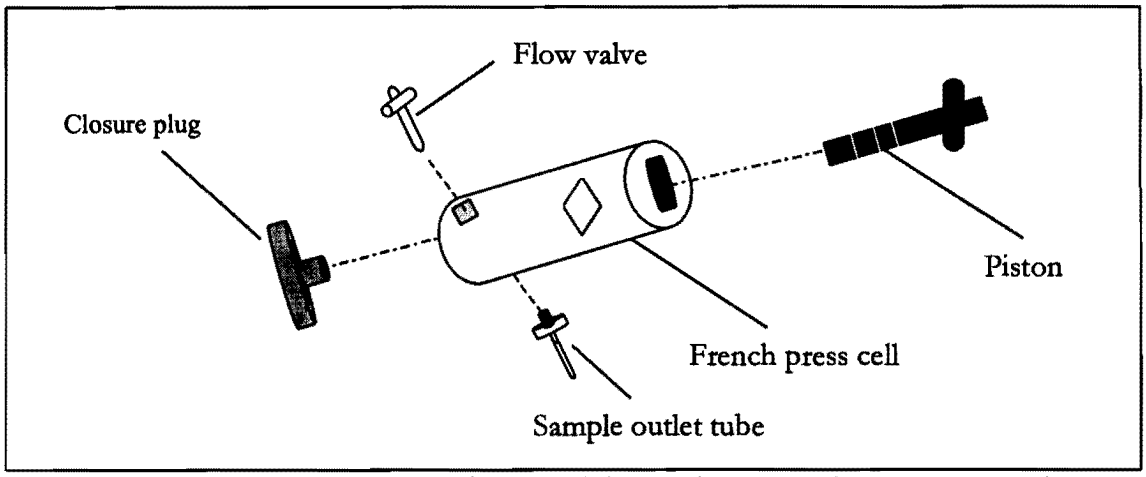


Figure 4.3 Exploded view of French press. Adapted from French Press Cells and Press Operator's Manual, SLM Instruments, Inc., Urbana, Illinois, USA.

4.3.4 YEAST METABOLIC ACTIVITY

The metabolic activity of yeast is dependent on environmental conditions and on the general state of the yeast cell (Walker, 1998a). It is therefore expected that a loss of yeast quality as a result of exposure to cold stress will influence yeast growth and substrate utilization on inoculation into nutrient media. Accelerated small-scale fermentations were selected to monitor changes in yeast vitality due to cooling.

4.3.4.1 ACCELERATED SMALL SCALE FERMENTATIONS

Yeast vitality can be assessed most comprehensively by conducting small-scale fermentations in which yeast is inoculated into the same medium under the same conditions used in brewery fermentations. These, however, take several days to complete and are not of use to predict the fermentation performance of pitching yeast in commercial brewing situations (Henschke and Eglinton, 1991). Several rapid small-scale fermentation techniques have been developed (D'Amore *et al.* 1991, King *et al.* 1981). These fermentations are generally conducted with agitation and at higher temperatures in order to accelerate the fermentation process. Up to 24 hours may be required before reliable and reproducible results are obtained (Lentini, 1993).

In the rapid fermentation technique selected for this study, the level of CO_2 production was used as an indicator of the physiological condition and fermentative ability of yeast (Lentini, 1993). The rate of CO_2 evolution was measured by mass loss during fermentation (see Page 52). The equipment required (mass balance, conical flasks and a constant temperature incubator) was readily available in the laboratory. Daoude and Searle (1986) report that the greatest drawback of CO_2 evolution tests is that yeast growth and the ability of yeast to adapt to the fermentation medium is neglected. For this study, the pre-inoculation glycogen content (Section 4.3.4.2), cell growth rate, substrate utilization and biomass yields were also monitored in order to gain a more accurate prediction of the effects of handling on yeast physiology. The procedure for the small-scale fermentations is outlined in Section 4.4.1 and Appendix A.6.

Substrate utilization was measured using the GOD-Perid kit (Boehringer Mannheimer GmbH Diagnostica, Catalogue No. 124036), based on the conversion of glucose to gluconate with the production of hydrogen peroxide. Hydrogen peroxide combines with di-ammonium 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonate), referred to as ABTS, to

form a green complex that is quantified spectrophotometrically at 620 nm (Appendix A.4). These reactions are represented below:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\textbf{GOD}} \text{Gluconate} + H_2O_2 \quad (4.1)$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\textbf{POD}} \text{Colored complex} + H_2O \quad (4.2)$$

where POD is peroxidase enzyme and GOD is glucose oxidase enzyme. The reproducibility study for the method is presented in Appendix A.7. The percentage error of samples with an absorbance of 0.7 was 2.8 %.

4.3.4.2 GLYCOGEN CONTENT

Glycogen is a polymer of glucose in which sub-units of 10 to 15 glucose molecules are linked by glycosidic bonds. It forms a readily available carbon and energy storage compound in yeast which can account for up to 40 % of cell dry mass (Oliver, 1991). Quain *et al.* (1981) report that the accumulation of glycogen in brewing yeast shows 3 distinct phases: rapid depletion during the initial hours of pitching, accumulation during the bulk of the fermentation and gradual depletion towards the end of fermentation. Decline in glycogen concentration at the end of fermentation is attributed to the provision of maintenance energy under conditions of starvation (Quain, 1988).

The availability of intracellular glycogen has been postulated to influence the initiation and subsequent progress of fermentation. Studies of relative rates of oxygen and carbohydrate uptake from medium illustrate that intracellular glycogen is the primary carbon and energy source during the first 4 hours of fermentation (Quain *et al.*, 1981). Metabolism of glycogen results in generation of ATP and the synthesis of sterols and fatty acids. These compounds are critical in maintaining the structure and function of the cell membrane and determining the extent of cell multiplication. Reduced glycogen levels on pitching correlate with a reduced rate of attenuation and yeast growth (Murray *et al.*, 1984).

Near–infrared reflectance (NIR) spectroscopy can be used for determining intracellular glycogen (Moonsamy *et al.* 1995). In mid-infrared spectra, peaks in the region 1000 to 700 cm^{-1} correspond to carbohydrate compounds. Differences in the second derivative of such spectra at 995, 965, 850 and 813 cm^{-1} have been attributed to variations in the mannan and glycogen content of yeast. Correlation between the peak height ratio at 994/965 cm^{-1} and 850/813 cm^{-1} and enzymatically determined glycogen concentrations have been shown (r^2= 0.81-0.83). This correlation is used to establish a calibration curve for the infrared spectrometer readings. Advantages of the technique include the simplicity of the procedure, the ability to perform the analysis on dried yeast samples that can be stored indefinitely and the rapidity with which the results can be generated. Sample preparation for this technique is given in Appendix A.8. The co-efficient of variance of replicate samples was calculated to be 4.5 %.

4.3.5 PHYSIOLOGICAL STRESS INDICATORS (COLD SHOCK)

Exposure of yeast to low temperature is reported to lead to changes in cell structure and expression of compounds that help preserve the cell integrity (Chapter 3). The degree of change is dependent on the intensity of the stress (Williams, 1990). In this study, cell morphology, trehalose and heat shock protein accumulation were used to indicate the extent of cold stress in yeast.

4.3.5.1 MORPHOLOGY

Two major physical and physico-chemical threats to cell integrity and function are postulated at temperatures close to 0 ^{0}C. The first is ice formation within the cell which may cause cell lysis due to the expansion of water upon freezing. The second is cellular desiccation as a result of ice formation outside the cell. Mindock *et al.* (2001) report that ice formation leads to the separation of pure water (ice) from the extracellular environment. The corresponding increase in salt concentration imposes an osmotic gradient across the cell membrane. Both phenomena (ice formation and extensive cell desiccation) can be counteracted by an increase of solute concentration inside the cell through the voluntary expulsion of water from the cell (Franks and Mathias, 1990). The high solute concentration formed inside the cell lowers the freezing point of the remaining water due to colligative effects, thus conferring cryoprotection (Mindock *et al.*, 2001).

In their experiments to evaluate the adaptive strategies for bacteria in sub-freezing conditions, Mindock *et al.* (2001) observed a 24 fold decrease in cell volume when comparing cells grown at 4 ^{0}C to those grown at 24 ^{0}C. Walker (1998a) also noted this apparent decrease in cell size at low temperatures in his review on the effects of cold stress on yeast. It is reported that this decrease in cell size may temporarily inhibit budding on returning the yeast to its growth temperature (Walker, 1998a). In this study, the size of yeast was determined using a Malvern Mastersizer (long bed version 2), as detailed in Section 4.3.3.1. The percentage error in the average yeast cell size of four replicate samples of yeast supernatant was found to be 0.74 %.

4.3.5.2 TREHALOSE

Trehalose is a non-reducing disaccharide of two glucose molecules (Oliver, 1991). It accumulates in the cytosol under conditions of nutrient depletion and was traditionally regarded as a reserve carbohydrate. However, Wiemken (1990), found trehalose to accumulate in yeast exposed to environmental stresses such as heat shock, low temperature and chemical induced desiccation (ethanol and hydrogen peroxide). It is hypothesized that trehalose acts as a protecting agent which preserves the structural integrity of the cytoplasm (Section 3.2.3.3).

In this investigation, the trehalose concentration was quantified to ascertain the extent of cold stress in yeast. It is postulated that the degree of change in membrane fluidity as a result of cold stress is related to trehalose concentration. The greater the change in membrane fluidity, the greater the amount of trehalose produced by the cell (Section 3.2.3.3). A decrease in fluidity may cause cell membrane to become more susceptible to developing leaks. NIR spectroscopy was used to determine trehalose content (Moonsamy *et al.* 1995). The method, together with sample preparation, is described in Appendix A.8. The percentage error for replicate samples was calculated to be 3.8 %.

4.3.5.3 HEAT SHOCK PROTEIN (HSP 12)

Yeast cells exhibit a rapid molecular response when exposed to elevated temperatures (Section 3.2.3.1). This is called the heat-shock response and leads to the induction of synthesis of a specific set of proteins, called heat shock proteins (hsp). Several hsp have been shown to perform molecular 'chaperoning' functions in yeast cells (hsp 60), while others are implicated in conferring thermotolerence (hsp 104, 90 and 70). It has been reported, however, that other environmental stresses (for example ethanol) also elicit the synthesis of hsp (Walker, 1998a).

Until recently, the cellular role of the family of small heat shock proteins hsp 12 was unknown (Walker, 1998a). Sales *et al.* (2000) observed that hsp 12 protect yeast membranes against desiccation. These proteins, located in the plasma membrane, are thought to interact electrostatically with charged groups present on the membrane surface. It is postulated that hsp 12 forms hydrogen bonds with proteins and glycolipids in the membrane, thereby providing a hydrophilic net that prohibits extensive water loss from the membrane under conditions causing desiccation. These water molecules contribute to membrane stabilization by preventing membrane phospholipid fusion (Sales *et al.*, 2000).

As low temperature stress may result in loss of cellular water and altered membrane configuration, concentrations of hsp 12 in cold stressed yeast were determined by SDS gel-electrophoresis of protein extract. This was carried out at room temperature with a 150-volt direct current. The method for preparing SDS gels is detailed in Appendix A.9.

4.4 RELATIONSHIP BETWEEN YEAST PHYSIOLOGICAL CONDITION AND FERMENTATION PERFORMANCE

The response of the yeast quality assays to differences in yeast quality resulting from physiological damage and their relationship with fermentation performance was investigated on healthy and stressed yeast. The yeast was stressed through a 72 hour storage period at elevated temperature followed by heat treatment at 80 ^{0}C.

4.4.1 EXPERIMENTAL PROCEDURE

A stationary phase yeast sample taken from a yeast collection vessel in a commercial brewery (4th generation) was diluted 1:1 (mass ratio) in phosphate buffered saline (PBS). This yeast was termed "healthy" and characterized by a methylene blue viability of 94 %. A second sample of stationary phase yeast diluted 1:1 in phosphate buffer solution (PBS) was incubated in an aerated 30 ^{0}C shaker for a 72 hour period over which a loss in yeast quality as a consequence of starvation was assessed. A sample of the physiologically stressed yeast was then placed in an 80 ^{0}C oven for a further 30 minutes. The resulting yeast had 32 % methylene blue viability and was termed "unhealthy". This, however, may have been an over estimation of viability in the heat treated yeast population as methylene blue does not correlate well at viabilities below 50 % (Section 4.3.2). Aliquots (5 mL) of healthy and unhealthy yeast were inoculated into 60 mL MYPG media of the following composition: 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone and 10g/L glucose. Each flask was stoppered and fitted with a glass tube containing silica gel to absorb any moisture evaporating from the flask (Figure 4.4). Further, as an added precaution, a flask containing water was also prepared to serve as an evaporation control.

These flasks were incubated in a 30 ^{0}C shaker for 24 hours and sampled every two hours for the first 8 hours of the 24 hours period, and thereafter at 24 hours. The mass of the flasks before and after sampling was recorded. Any mass loss observed between sampling was attributed to carbon dioxide evolution. Samples taken were analysed for glucose concentration, biomass concentration and methylene blue viability. The cell growth rate was determined from a plot of the integrated form of the Monod batch growth equation (Equation 4.3), where C_X is cell concentration, μ is growth rate, and t is the time.

$$\text{Ln}\,(C_x) = \mu\, t \qquad (4.3)$$

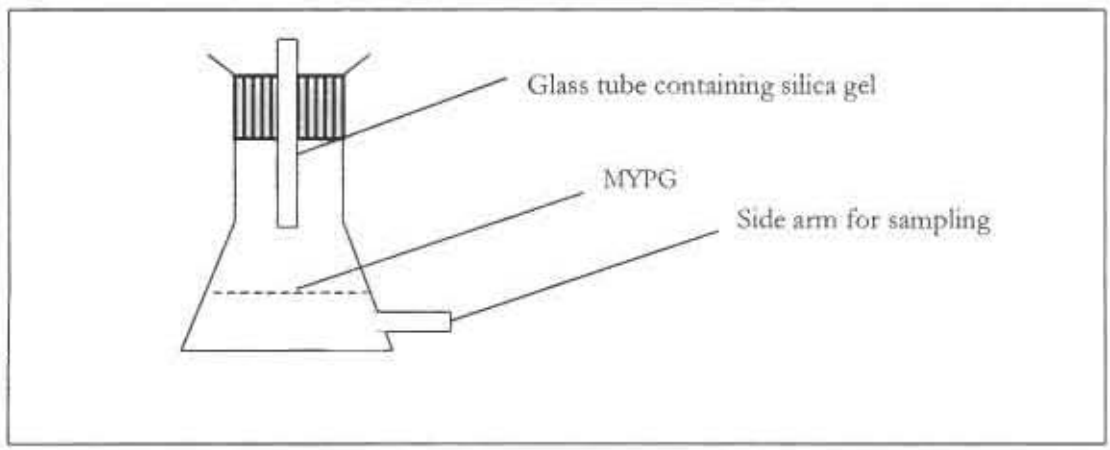


Figure 4.4 Apparatus used in small-scale fermentations to assess yeast vitality.

4.4.2 EFFECT OF STARVATION AND HEAT TREATMENT ON YEAST

The results of the yeast quality assays for the physiologically stressed yeast (starvation, induced by storage at 30 °C) are given in Figures 4.5 and 4.6. Figure 4.5 shows that there was no difference in total cell number in suspension with increasing physiological stress (starvation, induced by storage at 30 °C). Hence the physiological stress imposed was inadequate to cause cell autolysis. There was however an increase in the external protease absorbance (Figure 4.6), suggesting a loss of cell membrane integrity (partial cell disruption). The increase in methylene blue (MB) staining shows an increase in the number of membrane damaged and metabolically inactive cells, as indicated by the decrease in viability, with increasing exposure to starvation stress (Figure 4.6). The subsequent heat treatment of the physiologically stressed yeast resulted in further membrane damage and enzyme deactivation (Table 4.2). As discussed in Section 4.3.2.1, methylene blue is an indicator of both membrane integrity and the ability of the cell to hydrolyze the dye. The increasing protease absorbance observed with decreasing viability (Figure 4.5 and Table 4.2), further supports the hypothesis that methylene blue measures membrane intactness.

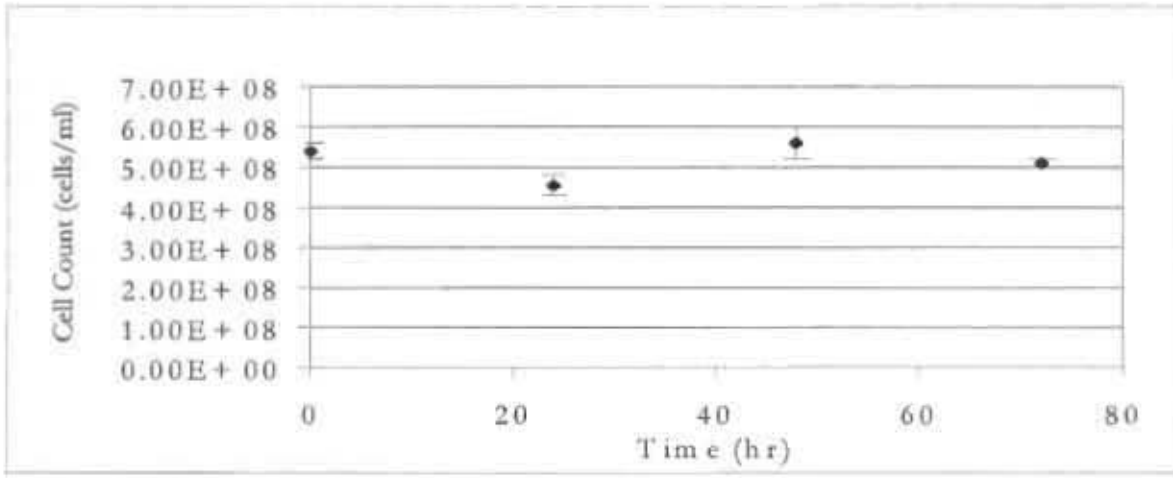


Figure 4.5 The extent of cell disruption on exposure to physiological stress (induced by storage at 30°C) determined by total cell count.

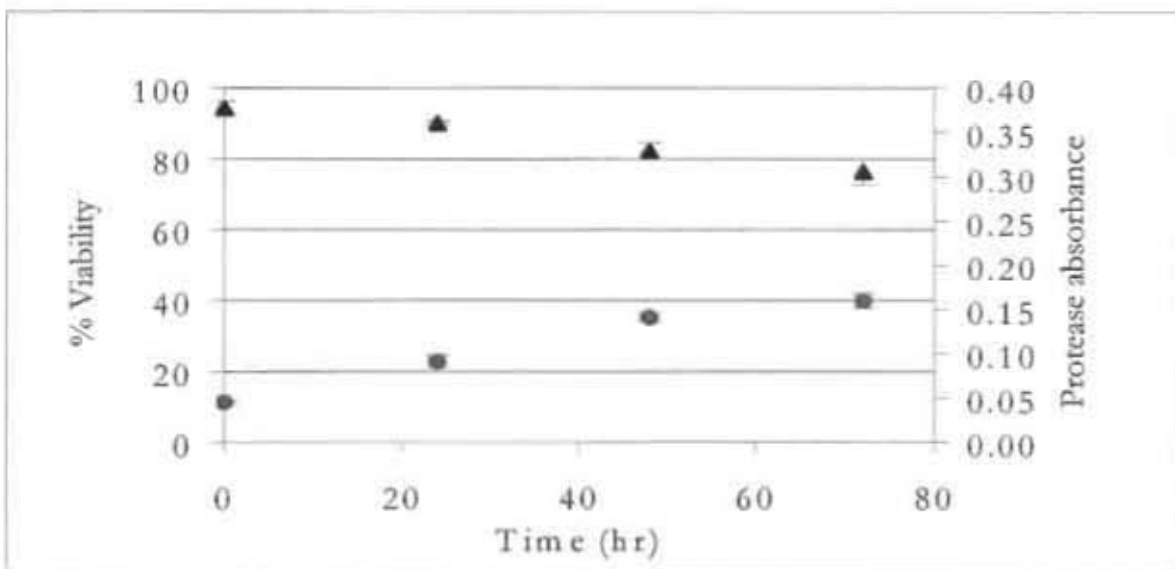


Figure 4.6 Extent cell membrane damage on exposure to physiological stress (induced by storage at 30°C) determined by methylene blue staining and protease absorbance.
▲ Methylene blue viability, ● protease absorbance.

Table 4.2 Summary of yeast quality assays of healthy, stressed and unhealthy yeast.

Yeast quality assay	Healthy yeast	Physiologically stressed Yeast	Heat treated yeast (Unhealthy yeast)
MB viability %	94	77	32
Protease absorbance	0.046	0.160	0.357

4.4.3 SMALL-SCALE FERMENTATION OF HEALTHY AND UNHEALTHY YEAST

Small scale fermentation results for healthy and unhealthy (starved and heat treated) yeast are presented in Figures 4.7 to 4.11. A strong correlation was illustrated between fermentation performance (biomass yield, cell growth rate, substrate utilization and CO_2 evolution) and the viability indicators. In Figure 4.7, unhealthy yeast (starved for 72 hours at 30 ^{0}C followed by 30 minutes of heat treatment at 80 ^{0}C), exhibited a reduced biomass yield compared to healthy yeast. The growth rates obtained over the 2 to 8 hour growth period were also lower in unhealthy yeast, 0.0086 hr^{-1} compared with 0.0343 hr^{-1} for healthy yeast (Figure 4.8 and 4.9). Jones (1987) reports that replicative competence in yeast is dependent on an intact and functioning cell membrane. Hence, the membrane damage observed on starving and heat treating the yeast may have affected its ability to reproduce The substrate utilization profiles, shown in Figure 4.10 show a decreased extent of sugar uptake in unhealthy yeast compared to the healthy yeast. The glucose concentrations at the end of fermentation were 1 g/L and 0.05 g/L respectively. The observed reduction in glucose uptake may have been due to the presence of damaged membranes and enzyme deactivation in unhealthy yeast (MB viability, Table 4.2). During the first two hours of the small-scale fermentations, healthy yeast evolved 3 times as much carbon dioxide (CO_2) as unhealthy yeast (Figure 4.11). Quain (1988) reports that yeast utilize their internal glycogen stores in the first stages of fermentation. Walker (1998a) reports that yeast in nutrient depleted media use intracellular glycogen to provide energy for growth and maintenance. Thus, it may be deduced that the pre-inoculation glycogen content of the healthy yeast was greater than that of the unhealthy yeast. The subsequent heat treatment to the yeast may have also lead to damage of enzymes essential for glucose metabolism, resulting in lower CO_2 production. After the 24 hour fermentation period, the healthy yeast evolved approximately twice as much CO_2 as unhealthy yeast. Mass loss due to evaporation was found to be negligible in comparison to that lost via CO_2 evolution.

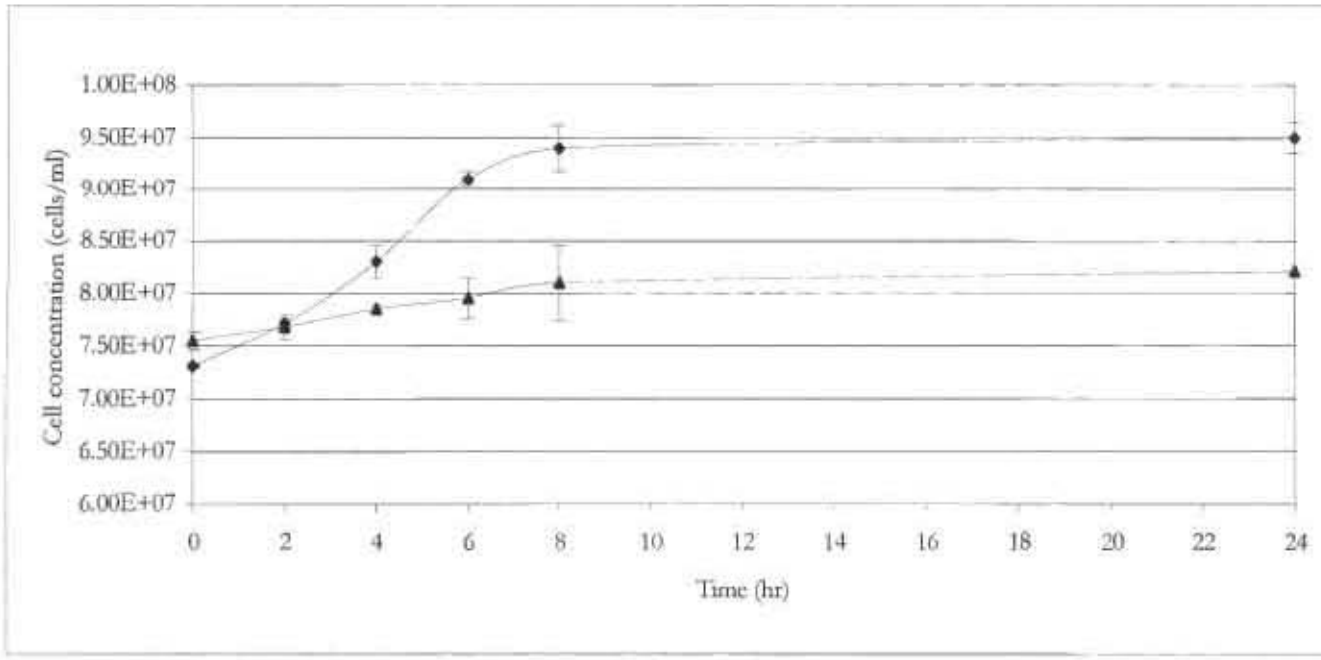


Figure 4.7 Cell growth profile for 24 hours of health and unhealthy yeast inoculated in growth media. ◆ Healthy yeast, ▲ unhealthy yeast.

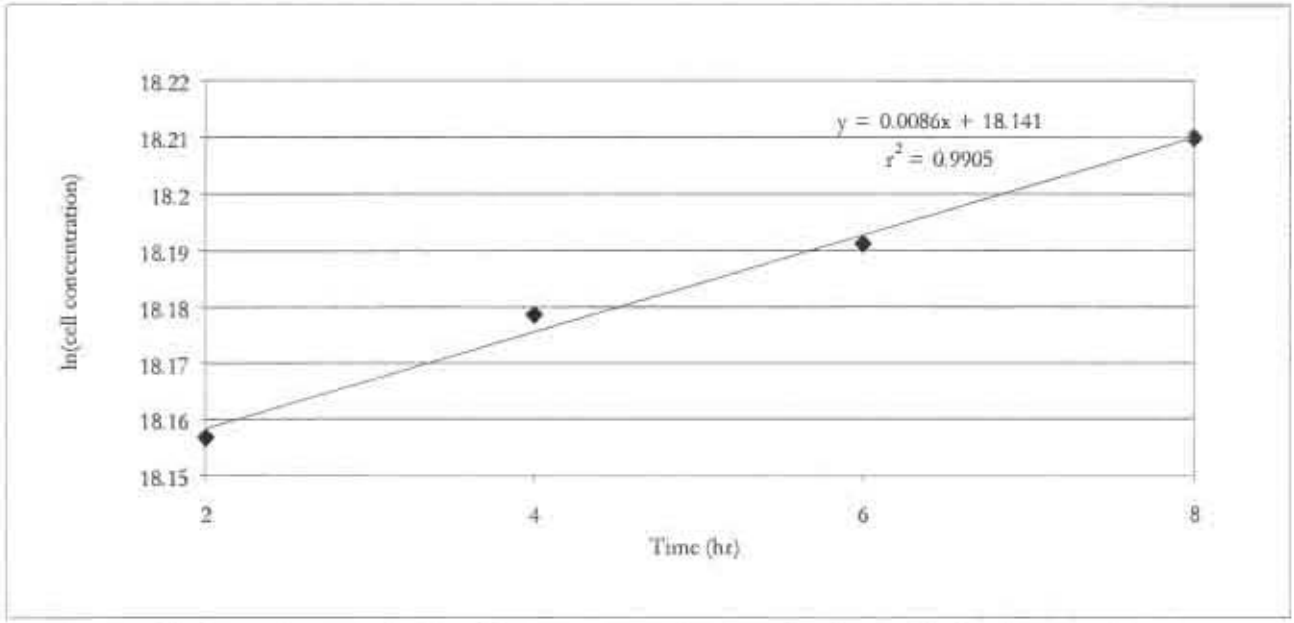


Figure 4.8 Growth rate of unhealthy yeast over the growth phase of the fermentation (from 2 to 8 hours after inoculation).

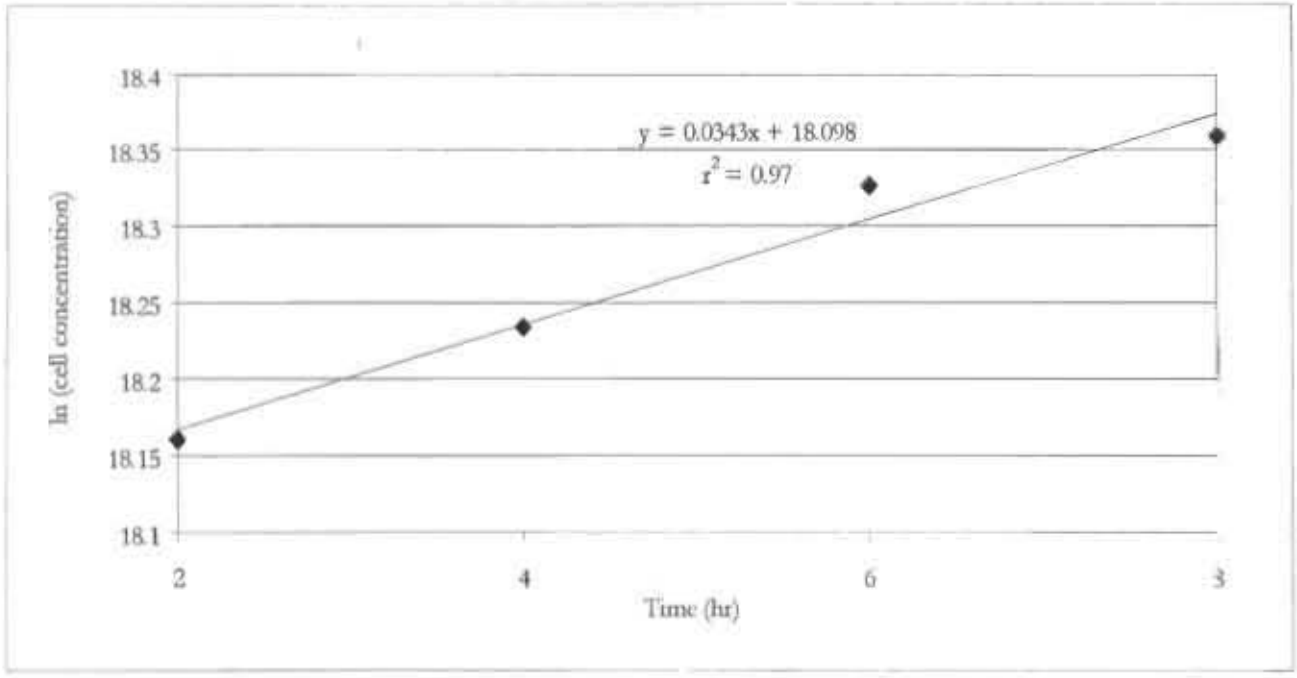


Figure 4.9 Growth rate of healthy yeast over the growth phase of the fermentation (from 2 to 8 hours after inoculation).

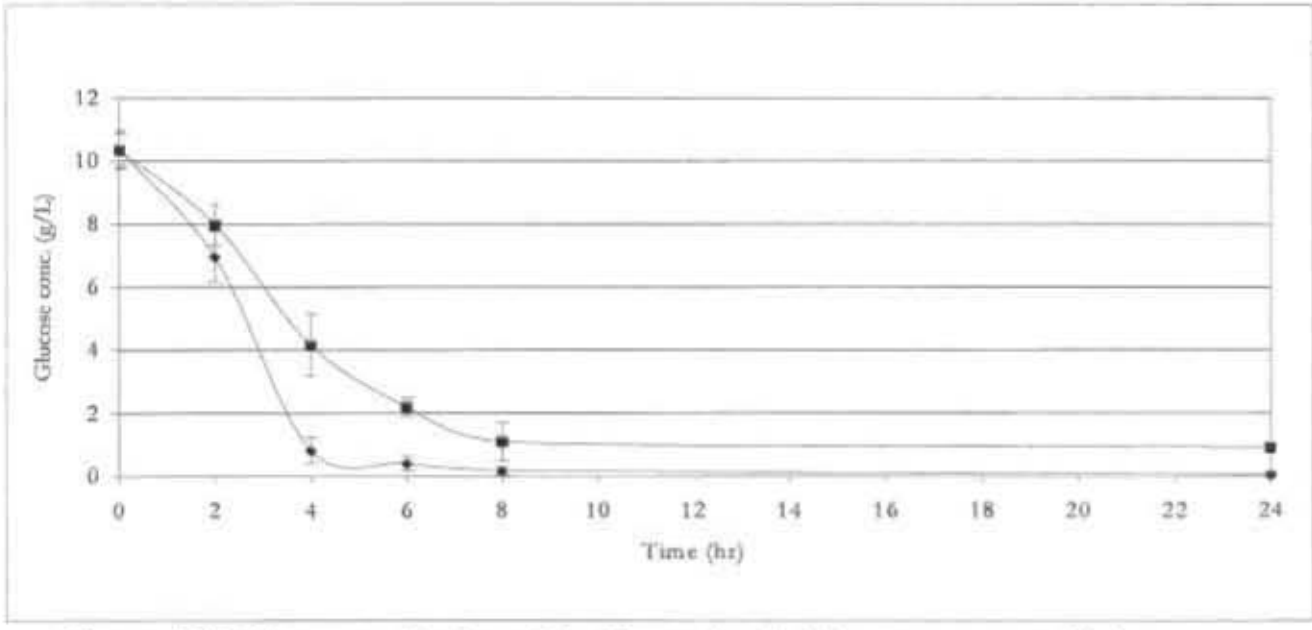


Figure 4.10 Substrate utilization of healthy and unhealthy yeast over a 24 hour fermentation period. ◆ Healthy yeast, ■ unhealthy yeast.

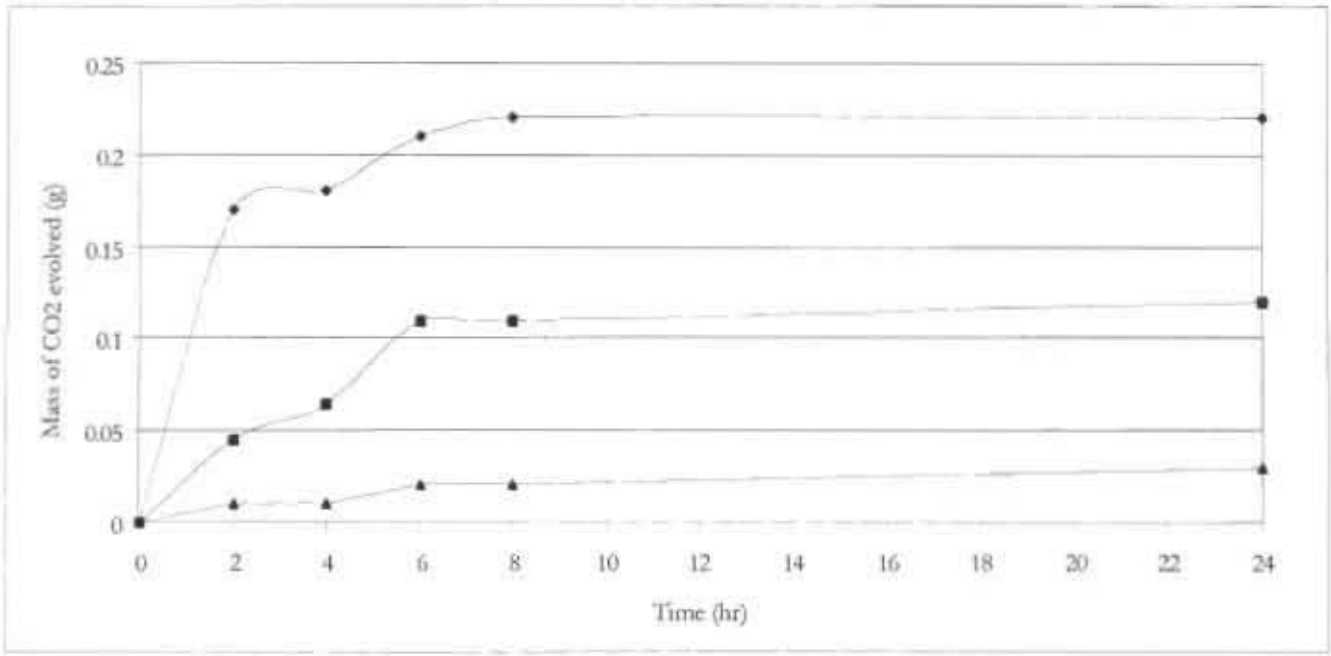


Figure 4.11 Changes in metabolic activity, measured as cumulative CO_2 evolution, of ◆ healthy and ■ unhealthy yeast over a 24 hour period. ▲ Evaporation control.

4.5 CHAPTER SUMMARY

Six analytical parameters to identify and quantify a loss of yeast quality have been presented. These include assays for the release of intracellular compounds (protease assay), cell death (methylene blue viability), cell reproduction and metabolic activity (small-scale fermentations), intracellular reserve indicators (glycogen assay), cold shock responses (trehalose, hsp, morphology) and the ability of the cell envelope to withstand hydrodynamic stress (fragility and haze analysis).

The relationship between yeast physiological condition and small-scale fermentation performance was investigated to validate choice of analytical methods. Methylene blue viability and protease release assays were used to assess a loss of yeast quality as a result of starvation and heat stress. The loss of yeast quality was related to membrane damage and enzyme deactivation, and affected subsequent fermentation performance of the yeast. Stressed yeast showed a reduced rate and extent of growth when compared to healthy yeast. The CO_2 and substrate utilization data from small-scale fermentations also showed that the healthy yeast was more metabolically active than stressed yeast. These results suggest that accelerated small-scale fermentations can be used effectively in determining the effects of physical stress on yeast growth and metabolism.

CHAPTER

5

EXPERIMENTAL PROCEDURES

This chapter gives a detailed account of the experiments performed to assess the potential for a loss of yeast quality as a consequence of cooling in a jacketed agitated vessel and in a heat exchanger.

5.1 INTRODUCTION

The potential for a loss in yeast quality during cooling is a function of (Fargher and Smith, 1995):

i) yeast cell condition,

ii) growth temperature,

iii) cell growth phase,

iv) temperature range over which cooling is performed,

v) cooling rates employed and

vi) level of low molecular weight protectants in the yeast.

The systems examined in this study are instantaneous cooling of yeast slurries by dilution, slow cooling in a jacketed agitated vessel, and rapid cooling in a heat exchanger. Parameters investigated include the effects of dilution, final temperature after cooling and cooling rates on yeast quality. Experiments were carried out on the commercial yeast strain, *Saccharomyces cerevisiae* SAB 5. The yeast was obtained from Castle

Lager yeast collection vessels (YCV) at the South African Breweries-Newlands. As a result, the yeast growth temperature (14 ^{0}C fermentation temperature) and cell growth phase (stationary phase) were kept constant. The generation number of yeast, defined as the number of times the yeast has been used in brewery fermentations, ranged from three to six.

5.2 COOLING WITH DILUTION

In the proposed cooling scheme (Chapter 3), cropped yeast at 14 ^{0}C is diluted with 12 ^{0}C deaerated, non-carbonated water. The ensuing mixture is agitated and cooled with glycol via cooling jackets to 4 ^{0}C at approximately 2 ^{0}C/hr (Wittl and Maier, 1980). The possible stresses exacted on the yeast are:

- Osmotic stress on dilution.
- Shear stress due to agitation.
- Cold stress from cooling.

The experimental procedures described in this section were used to ascertain the effects of dilution (as a function of osmotic stress), agitation, rapid cooling by dilution, and final cooling temperature on yeast quality.

5.2.1 EFFECT OF DILUTION ON YEAST QUALITY

A cropped yeast sample was obtained from a Castle Lager YCV at the South African Breweries. The yeast was centrifuged at 3000 g for 5 minutes in a Beckman J-25 Avanti centrifuge. The resulting supernatant was discarded and the yeast resuspended (to maximise any osmotic shock, Section 3.3) at a range of mass ratios (1:1, 2:1 and 1:2) in each of sterilised deaerated, deionised water, phosphate buffer saline solution (PBS)(control) and MYPG media at 14 ^{0}C. The 250 mL Erlenmeyer flasks containing the yeast slurries were sealed to prevent oxygen ingress and left at room temperature. The flasks were sampled every 20 minutes, for 4 hours, and analysed for cell membrane damage (protease release) and cell death associated with membrane damage (methylene blue viability). After 60 minutes, 5 mL aliquots of the yeast were sampled, assayed for glycogen content and then inoculated into fresh growth media (MYPG). The suspension was then incubated in a 30 ^{0}C shaker for metabolic activity testing (small-scale fermentations, Section 4.3.4).

5.2.2 EFFECT OF AGITATION ON YEAST QUALITY

The primary function of agitation is to achieve homogeneity of yeast slurry without any damage to yeast cells (Section 2.5.1). Injury to yeast as a result of agitation is a function of agitation rate and fluid viscosity (Lentini *et al.*, 1992). In order to investigate the effect of agitation on yeast quality, it is important to minimise physiological stress on the cells. This can be achieved by minimising metabolic processes in yeast, thus preventing the accumulation of CO_2 and ethanol to toxic levels and the generation of metabolic heat. These factors make yeast cells more susceptible to mechanical damage (Smart *et al.*, 1995).

Yeast was obtained from a brewery YCV and centrifuged at 3000 g for 5 minutes in a Beckman J-25 Avanti centrifuge. The supernatant was discarded and the yeast resuspended in 14 ^{0}C sterile deaerated, deionised water to form a nutrient depleted suspension of 30 % consistency (on a mass basis). A 200 mL aliquot of yeast slurry was then placed in each of two 250 mL beakers. The yeast in one vessel was agitated at 570 rpm (based on the average tip speed of brewery storage vessel agitators of approximately 1.5 m/s) using a pitched-blade impeller of 50 mm in diameter. To further prevent metabolic activity, anaerobic conditions were maintained in the vessels by circulating nitrogen gas through the headspace (Figure 5.1).

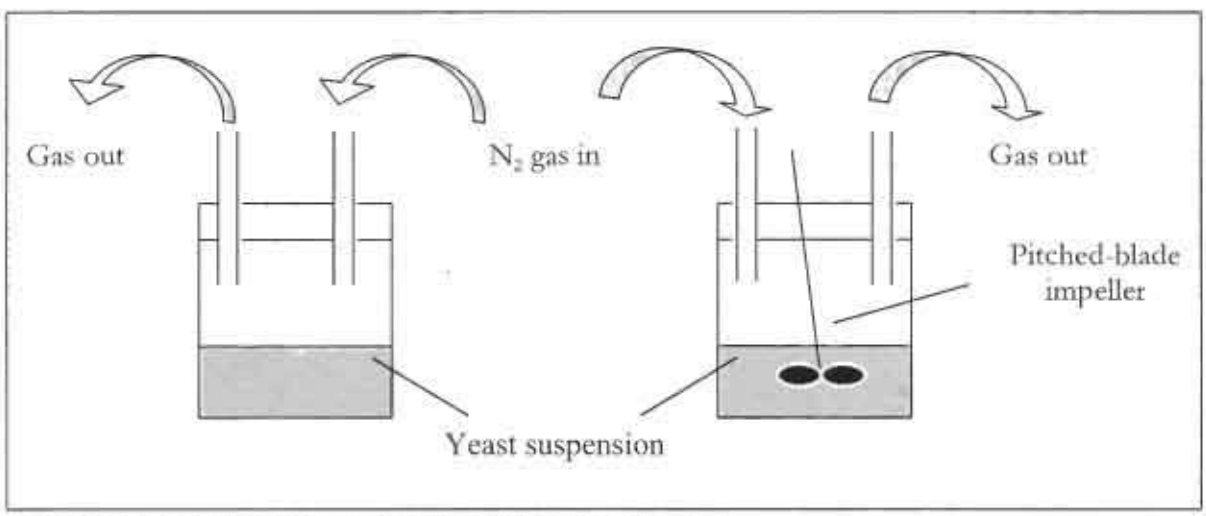


Figure 5.1 Equipment set-up for agitation experiments.

The experiment was carried out at room temperature over a four-hour period. The vessels were sampled every 20 minutes and analysed for cell membrane damage (protease release) and cell death associated with membrane damage (methylene blue viability). A

haze analysis was carried out on the yeast at the beginning and at the end of the experiment. After 60 minutes, 5 mL aliquots of yeast were sampled, assayed for glycogen content and then inoculated into MYPG media for metabolic activity testing.

5.2.3 EFFECT OF COLD STRESS ON YEAST QUALITY

Farghet and Smith (1995) report that a loss in yeast quality due to cold stress is affected by the temperature range over which cooling is performed. In this study, experiments were carried out to establish the effect of final cooling temperature on yeast quality. To determine the influence of cooling rate on the extent of cold injury experienced by yeast on cooling by dilution, an experiment comparing fast cooling (instantaneous) and slow cooling (2 °C/hr) was performed.

5.2.3.1 FINAL COOLING TEMPERATURE

Yeast from a brewery YCV was centrifuged at 3000 g for 5 minutes in a Beckman J-25 Avanti centrifuge. The supernatant was discarded and the yeast was resuspended (30 % consistency) in 14 °C, 12 °C, 8 °C and 4 °C sterile deionised deaerated water. The yeast was maintained at these temperatures for 4 hours by one of three programmable refrigerated circulators (Julabo F12-EC Chiller, FTC-3000 flow through Chiller or RTE-8 Endocal refrigerated circulator) (Figure 5.2).

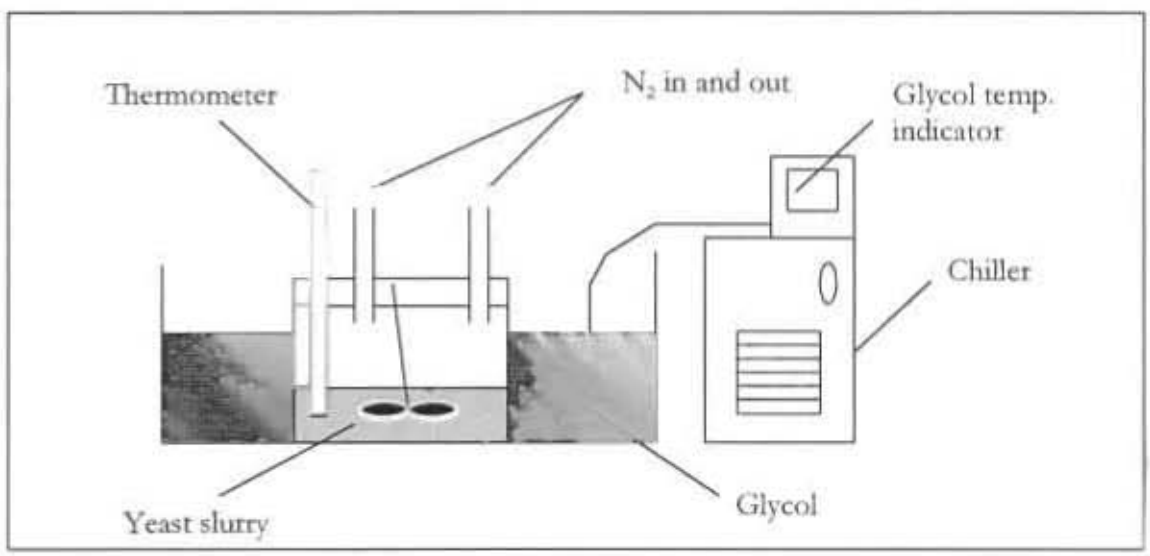


Figure 5.2 Equipment set-up to determine effects of cooling by dilution on yeast quality.

After 60 minutes, 5 mL aliquots of yeast were inoculated into MYPG media for vitality testing. Further samples were taken over the duration of the experiment and analysed for

cell membrane damage, cell death associated with membrane damage, cell wall damage (fragility), physiological stress indicators (morphology, trehalose, hsp 12), glycogen content and changes in metabolic activity (small-scale fermentation). Fragility tests were done on the yeast after 120 minutes at various final cooling temperatures.

5.2.3.2 SLOW AND FAST COOLING WITH DILUTION

Yeast was centrifuged at 3000 g for 5 minutes in a Beckman J-25 Avanti centrifuge. The supernatant was discarded and the yeast was resuspended (30 % consistency) in 12 ^{0}C sterile deionised deaerated water and cooled to 4 ^{0}C at 2 ^{0}C per hour in a jacketed agitated vessel by adjusting the glycol temperature with a programmable Julabo F12-EC Chiller. The yeast suspension was then held at 4 ^{0}C for a further 4 hours. Samples were taken over this period and analysed for cell membrane damage, cell death associated with membrane damage, cell wall damage (fragility), physiological stress indicators (morphology, trehalose, hsp 12) glycogen content and changes in metabolic activity (small-scale fermentation). Small-scale fermentations were conducted on samples collected after 60 minutes at 4 ^{0}C.

Another batch of centrifuged yeast was fast cooled (3 ^{0}C/sec cooling rate based on an estimated mixing time of 3 seconds in the jacketed agitated vessel) by resuspension (to 30 % consistency) in 4 ^{0}C sterile deionised deaerated water and maintained at 4 ^{0}C for 4 hours in a jacketed agitated vessel using a FTC– 3000 flow through Chiller. As a control, yeast was also diluted in 14 ^{0}C sterile deionised deaerated water and kept at that temperature with an RTE- 8 Endocal refrigerated circulator for 4 hours. Samples obtained were analysed in an identical manner to those of the slow cooled yeast.

5.3 COOLING IN A HEAT EXCHANGER

During yeast handling, cropped yeast is rapidly cooled from a fermentation temperature of 14 ^{0}C to a storage temperature approaching 0 ^{0}C by passage through a heat exchanger. Based on operating conditions at South African Breweries-Newlands (Horn, 2000), the yeast is cooled at a rate of approximately 2.3 ^{0}C/sec (Appendix B). In this study, work was conducted to investigate the effect of cooling rates employed in a heat exchanger on yeast quality. This was done in order to identify cooling rates (critical) above which cold injury to yeast will impinge on process performance. Work was also conducted on-line at

Newlands brewery to assess the potential for a loss of yeast quality during cooling under routine brewery operations.

5.3.1 COOLING RATE

Yeast was centrifuged at 3000 g for 5 minutes in a Beckman J-25 Avanti centrifuge and resuspended (30 % consistency) in 14- 18 °C deaerated deionised water. Using a Watson-Marlow 313S roller pump, the yeast suspension was pumped through 2 m copper tubing (6 mm internal diameter) twisted into coils (Figure 5.3). The coils were submerged in approximately -1 °C ethylene glycol. Cooling rates were varied by adjusting the yeast flowrate. The cooling rates achieved ranged from 1 to 4 °C/sec. The final cooling temperatures obtained at all flowrates varied between 2 and 5 °C.

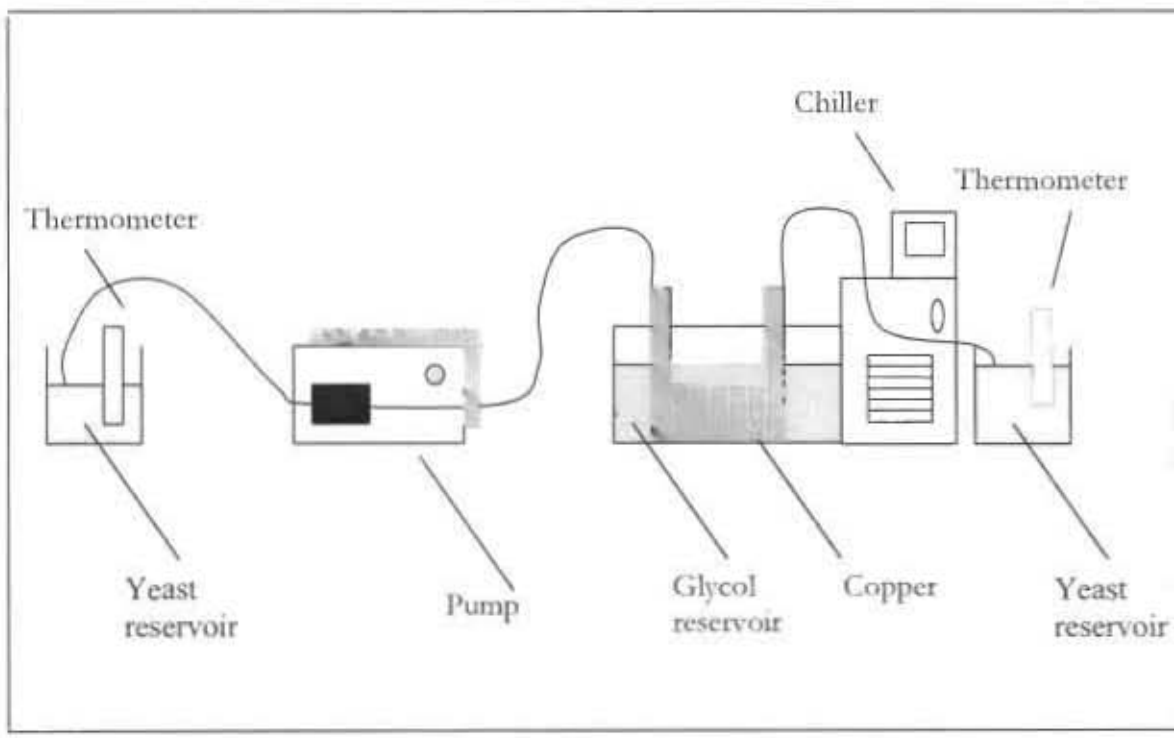


Figure 5.3 Experiment set-up used to simulate cooling in a heat exchanger.

Yeast samples were collected before and after cooling and analysed for cell membrane damage, cell death associated with membrane damage, cell wall damage (fragility, haze material), physiological stress indicators (trehalose), glycogen content and changes in metabolic activity (small-scale fermentation). Small-scale fermentations were conducted with the uncooled yeast (control), and yeast cooled at 1, 2, 3 and 4 °C/sec.

5.3.2 YEAST COOLING IN THE BREWERY

Positive displacement pumps are used in the brewery to transfer yeast from the fermentation vessel to yeast collection vessels. During cropping, these pumps may be operated at mass flow rates ranging from a minimum of 60 kg/min (57 L/min) to a maximum of 80 kg/min (75 L/min) (Theron, 2000). To assess the potential for loss of yeast quality on cooling, cropped yeast (14 ^{0}C) was pumped through a plate and frame heat exchanger at 40 (38 L/min), 60 and 80 kg/min (Figure 5.4).

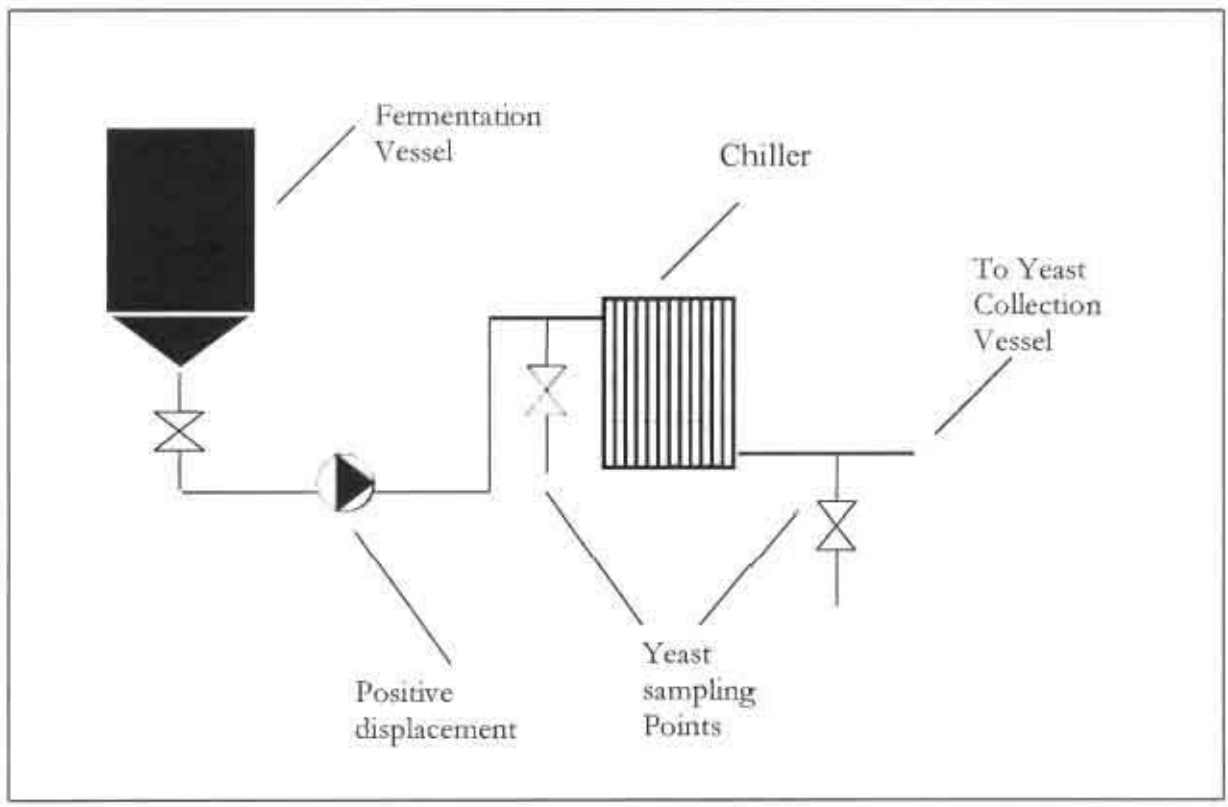


Figure 5.4 Experiment set-up to determine the potential for loss of yeast quality during rapid cooling in a brewery.

The final cooling temperature of the yeast was kept constant at 2.5 ^{0}C by an automated control system that varied the coolant (ethylene glycol) flow rate. Yeast quality (cell membrane damage, cell death associated with membrane damage and cell wall damage (haze)) was analysed prior to and after cooling at each flowrate. Yeast was also pumped through the heat exchanger without any cooling in order to assess any hydrodynamic effects on yeast quality. Metabolic activity tests were done on uncooled yeast, and yeast pumped through the heat exchanger at 40, 60 and 80 kg/min. These flowrates correspond to cooling rates approximately equal to 1, 2 and 3 ^{0}C/sec.

CHAPTER

6

RESULTS AND DISCUSSION

In this chapter, the results obtained from experiments to establish the effects of cooling of diluted yeast slurries in a jacketed agitated vessel and in a heat exchanger on yeast quality are presented and discussed.

6.1 INTRODUCTION

The experiments performed to assess the potential for a loss in yeast quality as a result of cooling of diluted yeast slurries in a jacketed agitated vessel and in a heat exchanger are detailed in Chapter 5. These experiments were carried out in order to ascertain the effects of osmotic shock, final cooling temperature and cooling rate on the physiological state of yeast and its ability to withstand subsequent mechanical handling. Critical cooling rates above which extensive cold injury will occur in yeast were sought.

6.2 THE EFFECT OF DILUTION ON YEAST QUALITY

To determine the effect of dilution on yeast quality, unwashed yeast at 14 °C was resuspended in each of sterilised deaerated, deionised water; PBS (control); and MYPG nutrient media at 14 °C at mass ratios of yeast to diluent of 1:1, 2:1, and 1:2. Samples were collected over a 4-hour period and analysed for cell membrane damage (protease release), cell death associated with membrane damage (methylene blue viability), and changes in growth and metabolic activity (small-scale fermentations in MYPG media with initial glucose concentration of approximately 10 g/L).

6.2.1 RESULTS

The results of the assays performed on yeast to determine the effect of dilution on cell integrity are given in Tables 6.1 to 6.3. A specific protease absorbance, defined as protease absorbance per cell (ΔA/cell count), was calculated in order to allow comparison across the experiment. The results in Tables 6.1 to 6.3 show that there was 1-3 % change in the total number of cells suspended in water and PBS (control) at a 1:1, 1:2 and 2:1 dilution ratio with time. Therefore, the osmotic stress produced by dilution did not cause cell disruption. There was however an increase in cell concentration (12-26 %) with yeast diluted in nutrient media at the various mass ratios. This increase was due to yeast growth. The integrity of a cell (intactness of the cell membrane) can be assessed by measuring the release of intracellular compounds (Mochaba *et al.*, 1993). The results in Tables 6.1 to 6.3 show that the protease excreted from yeast increased at low yeast to diluent ratios. Yeast diluted at a 1:2 ratio showed an almost 3 fold increase in specific protease release compared to yeast at 1:1 or 2:1, suggesting a decrease in intactness of the cell membrane with increased osmotic stress. On closer inspection of the data however, it can be seen that there is little difference in protease release of yeast diluted in water and PBS at the various dilution ratios. Physiologically buffered saline (PBS) is designed to prevent osmotic stress by its isotonic nature, hence overriding the hypothesis of loss of integrity due to osmotic stress. Slaughter and Nomura (1992) report the release of protease under conditions of nitrogen limitation. This release of proteases aids in the extracellular breakdown of proteins in the surrounding media to alleviate the limitation. Further evidence for this is the consistently lower changes in protease activity found on diluting yeast in MYPG (35-60 %). MYPG has both yeast extract (3 g/L) and peptone (5 g/L) as nitrogen sources. Hence protease release in these experiments is correlated to nitrogen limitation and not osmotic stress. The cell viability data in

Tables 6.1 to 6.3 confirm there was minor (<3 %) cell death associated with membrane damage and enzyme deactivation on resuspending yeast in the diluents in the absence of nutrients.

Table 6.1 Change in protease absorbance, methylene blue viability and cell concentration of yeast diluted (1:1) in Water, PBS and MYPG over a 4-hour period (Δ = Final – Initial).

Diluent	Δ Specific protease absorbance	Δ % Viability	Δ Cell concentration (%)
Water	1.15	- 1.5 ± 0.5	1.2
PBS	1.19	- 1.5 ± 0.5	0.8
MYPG	0.56	1.0 ± 0.7	26.1

Where the initial yeast concentration (cells/mL) in Water was 3.27 E+08 ± 4.50 E+06, PBS 3.17 E+08 ± 3.20 E+06, and in MYPG 3.26 E+08 ± 1.15 E+07.

Table 6.2 Change in protease absorbance, methylene blue viability and cell concentration of yeast diluted (2:1) in water, PBS and MYPG over a 4-hour period (Δ= Final – Initial).

Diluent	Δ Specific protease absorbance	Δ % Viability	Δ Cell concentration (%)
Water	1.89	- 2.0 ± 0.7	2.9
PBS	1.73	- 1.5 ± 0.5	1.4
MYPG	0.74	0.5 ± 0.5	17.2

Where the initial yeast concentration (cells/mL) in Water was 2.6 E+08 ± 3.0 E+06, PBS 2.8 E+08 ± 3.0 E+06, and in MYPG 3.6 E+08 ± 2.5 E+06.

Table 6.3 Change in protease absorbance, methylene blue viability and cell concentration of yeast diluted (1:2) in water, PBS and MYPG over 4-hour period (Δ= Final – Initial).

Diluent	Δ Specific protease absorbance	Δ % Viability	Δ Cell concentration (%)
Water	4.65	- 2.0 ± 0.7	2.9
PBS	5.07	- 2.5 ± 0.5	2.4
MYPG	3.03	0.7 ± 0.7	12.1

Where the initial yeast concentration (cells/mL) in Water was 1.2 E+08 ± 7.0 E+05, PBS 1.06 E+08 ± 1.12 E+06, and in MYPG 1.03 E+ 08 ± 1.12 E+06.

Results of small-scale fermentations tests carried out on the yeast after one hour incubation are summarised in Tables 6.4 to 6.6. Each fermentation was done in duplicate and the average result is presented in Tables 6.4 to 6.6. Typical cell growth and CO_2 evolution profiles obtained in the experiments are illustrated in Figure 6.1 and 6.2 respectively. Yeast diluted in water and PBS showed similar (within 1-5 % of each other) glycogen content on inoculation whilst yeast diluted in nutrient media showed comparatively higher glycogen concentrations (8-14 %). Sall *et al.* (1988) report that yeast cells in sugar-rich environment accumulate intracellular glycogen. Glycogen is a source of both metabolic energy and carbon-based intermediates that can be mobilised rapidly for biochemical reactions. Sall *et al.* (1988) also report that under conditions in which external carbon is limiting, internal glycogen concentrations decline. The MYPG media used in the experiments had an initial glucose concentration of approximately 10 g/L and an initial malt extract concentration of approximately 3 g/L. Results in Tables 6.4 to 6.6 also indicate that there was little difference in the CO_2 yields (<5 %) of the yeast on growth following dilution in water and PBS at the various dilution ratios. Yeast diluted in MYPG however showed higher CO_2 yields (7-16 %). This may have been due to its superior glycogen content on inoculation as the end of fermentation glucose concentrations in each flask were close to zero (<0.05 g/L). Yeast growth and the ability of the yeast to adapt to fermentation media were monitored by measuring the yeast growth rate and biomass yield. The growth rates of yeast in water and PBS were very similar (< 3 % difference) at each of the dilution ratios whilst yeast resuspended in MYPG showed slightly higher growth rates (6-13 %). Basson (1996) reports that physiological stress, such as prolonged exposure to a nutrient depleted environment, may lead to reduced growth potential in yeast. On conducting a two-sided t-test on the biomass yields obtained in the experiments, the null hypothesis being that there is no significant difference between the quantities, 't' values corresponding to the confidence intervals listed in Table 6.7 were found. It was therefore concluded that there is no significant difference in biomass yield obtained from yeast diluted in water, PBS or MYPG. Osmotic stress on dilution did not affect the yield of yeast in the fermentation medium, but did result in a small reduction in growth rate.

Table 6.4 Summary of vitality data of yeast diluted 1:1 in water, PBS and MYPG.

Diluent	Glycogen Content on inoculation (g/g d.w)	Initial cell concentration (cells/mL) $\times 10^5$	Final cell concentration (cells/mL) $\times 10^5$	Biomass Yield (cells/g glucose) $\times 10^7$	CO_2 evolved (g)	CO_2 Yield (g/g glucose)	Growth rate (hr^{-1})
Water	19.23 ± 0.43	640 ± 8	960 ± 16	290 ± 9	0.26 ± 0.01	0.41 ± 0.01	0.0447 ± 0.0001
PBS	18.95 ± 0.98	630 ± 12	941 ± 28	291 ± 11	0.27 ± 0.01	0.41 ± 0.01	0.0445 ± 0.0001
MYPG	20.88 ± 0.63	638 ± 12	950 ± 7	292 ± 17	0.28 ± 0.02	0.44 ± 0.02	0.0479 ± 0.0004

Table 6.5 Summary of vitality data of yeast diluted 2:1 in water, PBS and MYPG.

Diluent	Glycogen Content on inoculation (g/g d.w)	Initial cell concentration (cells/mL) $\times 10^5$	Final cell concentration (cells/mL) $\times 10^5$	Biomass Yield (cells/g glucose) $\times 10^7$	CO_2 Evolved (g)	CO_2 Yield (g/g glucose)	Growth rate (hr^{-1})
Water	21.58 ± 0.80	1010 ± 30	1210 ± 26	191 ± 15	0.25 ± 0.01	0.38 ± 0.01	0.0142 ± 0.0001
PBS	21.94 ± 0.40	900 ± 12	1200 ± 28	189 ± 25	0.26 ± 0.01	0.40 ± 0.01	0.0140 ± 0.0001
MYPG	23.69 ± 0.98	1000 ± 40	1220 ± 48	189 ± 28	0.28 ± 0.01	0.44 ± 0.01	0.0151 ± 0.0005

Table 6.6 Summary of vitality data of yeast diluted 1:2 in water, PBS and MYPG.

Diluent	Glycogen Content on inoculation (g/g d.w)	Initial cell concentration (cells/mL) $\times 10^5$	Final cell concentration (cells/mL) $\times 10^5$	Biomass Yield (cells/g glucose) $\times 10^7$	CO_2 evolved (g)	CO_2 Yield (g/g glucose)	Growth rate (hr^{-1})
Water	21.33 ± 0.58	540 ± 13	780 ± 30	219 ± 9	0.25 ± 0.01	0.39 ± 0.02	0.0262 ± 0.0003
PBS	22.41 ± 0.27	546 ± 24	780 ± 18	218 ± 12	0.24 ± 0.01	0.37 ± 0.02	0.0267 ± 0.0001
MYPG	24.15 ± 0.22	535 ± 15	700 ± 27	229 ± 17	0.27 ± 0.01	0.42 ± 0.02	0.0306 ± 0.0004

Table 6.7 Confidence intervals (%) obtained on comparing biomass yields obtained from yeast diluted in water, PBS and MYPG at the different ratios through the statistical t-test.

Biomass yields	1:1	2:1	1:2
Water and PBS	97 %	96 %	99 %
Water and MYPG	95 %	93 %	98 %
PBS and MYPG	95 %	98 %	97 %

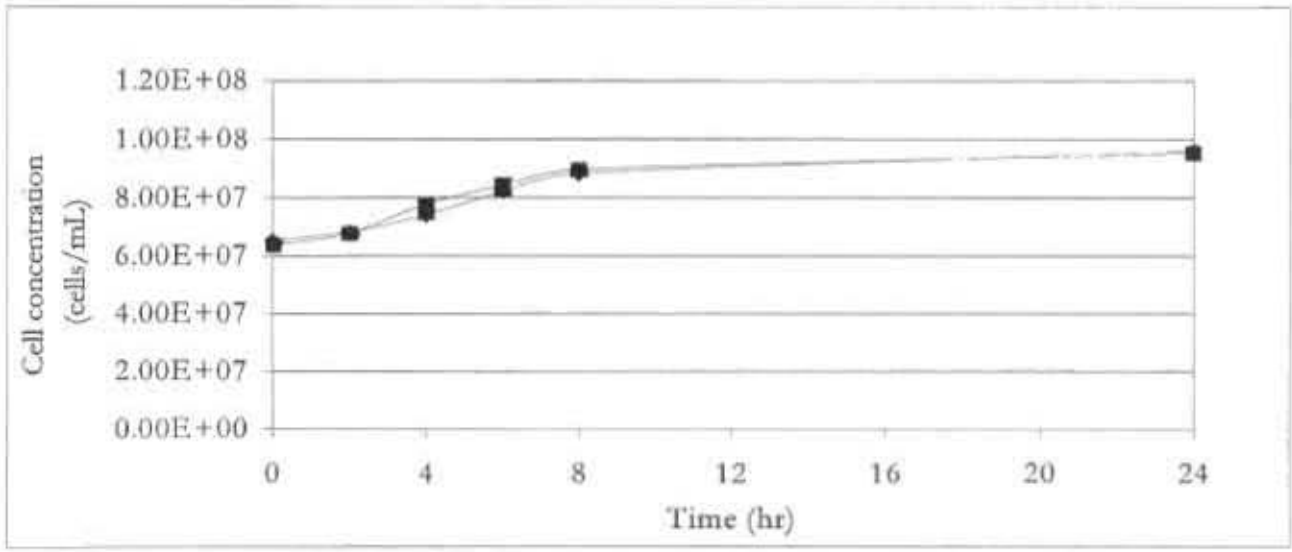


Figure 6.1 Cell growth profiles from duplicate small-scale fermentations of yeast slurries diluted (1:1) in Water.

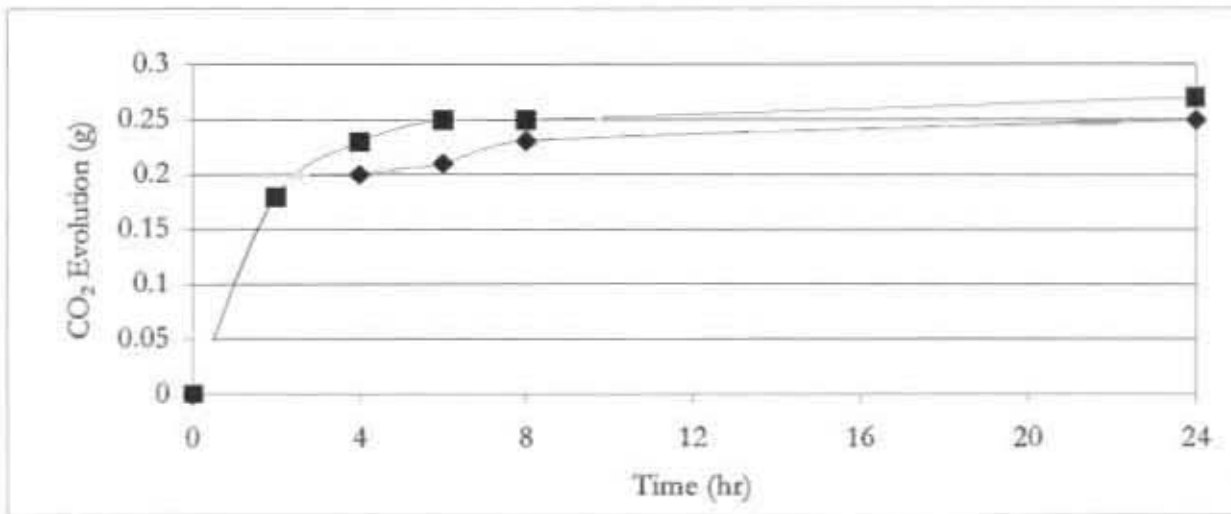


Figure 6.2 Carbon dioxide evolution data from duplicate small-scale fermentations of yeast slurries diluted (1:1) in Water.

6.2.2 SUMMARY

From the experiments presented in this section, it was found that the osmotic stress encountered on dilution had little or no effect on yeast cell integrity, growth and fermentative ability. The protease release observed on suspending yeast in water and PBS was attributed to the absence of a nitrogen source in the two diluents (Slaughter and Nomura, 1992) and not to the loss of membrane integrity due to osmotic stress. Prolonged storage of yeast in a nutrient depleted media may however lead to a decrease in internal glycogen content of the yeast.

6.3 EFFECT OF AGITATION ON YEAST QUALITY

The effect of agitation on yeast quality in the experiment set-up for final cooling temperature experiments (Section 5.2.3.1) was investigated. Unwashed yeast at 14 ^{0}C was resuspended in deaerated deionised water at 14 ^{0}C to a 30 % consistency (on mass basis). A 200 mL aliquot of yeast slurry was then placed in each of two 250 mL beakers. The yeast in one of the vessels was agitated at 570 rpm (based on the average tip speed of brewery storage vessel agitators, approximately 1.5 m/s) using a pitched-blade impeller of 50 mm in diameter over a 4-hour period. Samples were examined for cell membrane damage (protease release), cell death associated with membrane damage (methylene blue viability), cell wall damage (haze), and changes in cell growth and metabolic activity (small-scale fermentations in MYPG media, initial glucose concentration of approximately 10 g/L).

6.3.1 RESULTS

The results of the assays performed on yeast to determine the effects of agitation on cell integrity are summarised in Table 6.8. There was no difference in the protease absorbance of yeast in both the agitated and non-agitated systems (Table 6.8). This suggests that the cell-cell and cell-obstacle interactions caused by agitation did not cause membrane permeabilisation. The observed protease release may have been due to the absence of a nitrogen source in the yeast slurries (Slaughter and Nomura, 1992). The change in viability of agitated and non-agitated yeast was also small (< 3 %) over the 4-hour duration of the experiment, indicating little or no cell death associated with membrane damage and enzyme deactivation.

Table 6.8 Change in protease absorbance and methylene blue viability of agitated and non-agitated yeast under nitrogen over a 4-hour period at 14 °C (Δ = Final – Initial).

System	Δ Protease absorbance	Δ % Viability
Agitated	0.05 ± 0.01	-2.5 ± 1.0
Non-Agitated	0.04 ± 0.02	-1.0 ± 0.7

Particle size distributions of the agitated and non-agitated yeast are presented in Figure 6.3. Lewis and Poerwantaro (1991) observed the release of a haze-forming colloidal material from the cell wall of brewers yeast on mechanical agitation. Fischer *et al.* (2001) report that particles in the range 0.1 to 3 μm are related to haze and protein-polyphenol compounds. More specifically, 0.1 to 2 μm particles are classified as haze, and 2-3 μm particles as protein-polyphenol compounds. In Figure 6.3, there is negligible difference in the size distributions of the two yeasts in the sub-3 μm region. This shows that the applied agitation did not cause damage to the yeast cell wall or cell membrane over the 4 hour application. There is also no change in the mean yeast cell size (predominant yeast cell size in Figure 6.3) over the two systems (± 6.63 μm).

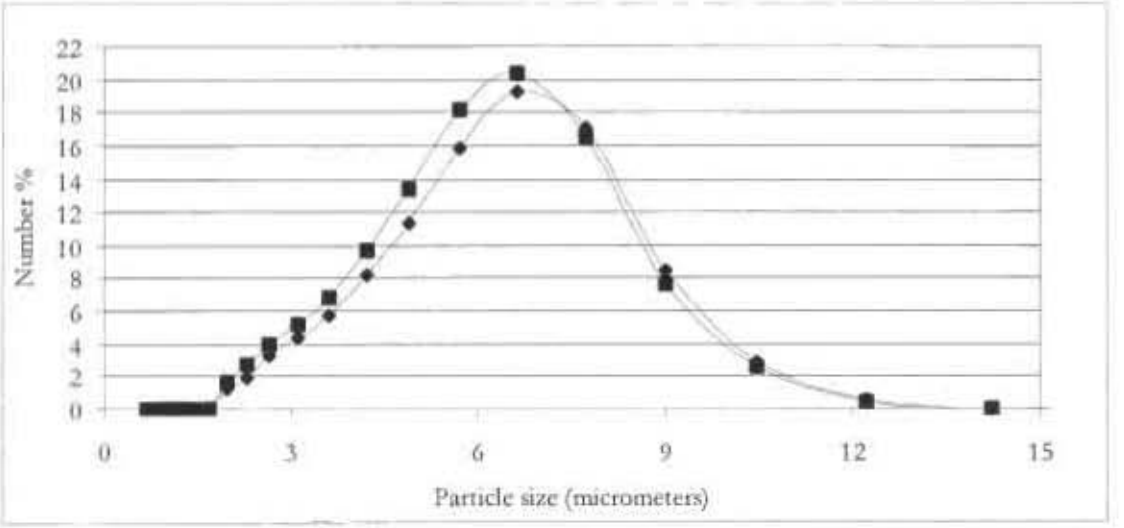


Figure 6.3 A comparison of the particle size distribution obtained from yeast exposed to 4 hours continuous agitation ■, and non-agitated control yeast ◆.

The results of the metabolic activity tests (small-scale fermentations) conducted on agitated and non-agitated yeast are summarised in Table 6.9. Glycogen content of the two yeasts was similar (<3 % difference) on inoculation following 1 hour agitation. Lentini (1993) reports that the level of CO_2 production can be used as an indicator of the physiological state and fermentative ability of yeast. The CO_2 evolution data shows that the agitation employed had no effect on the physiological state or fermentative ability of the yeast. End of fermentation glucose concentrations of agitated and non-agitated yeast approached zero (<0.05 g/L). A two-sided t-test was done on the biomass yields obtained in the experiments, with the null hypothesis being there is no significant difference between the yields. A 't' value corresponding to the 90 % confidence interval was found, indicating the null hypothesis to be true. There was no significant difference in yeast growth (assessed through final cell number or growth rate) over the two systems. Therefore, the agitation employed in the experiment set-up has no effect on yeast growth and the ability of the yeast to adapt to the fermentation media.

Table 6.9 Summary of vitality data of agitated and non-agitated yeast.

System	Glycogen Content on inoculation (g/g d.w)	Initial cell concentration (cells/mL) $\times 10^5$	Final cell concentration (cells/mL) $\times 10^5$	Biomass Yield (cells/g glucose) $\times 10^7$	CO_2 Evolved (g)	CO_2 Yield (g/g glucose)	Growth rate (hr^{-1})
Agitated	18.48 ± 0.28	604 ± 4	778 ± 16	162 ± 38	0.25 ± 0.01	0.38 ± 0.01	0.017 ± 0.001
Non-Agitated	18.96 ± 0.83	609 ± 34	768 ± 18	158 ± 15	0.25 ± 0.01	0.39 ± 0.02	0.017 ± 0.001

6.3.2 SUMMARY

The agitation employed in the experiment set-up for final cooling temperature experiments (Section 5.2.3.1) had no effect on cell envelope (wall and membrane) integrity. Yeast growth and the ability of the yeast to adapt to the fermentation media were also not affected by the agitation. Hence, any damage to yeast observed in the jacketed agitated vessel on cooling can be attributed to the influence of cold stress.

6.4 EFFECT OF COLD STRESS ON YEAST QUALITY

Results detailed in Sections 6.2 and 6.3 indicate that dilution (osmotic stress) and agitation of yeast had no effect on cell envelope integrity and yeast growth. Prolonged storage of yeast in a nutrient depleted media did however result in protease release and reduced glycogen levels. The cold stress encountered on cooling diluted yeast slurries in jacketed agitated vessels may make yeast more susceptible to damage. This section presents results obtained from experiments investigating the influence of final cooling temperature and cooling rate on structural and functional integrity of yeast.

6.4.1 FINAL COOLING TEMPERATURE

Unwashed yeast was resuspended into deaerated deionised water at 14 °C (control), 12 °C, 8 °C and 4 °C to a 30 % consistency (by mass). The diluted yeast was agitated over a 4-hour period at 570 rpm, using a pitched-blade impeller of 50 mm in diameter. The yeast was maintained at temperature by coolant-circulating refrigerated units. Samples taken over the duration of the experiment were analysed for cell membrane damage, cell death associated with membrane damage, cell wall damage (fragility), physiological stress indicators (morphology, trehalose, hsp 12), glycogen content and changes in growth and metabolic activity (small-scale fermentation in MYPG media with approximately 10 g/L glucose).

6.4.1.1 RESULTS

The results of the assays performed on yeast to determine the effects of final cooling temperature on cell integrity are summarised in Table 6.10. There was no decrease in the total number of cells in suspension at the various final-cooling temperatures. This suggests that the temperature stress to which the yeast was subjected did not cause total cell disruption. A general increase in the change in protease absorbance of yeast was also observed with increasing low temperature stress (Table 6.10). This observation cannot be attributed to the absence of a nitrogen source only. Nitrogen limitation causes similar changes in protease absorbance irrespective of final cooling temperature or decreasing release with decreasing temperature owing to reduced metabolic activity. Thus, it is postulated that increasing cell membrane damage was found as the final temperature decreased. Moreover, the results of the methylene blue staining (% viability, Table 6.10) indicate that cell death through membrane damage and enzyme deactivation was most prominent in yeast cooled to 4 °C.

Table 6.10 Change in protease absorbance, viability and cell concentration of yeast 4 hours after dilution into water to a 30 % consistency across temperature range 14-4 °C (Δ = Final – Initial).

Final temperature (°C)	Δ Specific protease absorbance	Δ % Viability	Δ Cell concentration (%)
14	1.36	-1.0 ± 0.9	15.2
12	1.26	-1.0 ± 0.5	4.9
8	2.04	-1.0 ± 0.5	13.9
4	2.38	- 4.0 ± 0.9	2.0

Where the initial yeast concentration (cells/mL) at 14 °C was 8.4 E+07 ± 3.2 E+06, 12 °C was 8.60 E+07 ± 7.10 E +06, 8 °C was 8.20 E+07 ± 1.40 E+06, and 4 °C was 8.40 E+07 ± 2.10 E+06.

The exposure of yeast to low temperature stress is reported to lead to changes in cell structure and the manufacture of compounds that help preserve cell integrity (Thiering *et al.*, 1998). Sales *et al.* (2000) report that trehalose is one such compound. Trehalose prevents the fusion between adjacent membrane phospholipids at low temperature thus maintaining lipid bi-layer fluidity. In Chapter 4, it was postulated that the degree of change in membrane fluidity as a result of cold stress could be related to trehalose concentration. The greater the cold stress, the greater the reduction in membrane fluidity, and therefore the greater the amount of trehalose required to counteract this change. However, it is also reported that trehalose may accumulate under conditions of nutrient depletion (Oliver, 1991). The trehalose content of the yeast at various final cooling temperatures is presented in Figure 6.4. There was a 30 %, 6 % and 3 % increase in trehalose content of yeast diluted in 4 °C, 8 °C and 12 °C water respectively over the first two hours of the experiment, compared to a 7 % decrease in trehalose over the same period in yeast at 14 °C (the co-efficient of variance obtained from the trehalose assays was 3.8 %). Trehalose content of yeast held at 4 °C was still comparatively higher after 4 hours. As temperature was the only parameter varied across the experiments, the observed change in trehalose content suggests increased membrane stress with increasing duration and magnitude of cold stress. Membranes with retarded fluidity are more susceptible to developing leaks (Williams, 1990). Formation of trehalose is induced in response to cold-mediated membrane stress to restrict decreases in membrane fluidity.

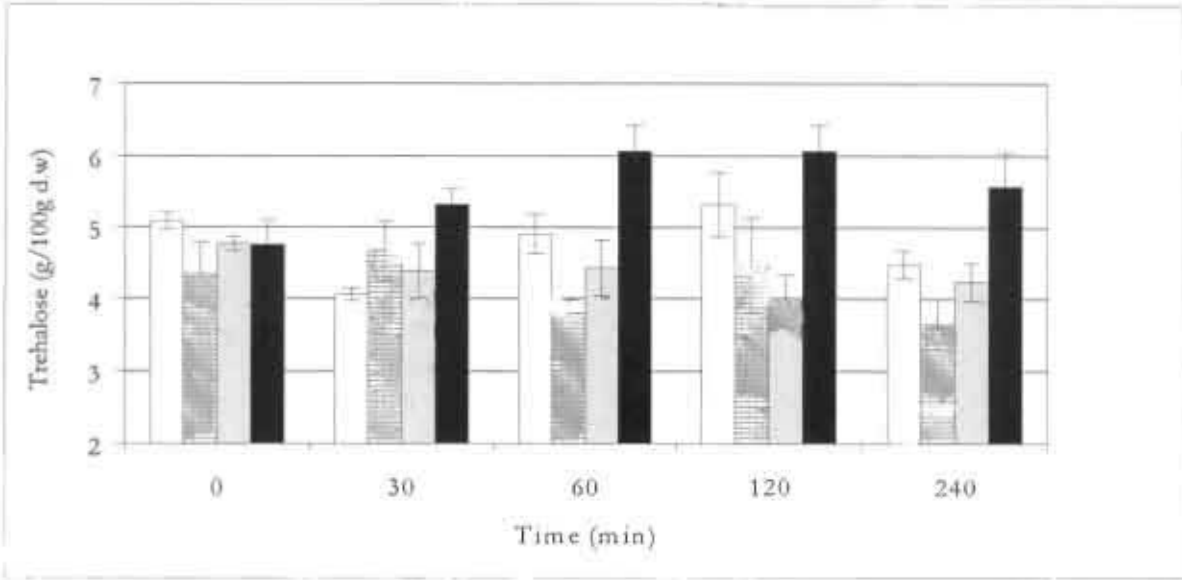


Figure 6.4 Changes in trehalose (content of yeast diluted in deionised dearated water at 14 °C □, 12 °C ▤, 8°C □ and 4 °C ■, and maintained at temperature for 4 hours.

Figure 6.5 shows the effect of final cooling temperatures on size distribution in yeast. There was little or no change in the amount of 0.1-3 µm particles detected with decreasing temperature of dilution. There was however a decrease in the predominant particle sizes present with decreasing temperature (morphology). Fischer *et al* (2001) report that these maxima correspond to the most common yeast cell size in the yeast slurries. The average yeast cell size was 5.69 µm at 4 °C, 6.63 µm at 8 °C, 6.63 µm at 12 °C, and 6.91 µm at 14 °C. In their experiments to evaluate the adaptive strategies for bacteria to near freezing conditions, Mindock *et al.* (2001) observed a 24 fold decrease in cell volume when comparing cells grown at 4 °C to those grown at 24 °C. This was attributed to the loss of cellular water from the bacteria at low temperatures. Furthermore, Mindock *et al.* (2001) report that this apparent desiccation leads to a high solute concentration in the cell, lowering the freezing point of the remaining water in the cytoplasm due to colligative effects, and thus confers cryoprotection. It is reported that this decrease in cell size may temporarily inhibit budding on returning the yeast to its growth temperature (Walker, 1998a).

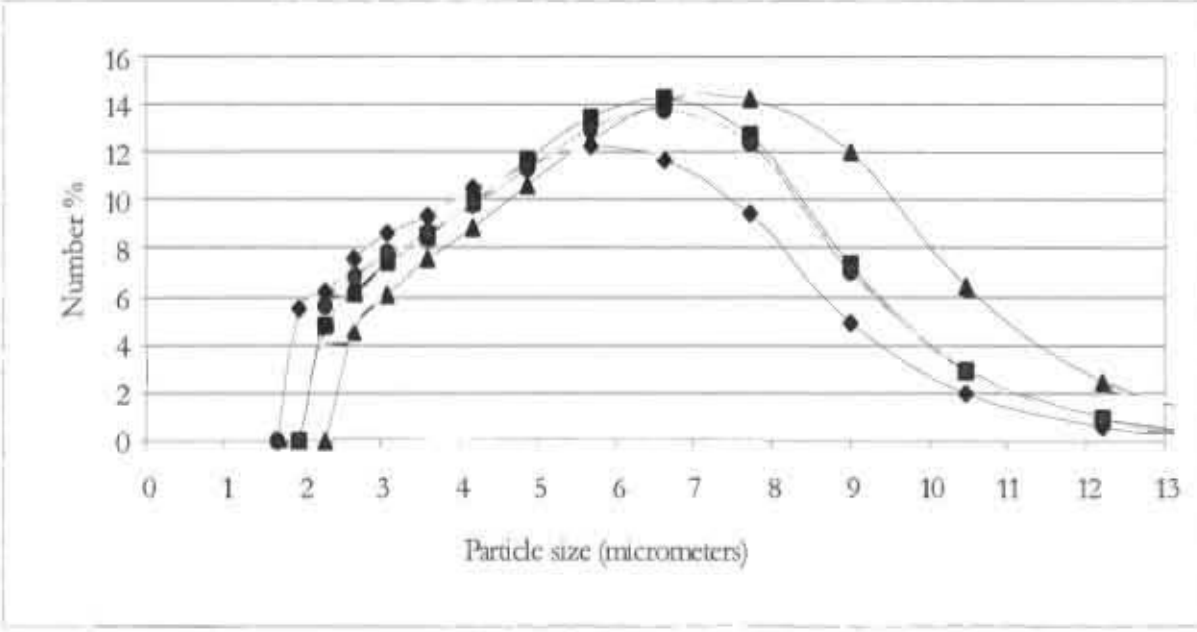
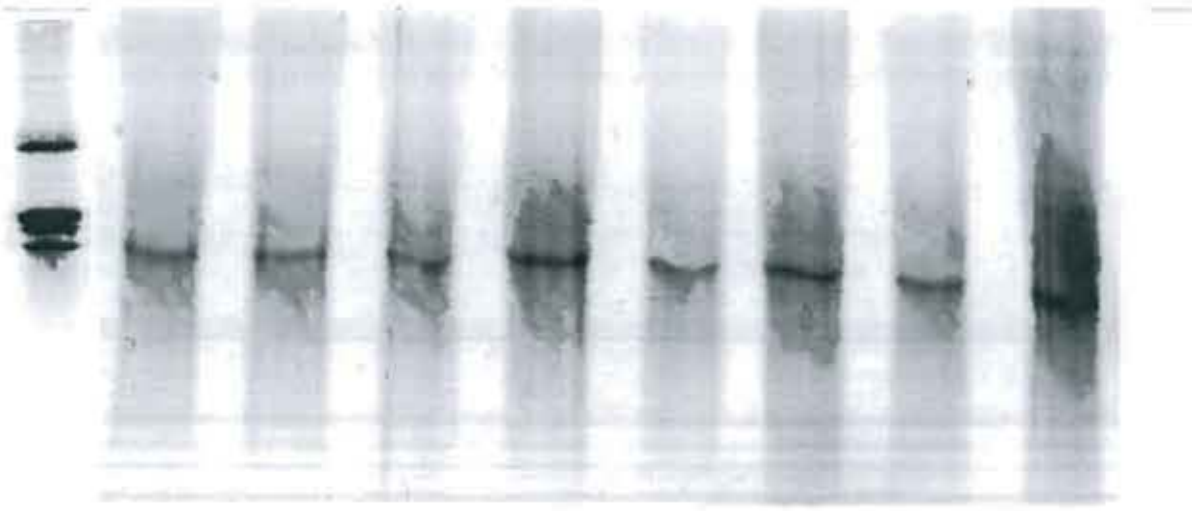


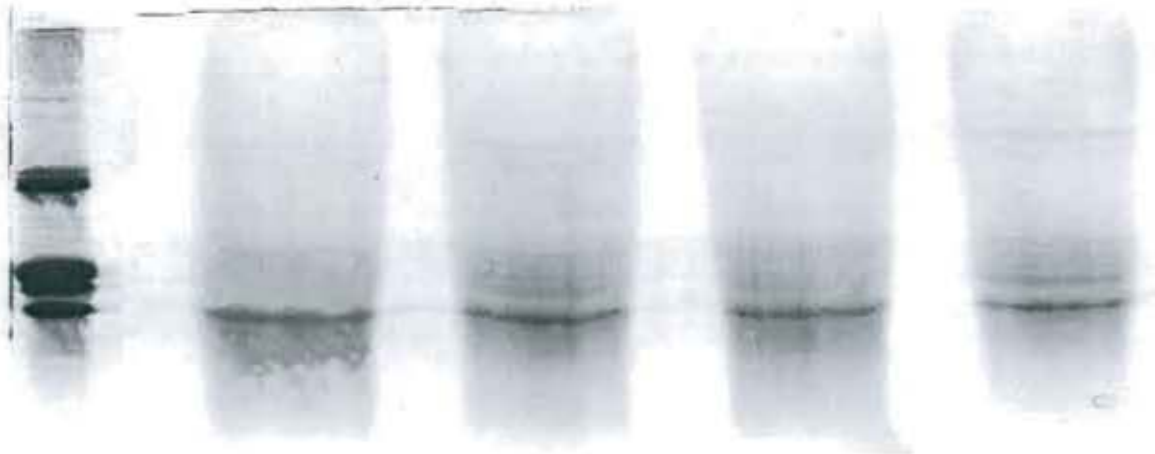
Figure 6.5 Particle size distribution of yeast diluted in deionised dearated water at 14 ^{0}C ▲, 12 ^{0}C ■, 8^{0}C ● and 4 ^{0}C ◆, and maintained at temperature for 4 hours.

Hsp 12 is a compound that is present in yeast cells under conditions that may lead to desiccation. This protein, identified as having a plasma membrane location, is postulated to interact electrostatically with charged groups present on the membrane surface. It is proposed that hsp 12 forms hydrogen bonds with proteins and glycolipids in the membrane, thereby providing a hydrophilic net to prevent membrane fusion in the desiccated state (Sales *et al.*, 2000). Gel electrophoresis was conducted to analyse for presence of hsp 12 in yeast resuspended in 14 ^{0}C, 12 ^{0}C, 8 ^{0}C and 4 ^{0}C water, following a 4 hour incubation (Figure 6.6). To determine whether osmotic stress aggravates desiccation at low temperature, hsp expression was compared in yeast diluted in 4 ^{0}C water and 4 ^{0}C sugar solution (MYPG). The protein gel obtained is shown in Figure 6.7.



(1) (2) (3) (4) (1') (2') (3') (4')

Figure 6.6 hsp 12 analysis by gel electrophoresis of yeast subjected to various temperatures. Lane (1) yeast at 12 °C, lane (2) 8 °C, lane (3) 14 °C and lane (4) 4 °C after 1hr. (') denotes analysis after 4 hours at temperature. 20 µL of protein was placed in each well.



(1) (2) (1') (2')

Figure 6.7 hsp 12 analysis by gel electrophoresis of yeast resuspended in water and MYPG. Lane (1) yeast after 1hr in 4 °C water, lane (2) yeast after 1 hr in 4 °C MYPG. (') denotes after 4 hours at temperature. 20 µL of protein was placed in each well.

The hsp 12 bands in Figure 6.6 and 6.7 were quantified using T-N Image Version 2.27 software (copyright T. Nelson 1995). The amount of protein in a particular band on the gel is proportional to the optical density of the band. The user selects the protein band to be quantified. The software then calculates the optical densities along the band. Unstained regions, such as those in-between adjacent bands, are automatically assigned an optical density of zero. The area underneath the curve representing optical density as a function of distance along the selected region is equivalent to the amount of protein present. The peak areas found are compared in Tables 6.11 and 6.12.

Table 6.11 Peak areas obtained on scanning hsp 12 bands from yeast diluted in 14, 12, 8 and 4 ^{0}C water.

Temperature (^{0}C)	Duration of exposure (hr)	Lane	Peak area
12	1	1	272
8	1	2	258
14	1	3	262
4	1	4	**413**
12	4	1'	237
8	4	2'	367
14	4	3'	295
4	4	4'	**510**

Table 6.12 Peak areas obtained on scanning hsp 12 bands from yeast diluted in cold MYPG and cold water.

Diluent	Duration of exposure (hr)	Lane	Peak area
Water	1	1	172
MYPG	1	2	**184**
Water	4	1'	185
MYPG	4	2'	**192**

In Table 6.11, the highest amount of hsp 12 was observed in yeast held at 4 ^{0}C. Particle size distribution results also show a marked decrease in yeast cell size at this temperature. It can therefore be argued that a 4 ^{0}C temperature stress may cause membrane dehydration. Loss of membrane bound water may lead to membrane fusion, compromising cell integrity. The expression of hsp 12 on cold shock at 4 ^{0}C corresponds to the desiccation observed, in accordance with the published role of hsp 12 in

conferring desiccation tolerance. The results in Table 6.12 show a marginal increase in the hsp 12 expression of yeast diluted in the cold MYPG than in cold water, suggesting a possible greater water loss in the lower water potential medium. Thus osmotic stress may enhance membrane dehydration at low temperature. A greater difference in hsp 12 levels is expected should a stronger osmolyte have been used. Sales *et al.* (2000) report a 14-fold increase in hsp 12 levels of yeast grown in 1.6 M mannitol compared with the same strain grown in 2 % (0.11 M) dextrose media. The loss of membrane bound water due to cold stress contributes to changes in membrane composition and fluidity.

The effect of final cooling temperature on the ability of the yeast cell envelope to withstand mechanical cell disruption (fragility) was measured in a French Press. The results obtained are illustrated in Figure 6.8. Yeast cooled to 4 ^{0}C consistently gave the highest protein release, corresponding to greater cell disruption, on increasing hydraulic pressure from 10 MPa to 30 MPa. There was however little difference in the protein release of yeast at 8 ^{0}C, 12 ^{0}C and 14 ^{0}C over this range. This suggests a weakening of the cell wall at low temperature (4 ^{0}C). To determine the nature of damage in the high-pressure homogeniser, the cell concentration and viability of cooled (4 hours at 4 ^{0}C) and control yeast (4 hours at 14 ^{0}C) were monitored before and after subjecting the yeast to a 30 MPa hydrostatic pressure in the French Press (Table 6.13). There was a 30 % decrease in cell concentration and a 15 % decrease in methylene blue viability of 14 ^{0}C yeast after exposure to the French Press. A 49 % decrease in cell concentration and a 35 % decrease in methylene blue viability of 4 ^{0}C yeast were found after exposure to the French press. Owing to the low methylene blue viabilities measured subsequent to the French Press, this decrease may be underestimated. The increased cell disruption at 4 ^{0}C confirms the hypothesis of a weakened yeast cell wall at low temperature. The decreased methylene blue viability at 4 ^{0}C indicates increased susceptibility of yeast cells to cell death associated with membrane damage and enzyme deactivation at low temperature.

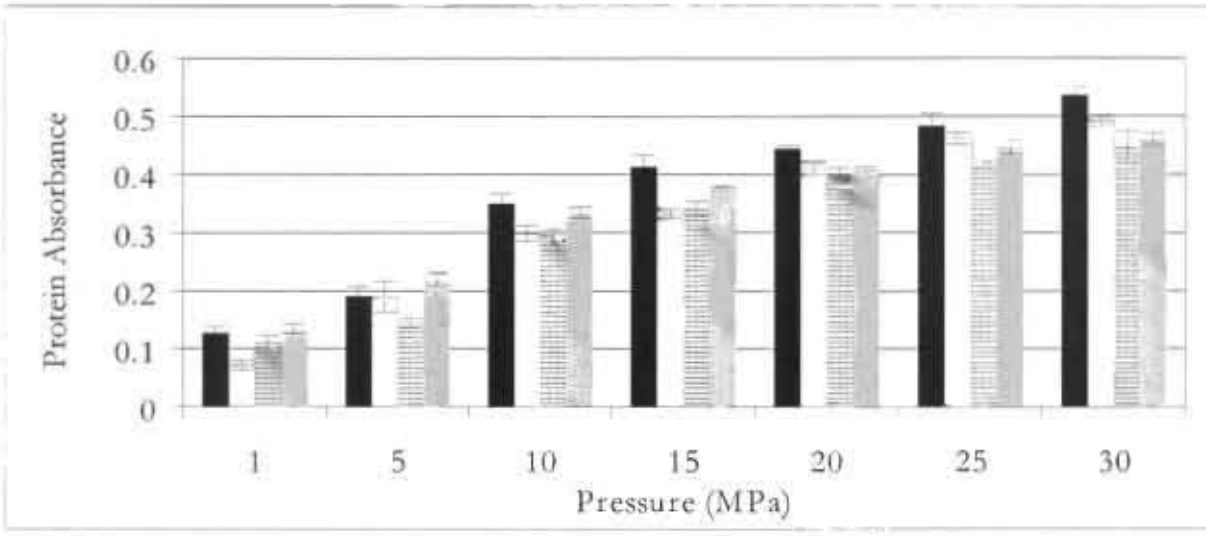


Figure 6.8 Changes in resilience of yeast cells (Fragility) diluted in deionised deaerated water at 14 °C ▭, 12 °C ▭ , 8°C ▭ and 4 °C ■ , maintained at temperature for 4 hours on exposure to various pressures in a French Press.

Table 6.13 Changes in cell concentration and % viability on subjecting yeast to mechanical stress in a French press.

Source of yeast	Cell concentration (cell/mL) $\times 10^3$	% Viability
Control yeast before French press	1580 ± 183	83.9 ± 6.1
Control yeast after French press	1110 ± 64	68.6 ± 4.1
Cooled yeast before French press	1560 ± 171	80.0 ± 2.5
Cooled yeast after French press	807 ± 70	44.5 ± 3.6

The glycogen content of the yeast at various final cooling temperatures is presented in Figure 6.9. There was little (1 %) difference in the glycogen content of yeast cooled to 4 °C with time. This suggests that there was no metabolism of the reserve carbohydrate at this temperature. However, there was an overall decrease in glycogen content of yeast held at the higher temperatures over the incubation period. Yeast at 8 °C, 12 °C and 14 °C showed a 11 %, 7 % and 22 % decrease in glycogen content over the 4-hour period respectively. This implies that yeast at temperatures of 8 °C and above is in a metabolically active state. In the absence of a suitable carbon source, glycogen is broken down to glucose, and further metabolized to provide energy for growth and maintenance (Hohmann and Mager, 1997).

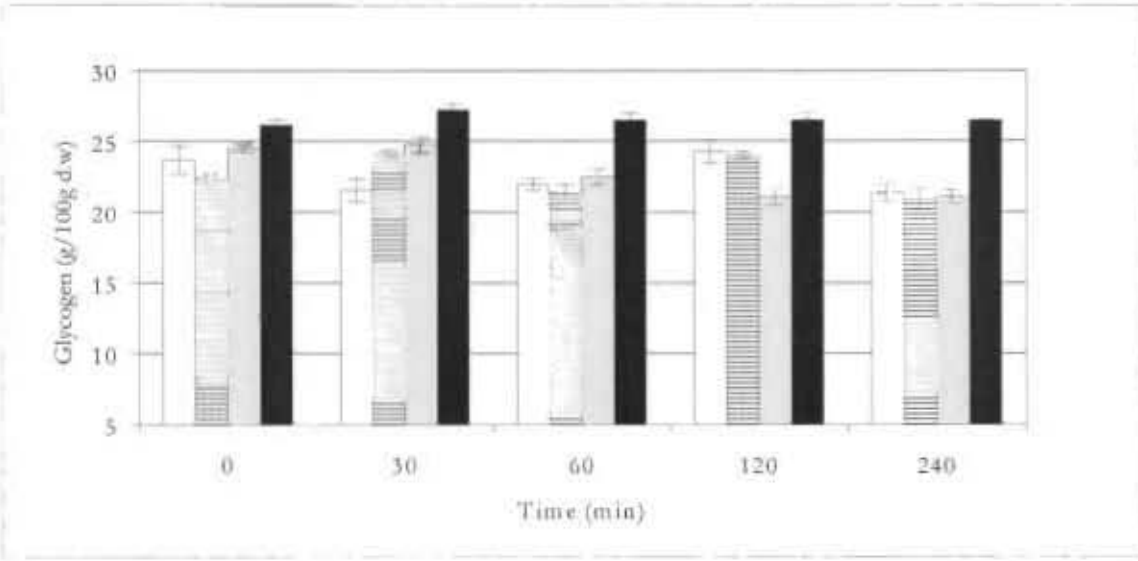


Figure 6.9 Changes in glycogen (content of yeast diluted in deionised dearated water at 14 °C ▨, 12 °C ▨, 8 °C □ and 4 °C ■, and maintained at temperature for 4 hours

Small-scale fermentation tests performed on yeast resuspended in deionised, deaerated water at 14 °C, 8 °C and 4 °C, after 1 hour at temperature are summarised in Table 6.14. The carbon dioxide evolution profiles of the yeast are given in Figure 6.10. Yeast at 4 °C showed a 16 % increase CO_2 yield compared with 8 °C and 14 °C yeast (Table 6.14). This may have been due to its superior glycogen content on inoculation (approximately 23 % higher than 8 °C and 14 °C yeast) as the end of fermentation glucose concentration in each flask was close to zero (<0.05 g/L). The CO_2 evolution profiles in Figure 6.10 are an indication of yeast metabolism over the course of the fermentation. Over the first 4 hours of fermentation, yeast held at 14 °C produced 40 % more CO_2 than 8 °C and 4 °C yeast. This indicates a lower metabolic activity in the cooled yeast over this period and suggests decreased ability to adapt to the fermentation medium. Pickerell *et al.* (1991) report that glycogen is the major endogenous source of energy for sterol and fatty acid production. These compounds are integral components of the cell membrane, and their concentration determines the extent of yeast cell growth. The results in Table 6.14 however indicate that 4 °C yeast had a 15 % lower biomass yield than 8 °C and 14 °C yeast. Lentini (1993) reports that damage to the cell membrane may affect the replicative ability of yeast. Protease release data (Table 6.10) shows possible membrane permeabilisation in the 4 °C yeast. Though there was no clear trend on the effect of final cooling temperature on growth rate, yeast held at 4 °C had the lowest growth rate. Walker (1998a) reports that the decrease in cell size observed at low temperatures may

temporarily inhibit budding on returning the yeast to its growth temperature. Yeast cooled to 4 °C showed a 14-18 % decrease in cell size compared to yeast at 8 °C and 14 °C.

Table 6.14 Summary of small-scale fermentation data of yeast in 14 °C, 8 °C and 4 °C deionised dearated water after 1 hour incubation.

Final Temp. (°C)	Glycogen Content on inoculation (g/g d.w)	Initial cell concentration (cells/mL) $\times 10^5$	Final cell concentration (cells/mL) $\times 10^5$	Biomass yield (cells/g glucose) $\times 10^7$	CO_2 Evolved (g)	CO_2 Yield (g/g glucose)	Growth rate (hr^{-1})
14	21.37 ± 0.39	195 ± 10	695 ± 21	467 ± 20	0.31 ± 0.01	0.48 ± 0.01	0.089 ± 0.009
8	22.50 ± 0.56	188 ± 25	685 ± 45	465 ± 17	0.31 ± 0.01	0.48 ± 0.01	0.099 ± 0.002
4	26.49 ± 0.47	181 ± 17	615 ± 22	405 ± 13	0.36 ± 0.01	0.55 ± 0.02	0.084 ± 0.001

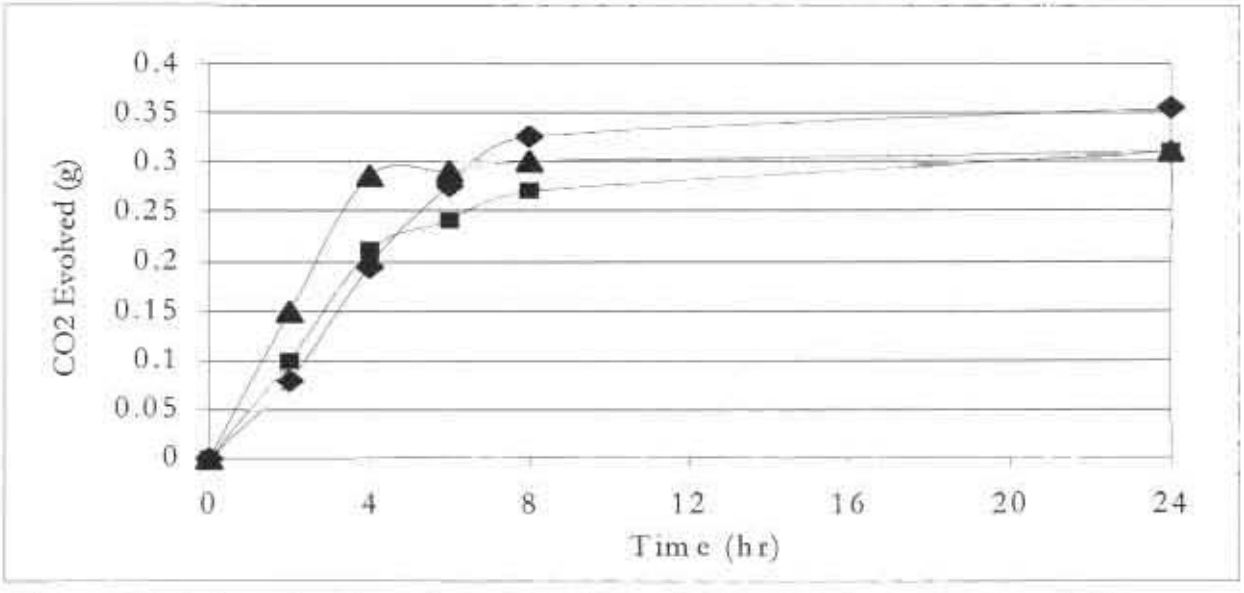


Figure 6.10 Carbon dioxide evolution data of yeast in deionised dearated water at 14 °C ▲, 8°C ■ and 4 °C ◆, and maintained at temperature for 4 hours, on vitality testing.

6.4.1.2 SUMMARY

Experiments performed to investigate the influence of final cooling temperature (14 °C (control), 12 °C, 8 °C and 4 °C) on yeast quality show that cold stress encountered on cooling diluted yeast slurries to 4 °C in jacketed agitated vessels may make yeast more susceptible to damage. There was an increase in protease absorbance of yeast with decreasing final cooling temperature at temperatures below 12 °C. Cold stress therefore made yeast more vulnerable to membrane damage. Moreover, methylene blue staining results indicate increased membrane permibilisation and enzyme deactivation in yeast subjected to 4 °C cold stress. In Chapter 4, it was postulated that the degree of change in membrane fluidity as a result of cold stress could be related to trehalose content. The greater the cold stress, the greater the change in membrane fluidity, and therefore the greater the amount of trehalose produced to counteract this change. Yeast held at 4 °C showed the most substantial increase in trehalose content (almost 30 %) suggesting increased membrane stress (decreased fluidity) at low final cooling temperature. Membranes with retarded fluidity are more susceptible to developing leaks (Williams, 1990). Particle size distribution data of yeast at the various final cooling temperatures showed a decrease in average yeast cell size with decreasing temperature. Mindock *et al.* (2001) report that the decrease in cell size is due to the loss of cellular water. This apparent desiccation plays an important role in conferring cryoprotection. Hsp 12 analysis of the yeast showed increased levels of the protein in yeast held at 4 °C. As hsp 12 is present in cells under conditions that may lead to membrane desiccation (Sales *et al.* 2000), it can be argued that low temperature stress may cause membrane dehydration, further reducing fluidity. The effect of final cooling temperature on the ability of the yeast cell envelope to withstand mechanical stress was measured in a French Press. The results show that a low final cooling temperature (4 °C) leads to an inherent weakening of both the cell membrane and cell wall. Small-scale fermentation tests performed on the yeast showed that yeast the yeast stored at 4 °C had reduced biomass yield and growth rate compared with control yeast.

6.4.2 SLOW AND FAST COOLING WITH DILUTION

To examine the effect of cooling rate on the extent of cold injury experienced by diluted yeast slurries during cooling in a jacketed agitated vessel, an experiment was performed in which washed yeast was resuspended in 4 °C water (fast cooling at an approximate cooling rate of 3.09 °C) to a 30 % consistency and maintained at 4 °C for 4 hours. Another batch of yeast was resuspended (30 % consistency) in 12 °C water and cooled to 4 °C at 2 °C/hr. The yeast was then held at 4 °C for 4 hours. As a control, yeast was also resuspended (30 % consistency) in 14 °C water and held at 14 °C for 4 hours. The yeast slurries were agitated at 570 rpm, using a pitched-blade impeller of 50 mm in diameter. Samples taken over the duration of the experiment were analysed for cell membrane damage, cell death associated with membrane damage, cell wall damage (fragility), physiological stress indicators (morphology, trehalose, hsp 12), glycogen content and changes in growth and metabolic activity (small-scale fermentation with approximately 10 g/L glucose).

6.4.2.1 RESULTS

The change in protease absorbance observed on slow and fast cooling of yeast is summarised in Table 6.15. The change in protease absorbance immediately on dilution is calculated as a difference between the protease absorbance found on resuspending yeast in 12 and 4 °C water (slow and fast cooling respectively) and that on resuspending yeast into 14 °C water (control). The change in protease absorbance induced by cold stress over the 4-hour incubation period is calculated as the difference in absorbance readings obtained after holding slow and fast cooled yeast at 4 °C for 4 hours and that of control yeast after 4 hours at 14 °C. No major difference was observed in protease absorbance of control, slow and fast cooled yeast immediately after dilution. Protease absorbance observed in slow cooled yeast over the 4 hour period required to achieve 4 °C was 3 fold greater than that of fast cooled yeast on reaching 4 °C. This does not necessarily entail membrane damage in slow cooled yeast as control yeast showed 29 % higher protease release over the same period. Thus, the protease release in slow cooled on achieving 4 °C may be largely due to the absence of a nitrogen source (Slaughter and Nomura, 1992). The protease release induced in fast cooled yeast over the 4 hour incubation was almost twice that induced in slow cooled yeast over the same period. This suggests that the extent of membrane damage on cooling yeast is influenced by the rate of cooling.

Table 6.15 Change in protease absorbance of control, fast and slow cooled yeast.

System	Protease absorbance on dilution	Protease absorbance on achieving 4 °C	Protease absorbance following 4hr incubation	Δ Protease absorbance on dilution	Δ Protease absorbance induced over 4hr incubation
Dilution at 14 °C (control)	0.04 ± 0.01	-	0.18 ± 0.01	0	0
Dilution at 12 °C (slow)	0.04 ± 0.01	0.14 ± 0.02	0.21 ± 0.01	0	0.03 ± 0.01
Dilution at 4 °C (fast)	0.05 ± 0.01	0.05 ± 0.01	0.25 ± 0.01	0.01 ± 0.01	0.07 ± 0.01

Figure 6.11 shows the change in trehalose content of slow and fast cooled yeast with time. There was a 30 % increase (relative to control) in the trehalose content of fast cooled yeast over its first 10 minutes at 4 °C. This indicates that the cell responded to counteract changes in membrane fluidity within this short period of exposure to cold stress. The trehalose content of the fast cooled yeast then remained constant over the 4-hour period (approximately 5.40 ± 0.50 g/100 g d.w). There was no difference in trehalose content of yeast on dilution into 12 °C water (beginning of the slow cooling process) and that of control yeast on dilution into 14 °C water (first 10 minutes, Figure 6.11). However, as slow cooling of the yeast progressed, its trehalose content also increased from 4.60 ± 0.10 g/100 g d.w to a maximum of 5.30 ± 0.03 g/100 g d.w on reaching 4 °C. This is postulated to concur with the counteracting of changes in membrane fluidity caused by the decreasing temperature in the vessel. The trehalose content of control yeast remained approximately 4.40 ± 0.05 g/100 g d.w throughout the experiment.

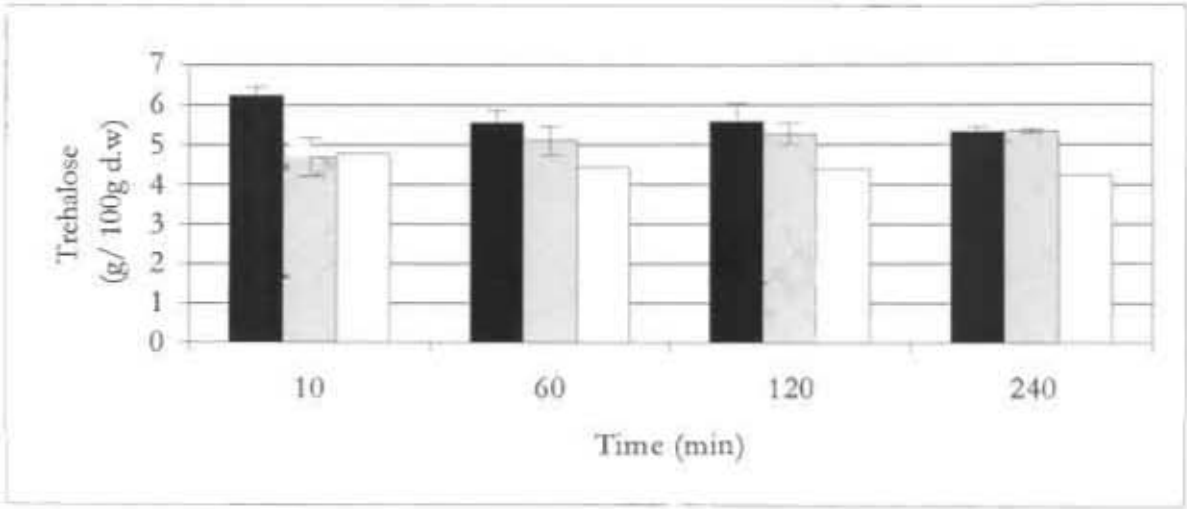


Figure 6.11 Changes in trehalose content of yeast during slow cooling □ , yeast being maintained at 4 °C after fast cooling ■, and control yeast □.

Changes in the particle size distribution of the control yeast (after 1 hour at 14 °C), fast cooled (after 1 hour at 4 °C) and slow cooled yeast (after 1 hour at 4 °C) are illustrated in Figure 6.12. There was negligible change in the number of 0.1-3 µm particles detected as a function of cooling or cooling rate. The predominant particle size was 6.63 µm in control yeast (after 1 hour at 14 °C), 5.70 µm in slow cooled yeast (after 1 hour at 4 °C), and 4.90 µm in fast cooled yeast (after 1 hour at 4 °C). This represents a decrease in mean yeast cell size with cooling, further augmented by an increased cooling rate. The additional reduction in size observed with fast cooled yeast either indicates a greater degree of desiccation or a greater loss of cellular material due to a more damaged membrane (Table 6.15).

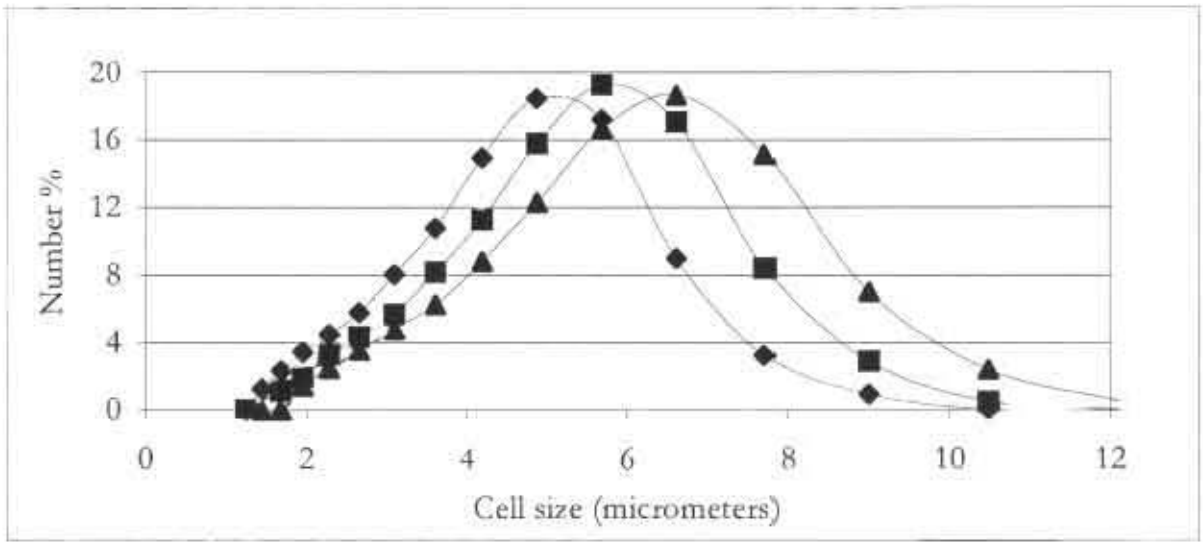


Figure 6.12 Particle size distribution of control yeast ▲, slow cooled yeast ■ and fast cooled yeast ◆.

Analysis of hsp 12 in slow and fast cooled yeast was conducted by gel electrophoresis, presented in Figure 6.13. Yeast samples for analysis were taken after 1 hour at 4 °C for slow cooled yeast, and after 1 hour at 4 °C for fast cooled yeast. In each case, further samples were taken following a 4 hour incubation at 4 °C. The hsp 12 bands on the gel were quantified using TN-Image Analysis software. The peak areas obtained on examining the hsp 12 bands are given in Table 6.16. It can be seen that the amount of hsp 12 expressed in the yeast was affected by the rate of cooling (Table 6.16). Fast cooled yeast produced 28 - 37 % more hsp 12 than slow cooled yeast over the 4-hour period at 4 °C. This suggests that the extent of desiccation experienced by the membrane was influenced by the rate of cooling. Loss of water from the cell membrane leads to

decreased fluidity (Sales *et al.* 2000). Retarded fluidity weakens membranes, reducing the membrane integrity (Walker, 1998a).

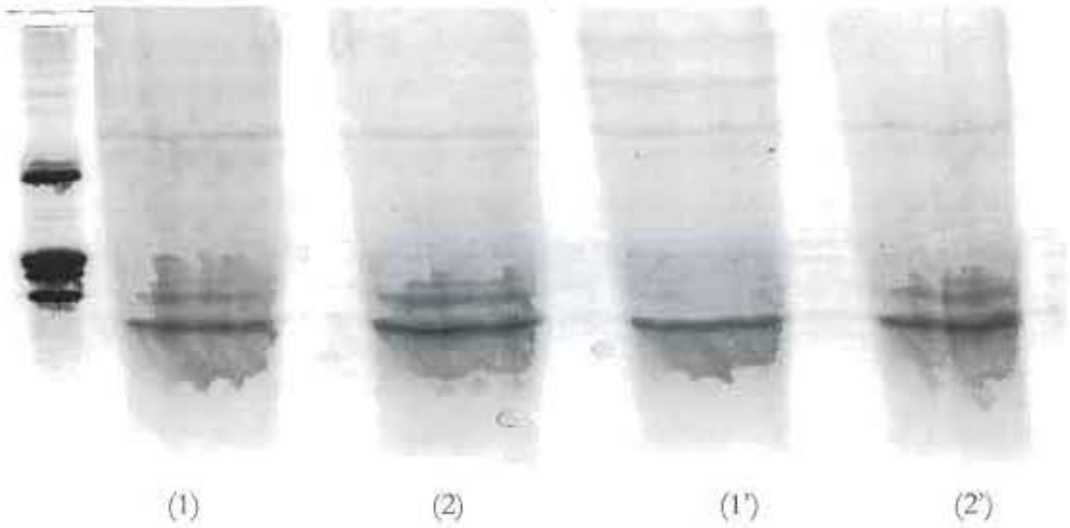


Figure 6.13 Protein gel used in hsp 12 analysis of fast and slow cooled yeast.(20 µL) Lane (1) slow cooled yeast after 1 hour, lane (2) fast cooled yeast after 1 hour. (1') slow cooled yeast after 4 hours at 4 ^{0}C. (2') fast cooled yeast after 4 hours at 4 ^{0}C.

Table 6.16 Peak areas obtained on quantifying hsp 12 bands from the protein gel of slow and fast cooled yeast.

System	Duration of exposure(hr)	Lane	Peak Areas
Slow cooling	1	1	164
Fast cooling	1	2	**211**
Slow cooling	4	1'	182
Fast cooling	4	2'	**249**

The effect of cooling rate on yeast resilience was quantified by resilience to mechanical cell disruption through exposure to the French Press (Figure 6.14). There was an increase in cell envelope (membrane and wall) fragility (increased protein release due to cell breakage on sudden pressure release) with cooling and cooling rate. Fargher and Smith (1995) report that if the rate of cooling is greater than the rate at which the cells can modify their membrane composition, then the cell membrane may become fixed in a random disorganised form, compromising integrity. The literature did not provide explanation for diminishing cell wall strength on low temperature stress. Moreover, in their work on the use of hydrodynamic cavitation for large scale microbial cell disruption, Save *et al.* (1997) observed that an increase in temperature weakens the cell wall and the

cell becomes more susceptible to breakage. Nonetheless, the results of the experiment indicate a decrease in cell wall strength with cooling and with cooling rate.

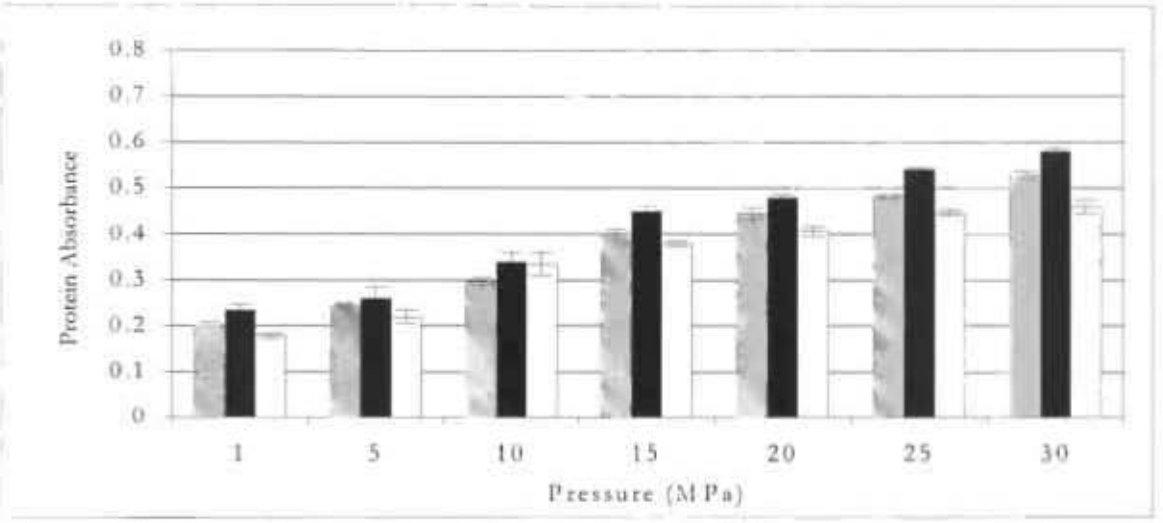


Figure 6.14 Comparison of yeast resilience to mechanical stress after slow cooling to 4 °C and being held there for 1hr □, after fast cooling to 4 °C and being held there for 1 hr ■, and after 1 hour at 14 °C (Control)□.

Glycogen data of control, slow and fast cooled yeast over a 4- hour period is presented in Figure 6.15. There was a 20 % decrease in glycogen content of slow cooled and control yeast over the first 4 hours of the experiment, indicating that the yeast was in a metabolically active state. Slow cooling therefore prolonged the exposure of metabolically active yeast to a nutrient deficient environment. Murray *et al.* (1984) report that reduced glycogen levels on inoculation correlate with reduced rate of attenuation and yeast growth. No change in glycogen content of fast cooled yeast was found over the duration of the experiment

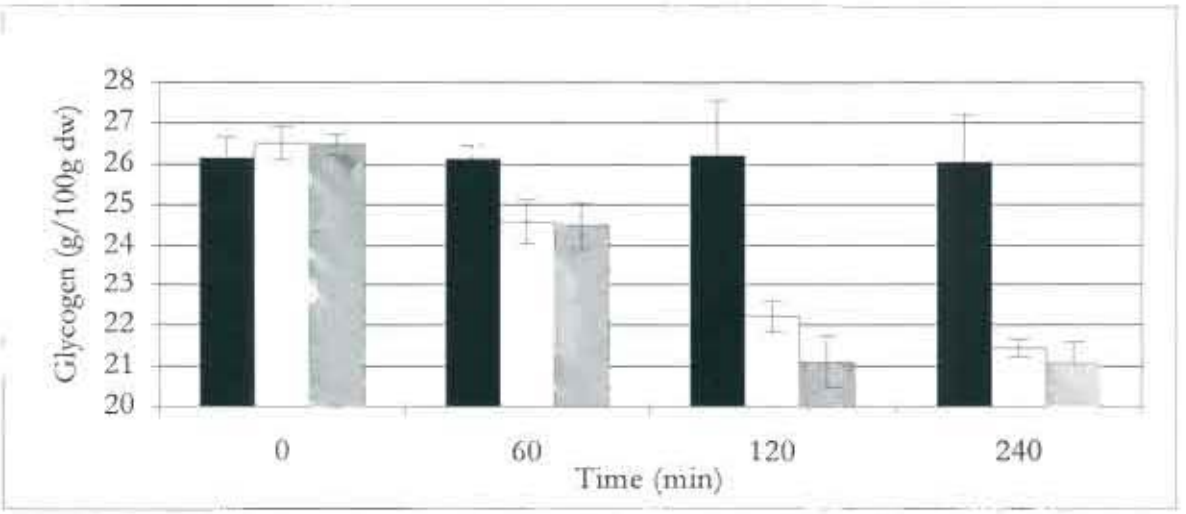


Figure 6.15 Changes in glycogen content of yeast during slow cooling □, yeast being maintained at 4 °C after fast cooling ■, and control yeast □.

Small-scale fermentations performed on slow cooled and fast cooled yeast are summarised in Table 6.17. The carbon dioxide evolution profiles of the yeast are given in Figure 6.16. The results suggest that there was no major difference in biomass yield with cooling rate. Slow cooled yeast did however exhibit a 5 % increase in cell growth rate (Table 6.17). Walker (1998a) reports that decrease in cell size may temporarily inhibit budding on returning the yeast to its growth temperature. The average cell size of slow cooled yeast was 5.70 µm whilst that of fast cooled yeast was 4.90 µm. In accordance, the growth rate of slow cooled yeast was slightly higher. The CO_2 evolution profiles in Figure 6.16 show that fast cooled yeast had a lower metabolic activity than slow cooled yeast during the early stages of fermentation (first 4 hours). The CO_2 evolved by fast and slow cooled yeast over this period of fermentation were 0.23 g and 0.30 g respectively. However, after 24 hours of fermentation, fast cooled yeast produced 10 % more CO_2 than slow cooled yeast. This may have been due to its higher levels of glycogen on inoculation (26 .10 ± 0.30 g/100 g d.w) than slow cooled yeast (21.00 ± 0.50 g/100 g d.w) as the end of fermentation glucose concentration in each flask was close to zero (<0.05 g/L).

Table 6.17 Small-scale fermentation data for slow cooled and fast cooled yeast following 1 hour incubation on attaining 4 ^{0}C.

System	Glycogen content on inoculation (g/g d.w)	Initial cell concentration (cells/mL) $\times 10^5$	Final cell concentration (cells/mL) $\times 10^5$	Biomass yield (cells/g glucose) $\times 10^7$	CO_2 Evolved (g)	CO_2 yield (g/g glucose)	Growth rate (hr^{-1})
Slow Cooled	21.00 ± 0.50	175 ± 16	625 ± 36	420 ± 6	0.40 ± 0.01	0.65 ± 0.02	0.125 ± 0.004
Fast Cooled	26.10 ± 0.30	149 ± 26	603 ± 34	424 ± 9	0.44 ± 0.01	0.68 ± 0.02	0.119 ± 0.001

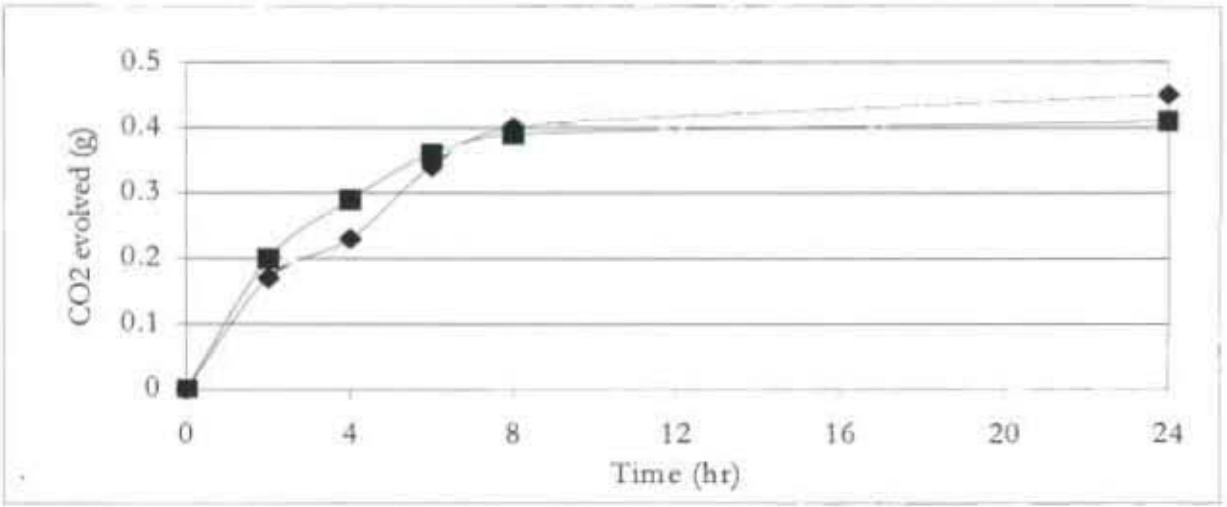


Figure 6.16 Carbon dioxide evolution data of fast ◆ and slow cooled ■ yeast on vitality testing following 1 hour incubation on attaining 4 ^{0}C.

6.4.2.2 SUMMARY

To determine the effect of cooling rate on extent of cold injury experienced by yeast slurries during cooling, an experiment was performed in which washed yeast (at 12 ^{0}C and 14 ^{0}C) was slow (2 ^{0}C/hr) and fast cooled (3.09 ^{0}C/sec) to 4 ^{0}C and held at temperature for 4 hours in a jacketed agitated vessel. As a control, yeast was also resuspended (30 % consistency) in 14 ^{0}C water and held at temperature for 4 hours. After 4 hours at 4 ^{0}C, fast cooled yeast showed an almost 200 % increase in protease absorbance compared with slow cooled yeast. This suggests that the extent of cold injury to the cell membrane increases with increasing cooling rate. Fast cooled yeast also showed a greater decrease in yeast cell size relative to slow cooled and control yeast. This indicates greater degree of desiccation with cooling rate. This observation was validated by the increase (approximately 37 %) in hsp 12 expression of fast cooled yeast compared

cooled yeast after 4 hours at 4 °C. Cell wall fragility also increased with fast cooling yeast. The small-scale fermentation tests performed on slow and fast cooled yeast however showed little or no difference in the growth or fermentative ability of the two yeasts.

6.4.3 RAPID COOLING IN A HEAT EXCHANGER

Having established that the extent of damage to yeast at low temperatures is influenced by the rate of cooling, work was conducted to investigate the effect of cooling rates employed in brewery heat exchangers on yeast quality. This was done in order to identify critical rates above which extensive cold injury will occur in yeast. The brewery cooling rates were simulated using laboratory scale cooling coils. The results of the experiments performed on unwashed yeast (resuspended in 14-18 °C water to a 30 % consistency) are presented here.

6.4.3.1 RESULTS

The change in protease absorbance observed as a function of cooling rate on rapid cooling of yeast is summarised in Table 6.18. This change was calculated as a difference between the protease absorbance of the yeast after passage through the cooling coils (post-cooling) and that before cooling (Section 5.3). Fargher and Smith (1995) report that the exposure of yeast to a rapid decrease in temperature may cause membrane components to become fixed in a random and disorganised fashion. In this state, the cell membrane may develop leaks resulting in the loss of cellular material. Table 6.18 shows there was an increase in protease release with increasing cooling rate. The release of cellular protease was however most prominent at cooling rates above 2.08 °C/sec.

Table 6.18 Change in protease absorbance of yeast as a function of cooling rate on rapid cooling.

Cooling rate (°C/sec)	Δ Protease absorbance
1.28	0.029 ± 0.012
2.08	0.025 ± 0.004
3.09	0.069 ± 0.007
3.35	0.146 ± 0.008
4.03	0.164 ± 0.006

The particle size distributions of yeast cooled at the various cooling rates are presented in Figure 6.17. To isolate yeast damage due to hydrodynamic effects (flow through the pump and the copper coils) from that due to rapid cooling, yeast was pumped through the coils at the highest flow rate achievable using the Marlow S40 pump (1.2 L/min) without any cooling. This "hydrodynamic run" was used as a control and thus any change in particle size distribution observed with cooling was attributed to the effects of cold stress. Fischer *et al* (2001) report that particles in the 0.1-2 μm size range can be classified as haze material and 2-3 μm particles as protein-polyphenol compounds. In order to compare the number of sub-3 μm particles generated at the various cooling rates effectively (Figure 6.17), the ratio of the areas underneath each peak in the 0.1-3 μm size range (Area 1) to that beneath the curve in the 3-10 μm range (Area 2) was calculated. The results obtained are displayed in Table 6.19. Figure 6.17 exhibits a common maximum cell size at approximately 7 μm across all cooling rates. This indicates that there was no change in the average yeast cell size on cooling and with increasing cooling rate, and therefore no cellular desiccation observed. There was however an increase in the proportion of sub-3 μm particles generated with increasing cooling rate (Table 6.19). Yeast cooled at rates above 1.28 ^{0}C/sec gave a 6-9 fold increase in sub-3 μm particles compared to control yeast. This shows that cold stress made the cell envelope more sensitive to hydrodynamic shear on flow through the copper coils and associated piping. The sensitivity of yeast to hydrodynamic shear increased with increased yeast cooling rate.

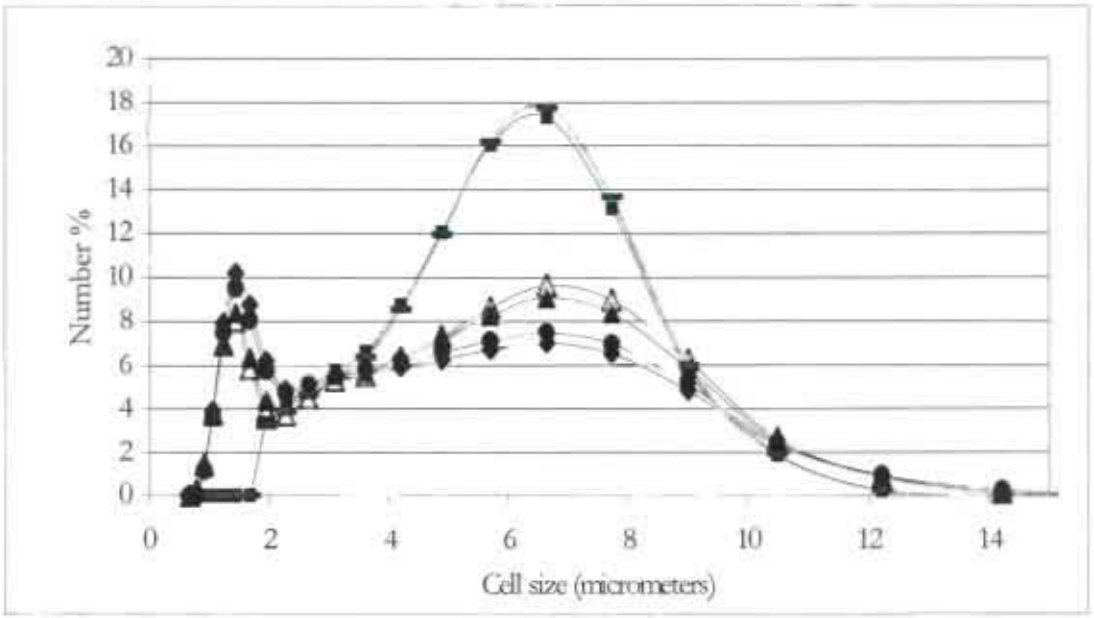


Figure 6.17 Effect of rapid cooling on particle size distribution. _ Hydrodynamic run (no cooling); ■ 1.28 °C/sec; △ 2.08 °C/sec; ▲ 3.09 °C/sec; ● 3.35 °C/sec; and ◆ 4.03 °C/sec.

Table 6.19 Effect of cooling rate on the ratio of sub3 micron to 3-10 micron particle.

Cooling rate (°C/sec)	Ratio (Area 1/Area 2)
Hydrodynamic	0.077
1.28	0.082
2.08	0.522
3.09	0.556
3.35	0.638
4.03	0.774

A further experiment to examine the influence of cold stress on the ability of yeast to withstand any subsequent hydrodynamic stress was conducted. A hydrodynamic run was performed with a batch of yeast at the highest achievable flow rate using the Marlow S40 pump (1.2 L/min). The yeast was then pumped through the copper coils at 1.2 L/min with cooling. The cooled yeast was recovered and pumped through the copper coils again, without any cooling (2nd hydrodynamic run). The particle size distributions of the yeast are given in Figure 6.18. The ratios of sub-3 μm to 3-10 μm particles in the individual curves of Figure 6.18 are given in Table 6.20. There was an 18 % increase (relative to control yeast) in the proportion of sub-3μm material present on rapidly chilling the yeast (cooling run). Yeast passed through the pump, coils and associated

tubing in the 2nd hydrodynamic run showed a 40 % increase (relative to control yeast) in the proportion of sub-3µm material present.

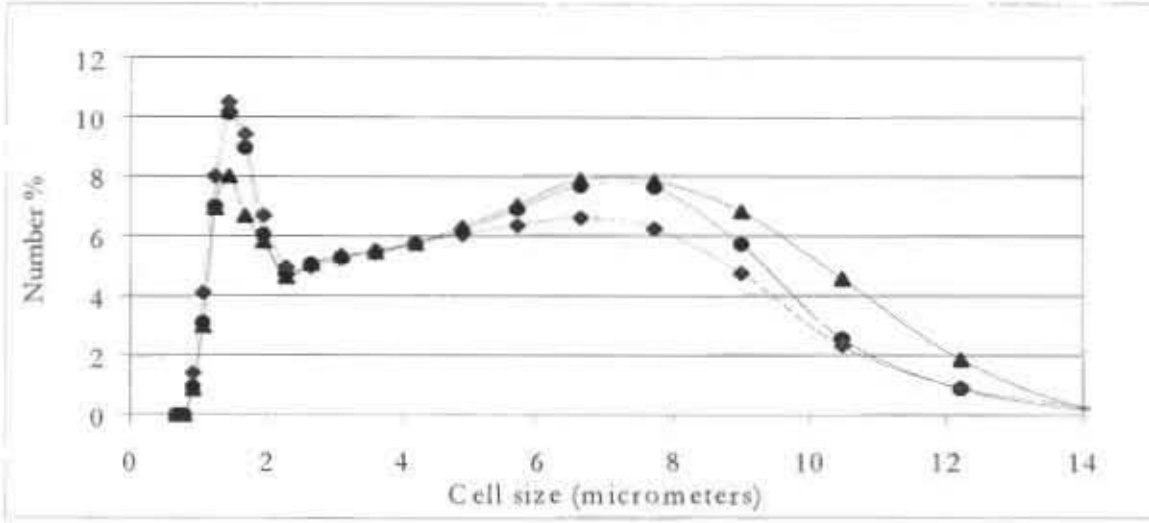


Figure 6.18 Effect of cold stress and hydrodynamic stress on particle size distribution. ▲ 1 pass through coils (no cooling), ● 1 pass through coils (cooling), followed by ◆ 1 pass through coils without any cooling.

Table 6.20 Effect of cooling rate on the ratio of sub2 micron to 3-10 micron particle.

Cooling rate (°C/sec)	Ratio (Area 1/Area 2)
Hydrodynamic run	0.626
Cooling run	0.736
2nd Hydrodynamic run	0.876

Efforts were made to determine the origin of this sub-3 µm material by staining techniques. Eosin yellow is reported to stain dextrin and proteinaceous matter (intracellular components), whilst lactophenol blue is specific for the cell wall material of fungi and yeast (Siebert *et al*, 1981). Aliquots of rapidly cooled yeast were placed in two test tubes with equal volumes of eosin yellow and lactophenol blue staining solutions respectively. After mixing, the samples were viewed as wet preparation under the following conditions with a light microscope: (1) Lactophenol Blue stain, brightfield illumination at ×400 magnification, (2) Eosin yellow stain, brightfield illumination at ×400 magnification. The photomicrographs obtained are presented in Figure 6.19 and 6.20.

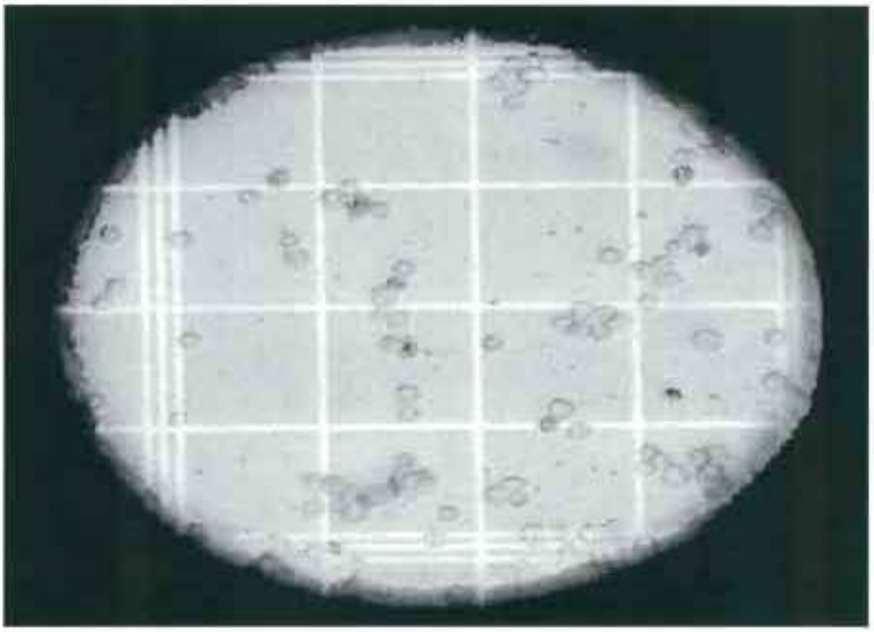


Figure 6.19 Photograph of haze stained with Lactophenol Blue stain. Brightfield illumination at ×400 magnification.

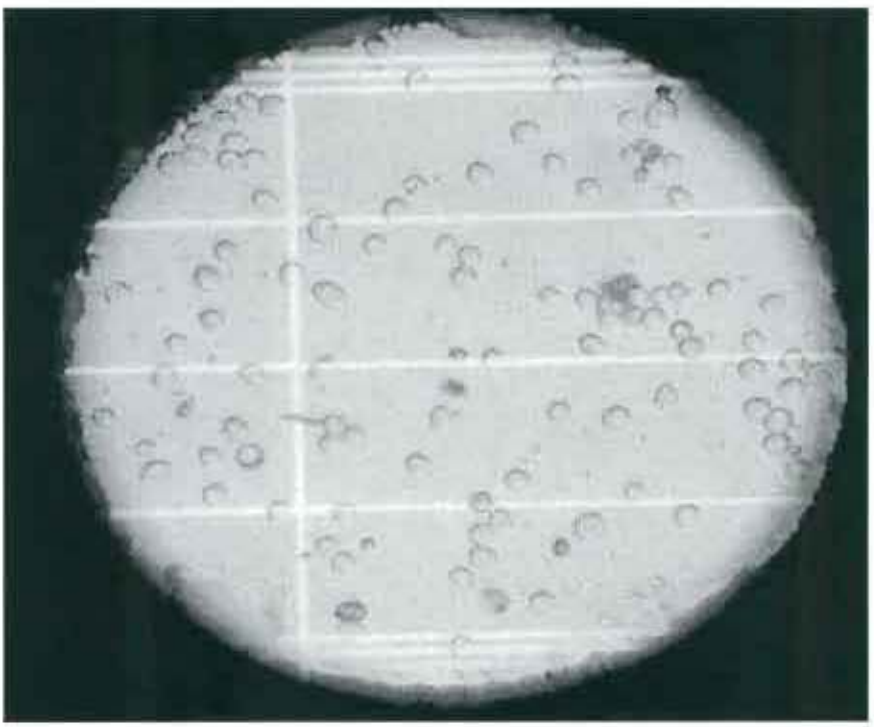


Figure 6.20 Photograph of haze stained with Eosin yellow stain. Brightfield illumination at ×400 magnification.

The photomicrographs in Figure 6.19 and 6.20 indicate that the amorphous material is both from the cytosol and the cell wall. This finding is in agreement with Fischer *et al.* (2001) who report sub-3 µm material as components from both the cell wall and the cytosol. Figure 6.19 and 6.20 also confirm the observation that the act of cooling leads to an inherent weakening of the cell wall and the cell membrane, the extent of which is dependent on the cooling rate. This manifests itself as the release of intracellular components through leaks in the cell membrane and the abrasion of the cell wall on exposure to hydrodynamic stress. Further evidence for this can be seen in Table 6.20 where additional damage to the cell envelope was observed on pumping chilled yeast through the through the experiment apparatus without any cooling (2nd hydrodynamic run). In the photomicrographs, there are some yeast cells which appear intact but are stained. The inability of these "intact" yeast cells to exclude the stains verifies the presence of membrane damaged cells. The presence of haze material and protein-polyphenol compounds in the yeast slurry may compromise beer quality.

Results of French Press experiments to determine the effects of rapid cooling on yeast resilience to mechanical cell disruption are given in Figure 6.21. There was an increase in cell envelope fragility with increasing cooling rate. Cell disruption and loss of membrane integrity represent the most severe types of injury occurring to cells in the French Press. This result is in accordance with the data presented in Figure 6.17 and 6.18.

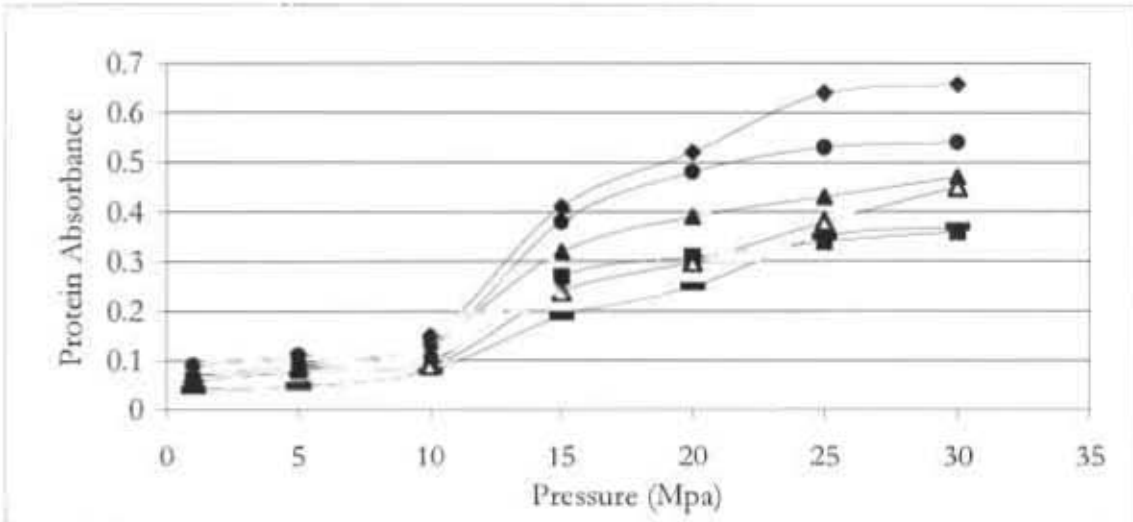


Figure 6.21 Effect of rapid cooling on yeast resilience. _ Hydrodynamic run (no cooling); ■ 1.28 °C/sec; △ 2.08 °C/sec; ▲ 3.09 °C/sec; ● 3.35 °C/sec; and ◆ 4.03 °C/sec.

Results obtained on conducting small-scale fermentations on the rapidly cooled yeast are summarised in Table 6.21 and the carbon dioxide evolution profiles obtained are given in Figure 6.22. The results in Table 6.21 show that there was no major difference in the biomass yields of control yeast and yeast exposed to 1.28 ^{0}C/sec and 2.08 ^{0}C/sec cooling rates. Yeast cooled at 3.09 ^{0}C/sec and 4.03 ^{0}C/sec however had a 29 % and 23 % lower yield respectively compared to control yeast, indicating subtle injury to the mechanisms that control the cells' ability to replicate with increasing cooling rates (cell membrane integrity, Table 6.18). The CO_2 evolution profiles in Figure 6.22 are an indication of yeast metabolism over the course of the fermentation. Yeast cooled at cooling rates above 2.08 ^{0}C/sec showed a 24 % decrease in carbon dioxide evolution compared to control yeast over the first 4 hours of the fermentation. This shows a lowered metabolic activity of the yeast over this period, indicating a reduced ability of yeast to adapt to the fermentation media. Yeast cooled at cooling rates at and above 2.08 ^{0}C/sec showed 8-14 % reduction in the overall carbon dioxide yields relative to control yeast. This may have been due to the 8-9 % lower glycogen content of the yeast on inoculation as the end of fermentation glucose concentration in each flask was close to zero (<0.05 g/L).

Table 6.21 Summary of vitality data of yeast cooled at various rates.

Cooling rate (^{0}C/sec)	Glycogen content on inoculation (g/g d.w)	Initial cell concentration (cells/mL) $\times10^5$	Final cell concentration (cells/mL) $\times10^5$	Biomass Yield (cells/g glucose) $\times10^7$	CO_2 Evolved (g)	CO_2 yield (g/g glucose)	Growth rate (hr^{-1})
No Cooling	27.37 ± 0.80	258 ± 10	356 ± 25	64 ± 3	0.41 ± 0.01	0.55 ± 0.01	0.025 ± 0.001
1.28	27.86 ± 1.69	279 ± 53	361 ± 22	66 ± 3	0.40 ± 0.02	0.54 ± 0.02	0.025 ± 0.001
2.08	25.22 ± 2.28	270 ± 8	350 ± 63	65 ± 2	0.38 ± 0.01	0.51 ± 0.01	0.025 ± 0.003
3.09	24.89 ± 1.69	280 ± 38	340 ± 35	50 ± 4	0.34 ± 0.01	0.46 ± 0.01	0.023 ± 0.002
4.03	24.93 ± 1.44	269 ± 40	370 ± 11	52 ± 6	0.35 ± 0.02	0.47 ± 0.02	0.023 ± 0.001

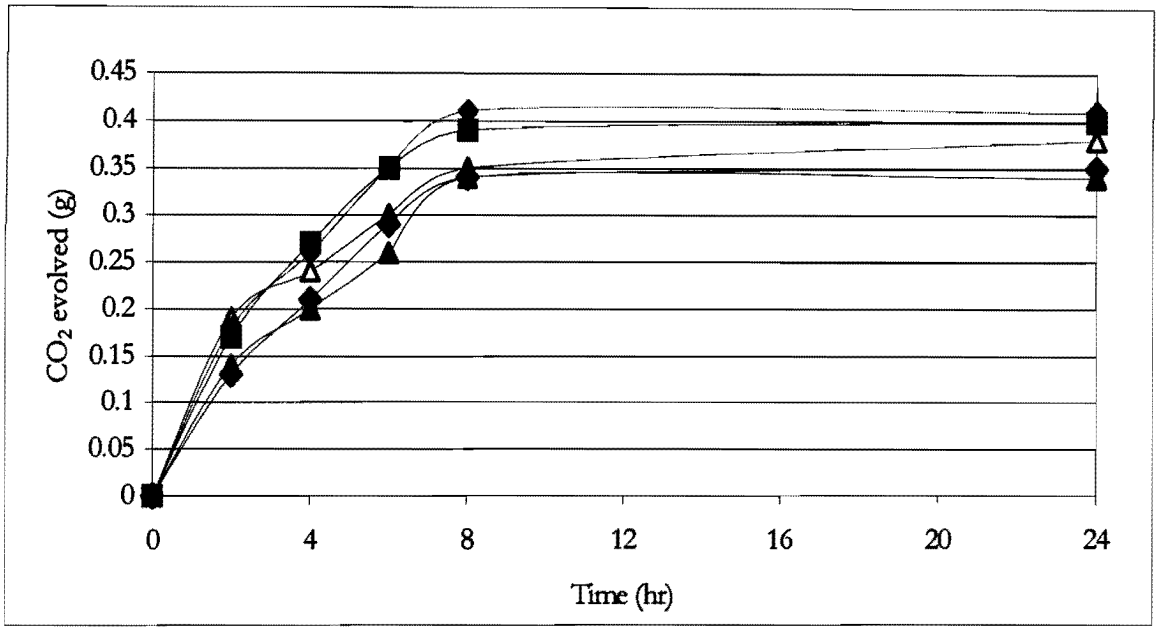


Figure 6.22 Carbon dioxide evolution data of yeast after ◆Hydrodynamic run (no cooling); cooling at ■ 1.28 ^{0}C/sec; Δ 2.08 ^{0}C/sec; ▲ 3.09 ^{0}C/sec; and ◆ 4.03 ^{0}C/sec.

6.4.3.2 SUMMARY

Work was conducted in the laboratory to investigate the effect of cooling rates employed in brewery heat exchangers on yeast quality. This was done in order to identify cooling rates above which extensive cold injury occurs in yeast. The cooling rates used in the experiments ranged from 1.28 to approximately 4.03 ^{0}C/sec. There was an increase in membrane damage (protease release) with increasing cooling rate. The release of cellular protease was however most significant at cooling rates above 2.08 ^{0}C/sec. The yeast cell wall also became more susceptible to shear damage (haze formation) with increasing cooling rate. This was further confirmed by French Press experiments in which the extent of cell disruption (weakening of the cell wall) increased with increasing cooling rate. This apparent weakening of the cell wall was again most prominent at cooling rates above 2.08 ^{0}C/sec. Further, small-scale fermentation results conducted on the yeast showed lowered biomass yield and reduction in CO_2 yield (fermentative ability) with increasing cooling rate above 2.08 ^{0}C/sec.

6.4.4 YEAST COOLING IN THE BREWERY

Laboratory experiments on the effects of rapid cooling in a heat exchanger on yeast indicate an overall weakening of the cell envelope as well as reduced growth and fermentative ability with increasing cooling rate. To assess whether similar effects would be observed in the brewery, on-line cooling trials were conducted at SAB-Newlands. Brewery cropped yeast (14 ^{0}C) was pumped through a plate and frame heat exchanger at cooling rates ranging from approximately 1.4 to 3.0 ^{0}C/sec.

6.4.4.1 RESULTS

The change in protease absorbance observed on cooling yeast in the brewery was calculated as a difference between the protease absorbance of the yeast before and after flowing through the plate and frame heat exchanger (Table 6.22). During the hydrodynamic run, there was a general increase in the protease release with increasing flow rate through the chiller (Table 6.22). This suggests that the shear stress experienced by the yeast as it passed through the bends and constrictions of the heat exchanger increased with increasing flow rate. This hydrodynamic stress led to cell membrane damage. On introducing cooling, there was an overall increase in protease release (compared with hydrodynamic run), the most prominent of which was by almost 200 % at a mass flow of 80 kg/min. On-line brewery experiments conducted by Robinson (2001) showed a 40 % increase in protease release of on passage through the heat exchanger (65 Kg/min). Thus, the intactness of the cell membrane showed a decrease on cooling in the heat exchanger. Table 6.22 shows that as the flow rate, and therefore cooling rate, was increased, so did the protease release from the yeast. Rapid cooling of yeast may lead to detrimental changes in cell membrane structure, making it prone to damage (Fargher and Smith, 1995). The release of cellular protease was highest at a cooling rate of 3.03 ^{0}C/sec (the highest rate tested).

Table 6.22 Change in protease absorbance on cooling yeast in a brewery heat exchanger.

Mass flow (kg/min)	Cooling rate (^{0}C/sec)	Δ Protease absorbance (hydrodynamic run)	Δ Protease absorbance (cooling)	Combined effect of cooling and hydrodynamic run
40	1.43	0.017 ± 0.005	0.004 ± 0.003	0.021 ± 0.006
60	2.31	0.033 ± 0.003	0.044 ± 0.006	0.077 ± 0.007
80	3.03	0.035 ± 0.003	0.065 ± 0.004	0.100 ± 0.005

The effect of cooling rate on the particle size distribution of yeast is presented in Figure 6.23, 6.24 and 6.25. Figure 6.23 shows that the hydrodynamic effects encountered by the yeast on passage through the positive displacement pump and the heat exchanger, in the absence of cooling, did not generate haze from the yeast. There was however an increase in protein-polyphenol compounds, particles in the 2-3 μm size range (Fischer *et al*, 2001), and a 14 % decrease in average yeast cell size with cooling at 1.4 ^{0}C/sec (from 5.70 μm in the hydrodynamic run to 4.88 μm on cooling). The apparent decrease in average yeast cell size is postulated to be due to the voluntary expulsion of cellular water at low temperatures as reported by Franks and Mathias (1990) or as a result of the loss of intracellular material through the damaged cell membrane. The effect of hydrodynamic stress on the particle size distribution due to flow through the positive displacement pump only, and flow through the pump and heat exchanger with and without cooling can be seen in Figure 6.24. There was an increase in 2-3 μm particles on passing the yeast through the heat exchanger (hydrodynamic run), suggesting some degree of damage to the yeast cell membrane. Therefore, the hydrodynamic stress experienced by the yeast, at 60 Kg/min, in the heat exchanger is greater than that due to passage through the pump. The appearance of 0.1-2 μm particles (haze) with the introduction of cooling suggests cell wall damage. Comparing Figure 6.24 to 6.23 shows an increase in cell envelope fragility with increasing cooling rate. The average cell size (approximately 6.60 μm) of the yeast in Figure 6.24 decreased to 5.75 μm on cooling. Figure 6.25 shows that increasing the flow rate further leads to an even greater presence of sub-3 micron particles. There was an increase in the amount of haze generated suggesting increased injury to the yeast cell wall. The yeast cell size also decreased from approximately 6.3 μm to 5.65 μm. This data represents the most significant damage to the yeast cell envelope in the brewery trials.

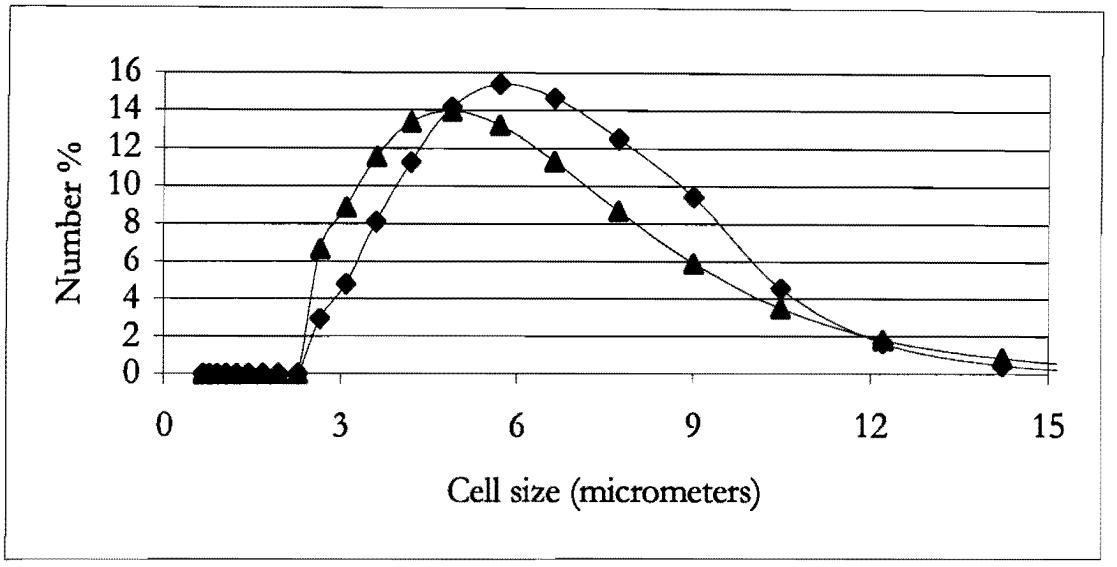


Figure 6.23 Particle size distribution of yeast; ◆Hydrodynamic run at 40 Kg/min, ▲ cooling run at 40 Kg/min (1.43 ^{0}C/sec).

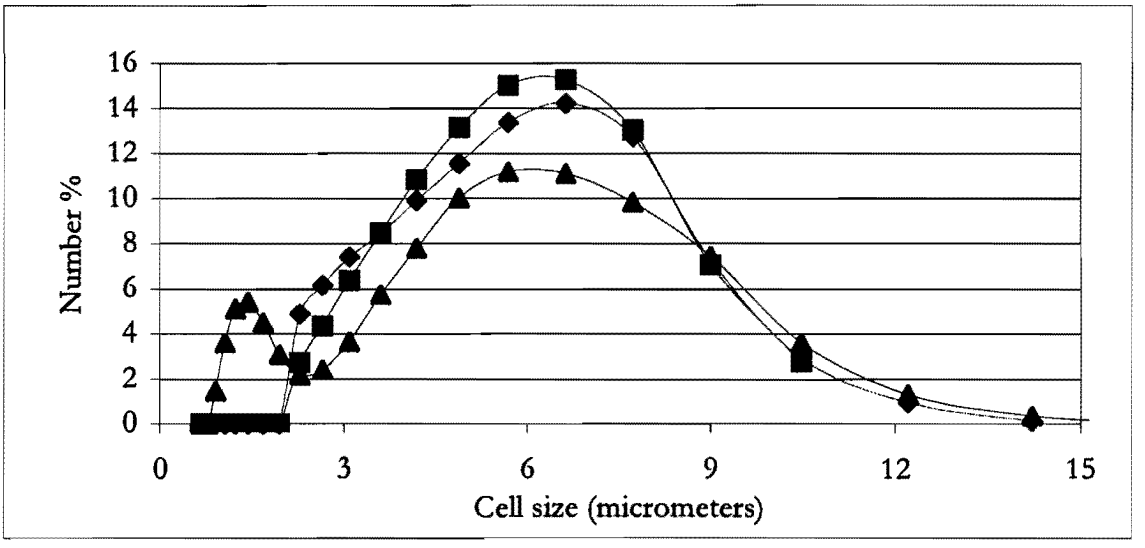


Figure 6.24 Particle size distribution of yeast; ■ After passage through pump at 60 Kg/min, ◆Hydrodynamic run (passage through pump and heat exchanger) at 60 Kg/min, ▲ cooling run at 60 Kg/min (2.31 ^{0}C/sec).

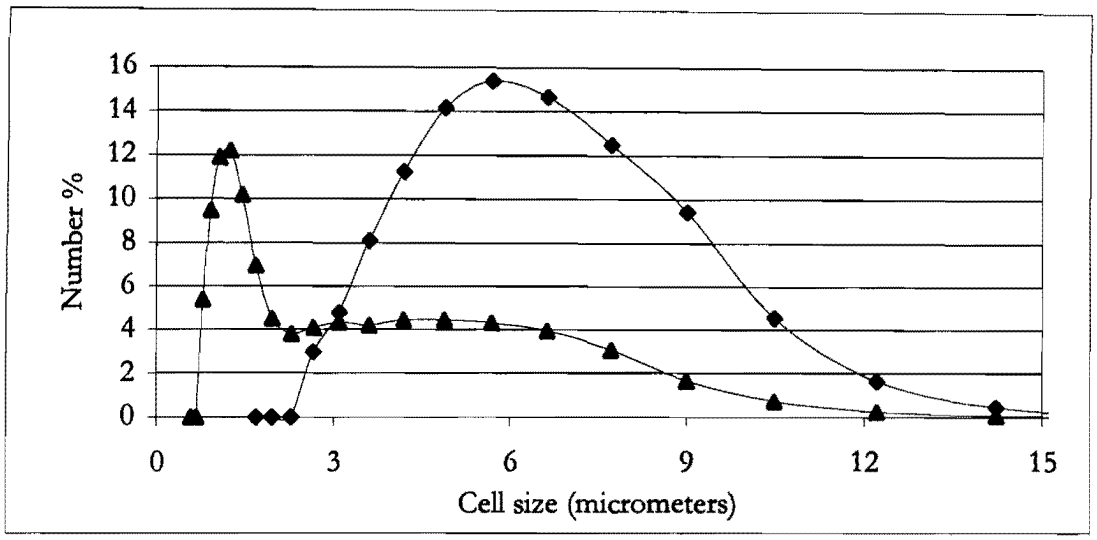


Figure 6.25 Particle size distribution of yeast; ◆Hydrodynamic run at 80 Kg/min, ▲ cooling run at 80 Kg/min (3.03 ^{0}C/sec).

Results obtained on conducting vitality tests on the yeast are summarised in Table 6.23. Yeast passed through the heat exchanger at 60 kg/min without any cooling was used as a control. The carbon dioxide evolution profiles of the yeast are given in Figure 6.26. The end of fermentation glucose concentration in each flask was close to zero (<0.05 g/L). The results of the small-scale fermentation suggest that there was a decrease in biomass yield on cooling and with increasing cooling rate (Table 6.23). Yeast cooled at 1.43 ^{0}C/sec and 3.03 ^{0}C/sec showed a 2 % and 12 % decrease in biomass yield respectively compared to control yeast (no cooling) where the typical co-efficient of variance in biomass data was 4.5 %. There was little or no difference in the growth rate of control yeast and yeast cooled at 1.43 ^{0}C/sec and 2.31 ^{0}C/sec. However, the growth rate following cooling at 3.03 ^{0}C/sec was reduced by 14 % with respect to control. The amount of CO_2 evolved decreased on cooling and with increasing cooling rate. Figure 6.26 shows that yeast cooled at 1.43 ^{0}C/sec and 2.31 ^{0}C/sec produced 21 % less CO_2 than control yeast during the first 4 hours of fermentation. Over the same period, yeast cooled at 3.03 ^{0}C/sec showed a 33 % decrease in CO_2 evolved compared to control yeast. These findings agree with those observed in laboratory tests (Section 6.4.3.1). Fargher and Smith (1995) report that membrane damage observed on cold shocking yeast may result in reduced growth rate and biomass yields. They report vacuole re-arrangement on fast cooling yeast, which has a detrimental effect on the ability of the yeast to metabolise glucose.

Table 6.23 Vitality data for brewery cooled yeast.

Cooling rate (°C/cec)	Initial cell concentration (cells/mL) $\times 10^5$	Final cell concentration (cells/mL) $\times 10^5$	Biomass yield (cells/g glucose) $\times 10^7$	CO_2 Evolved (g)	CO_2 yield (g/g glucose)	Growth rate (cells/mL hr)
No cooling	76 ± 3	140 ± 5	50 ± 2	0.52 ± 0.02	0.70 ± 0.03	0.063 ± 0.003
1.43	73 ± 2	134 ± 8	49 ± 5	0.48 ± 0.01	0.64 ± 0.01	0.062 ± 0.002
2.31	71 ± 4	130 ± 5	46 ± 2	0.42 ± 0.01	0.56 ± 0.01	0.062 ± 0.002
3.03	68 ± 5	100 ± 4	45 ± 3	0.35 ± 0.01	0.47 ± 0.01	0.054 ± 0.003

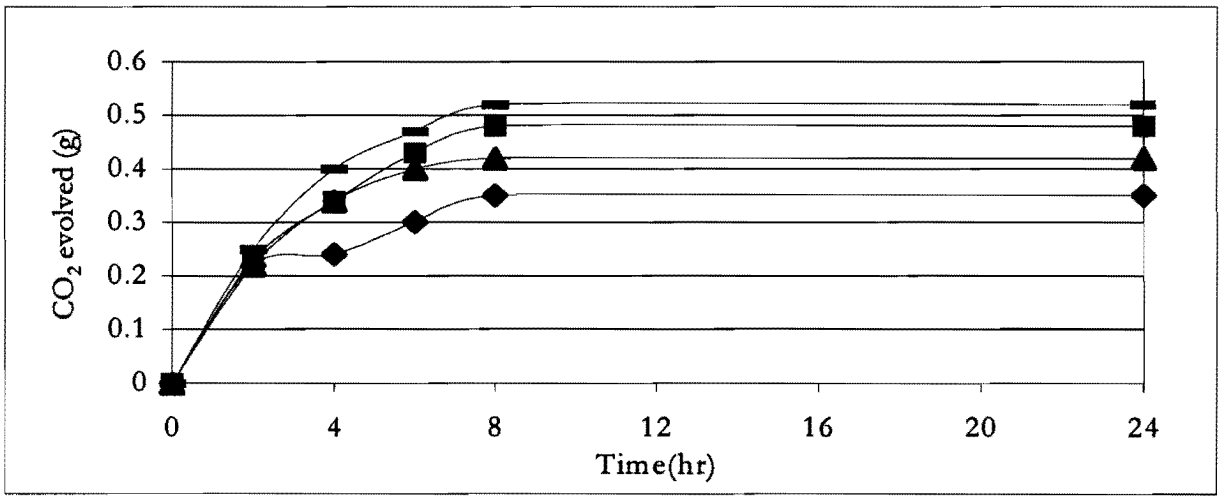


Figure 6.26 Carbon dioxide evolution data of yeast after _ Hydrodynamic run (no cooling); cooling at ■ 1.43 °C/sec; ▲ 2.31 °C/sec; and ◆ 3.03 °C/sec.

6.4.4.2 SUMMARY

Laboratory scale experiments on the effects of brewery cooling rates on yeast quality identified cooling rates above 2.08 °C/sec as critical with respect to extent of cold injury in yeast. On-line trials were conducted at SAB-Newlands in order to investigate whether similar effects would be observed in the brewery. Cropped yeast (14 °C) was pumped through a plate and frame heat exchanger at cooling rates ranging from 1.4 to approximately 3.0 °C/sec. Again, cell membrane (protease release) and cell wall fragility (haze formation) increased with increasing cooling rate, the most prominent of which was at 3.03 °C/sec (highest flow rate tested). Biomass yield, yeast growth rate and CO_2 evolution also decreased with increasing cooling rate.

6.5 CHAPTER SUMMARY

The results detailed in the chapter were obtained from experiments performed to assess the potential for a loss in yeast quality as a result of cooling of diluted yeast slurries in a jacketed agitated vessel and in a heat exchanger. These experiments were carried out in order to determine the effects of osmotic shock, agitation, final cooling temperature and rate of cooling on the physiological state of yeast and its ability to withstand subsequent mechanical handling. Key findings from this work are outlined below.

6.5.1 THE EFFECT OF OSMOTIC STRESS AND AGITATION IN A JACKETED AGITATED VESSEL

To determine the effect of osmotic stress on yeast quality, washed yeast at 14 ^{0}C was resuspended in each of sterilised deaerated, deionised water; PBS (control); and MYPG nutrient media at 14 ^{0}C at 1:1, 2:1, and 1:2 yeast to diluent mass ratios. Samples were collected over a 4-hour period and analysed for cell membrane damage (protease release), cell death associated with membrane damage (methylene blue viability), and changes in growth and metabolic activity (small-scale fermentations). From the experiments, it was found that the osmotic stress encountered on dilution had little or no effect on yeast cell integrity, growth and fermentative ability. The protease release observed on suspending yeast in water and PBS was attributed to the absence of a nitrogen source in the two diluents (Slaughter and Nomura, 1992) and not to the loss of membrane integrity due to osmotic stress. Prolonged storage of yeast at 14 ^{0}C in a nutrient depleted media may however lead to a decrease in internal glycogen content of the yeast.

The effect of agitation in a jacketed agitated vessel on yeast quality was investigated. Washed yeast at 14 ^{0}C was resuspended in deaerated deionised water at 14 ^{0}C to a 30 % consistency (on mass basis). A 200 mL aliquot of yeast slurry was then placed in each of two 250 mL beakers. The yeast in one of the vessels was agitated at 570 rpm (based on the average tip speed of brewery storage vessel agitators, approximately 1.5 m/s) using a pitched-blade impeller of 50 mm in diameter over a 4-hour period. Samples were examined for cell membrane damage (protease release), cell death associated with membrane damage (methylene blue viability), cell wall damage (haze), and changes in cell growth and metabolic activity (small-scale fermentations). The agitation employed in the vessel had no effect on cell envelope (wall and membrane) integrity. Yeast growth and the ability of the yeast to adapt to the fermentation media were also not affected by the

agitation. Hence, damage to yeast observed on cooling yeast in the jacketed agitated vessel can be attributed to the influence of cold stress.

6.5.2 THE EFFECT OF FINAL COOLING TEMPERATURE ON COOLING DILUTED YEAST SLURRIES IN A JACKETED AGITATED VESSEL

Washed yeast was resuspended into deaerated deionised water at 14 ^{0}C (control), 12 ^{0}C, 8 ^{0}C and 4 ^{0}C to a 30 % consistency (by mass). The diluted yeast was agitated over a 4-hour period at 570 rpm, using a pitched-blade impeller of 50 mm in diameter. The yeast was maintained at temperature by coolant-circulating refrigerated units. Samples taken over the duration of the experiment were analysed for the presence of physiological stress indicators (morphology, trehalose, hsp 12), cell membrane damage, cell death associated with membrane damage, cell wall damage (fragility), glycogen content and changes in growth and metabolic activity (small-scale fermentation). There was an increase in the level of expression of membrane stress compounds (trehalose and hsp) in the yeast with decreasing final cooling temperature (Figure 6.27). Cellular and membrane desiccation (cell size and hsp 12) also increased with final cooling temperature. These findings support the hypothesis that membrane composition is altered at low temperature, the extent of which is dependent on the magnitude of the low temperature stress.

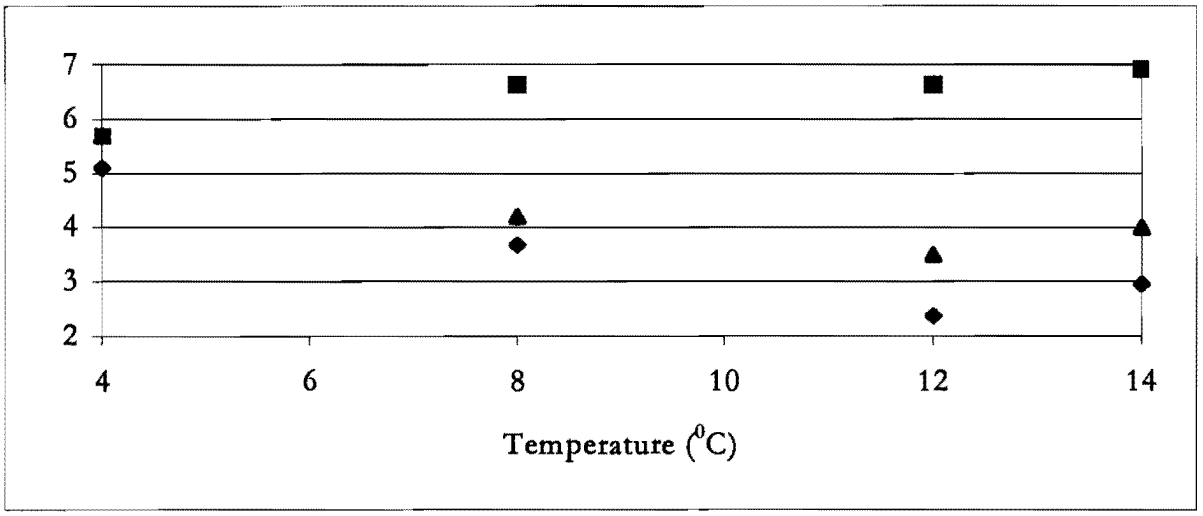


Figure 6.27 Effect of final cooling temperature on physiological stress indicators. ■ Yeast cell size (μm), ▲ trehalose content (g/g d.w), ◆ Hsp 12 expression (/100).

Exposure of yeast to low temperature in the jacketed agitated vessel led cell membrane permeabilisation and a decrease in cell wall strength (Figure 6.28). Damage to the cell envelope was dependent on the intensity of the cold stress. Lewis (1994) and Fargher and Smith (1995) report that loss in membrane fluidity at low temperature may compromise membrane integrity. The literature however did not provide explanation for diminishing cell wall strength on low temperature stress.

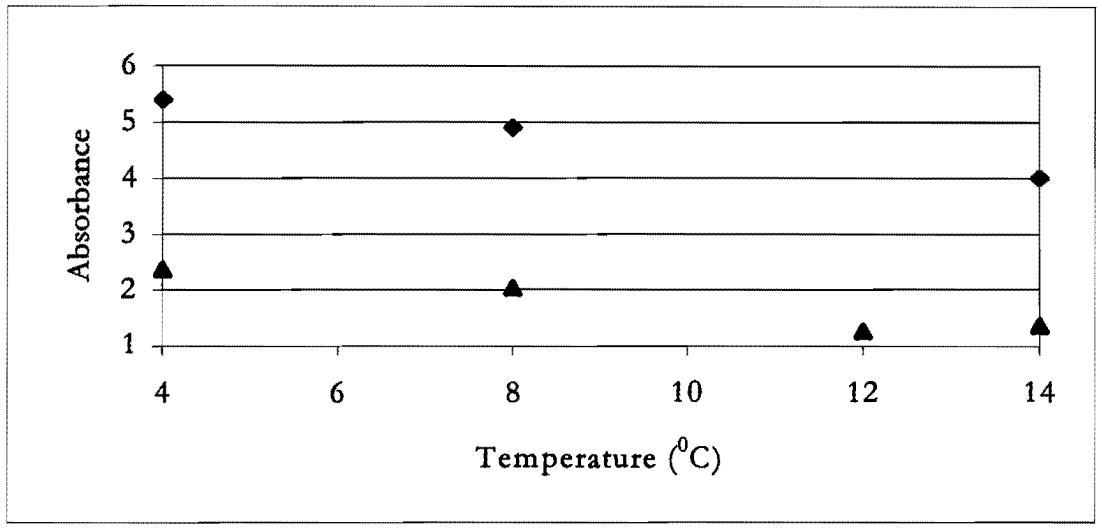


Figure 6.28 Effect of final cooling temperature on cell envelope integrity. ◆ Total protein release from French Press fragility analysis of yeast incubated for 4 hours at temperature in a jacketed agitated vessel and ▲ protease release after 4 hours at temperature in a jacketed agitated vessel.

Changes in yeast growth and metabolism were also observed with decreasing final cooling temperature in the small-scale fermentations (Figure 6.29). The reduction in biomass yield may have been due to the increased presence of membrane damaged cells at low temperature (protease absorbance in Figure 6.28). Lentini (1993) reports that damage to the cell membrane may affect the replicative ability of yeast. The CO_2 yield is an indicator of yeast metabolic activity throughout the fermentation. The increase in CO_2 yield of yeast held at 4 ^{0}C may have been due to its higher glycogen content on inoculation as the end of fermentation glucose concentration in the fermentation media was close to zero for all yeast (<0.05 g/L). Storage of yeast in the jacketed agitated vessel at temperatures above 8 ^{0}C led a decrease in internal glycogen content.

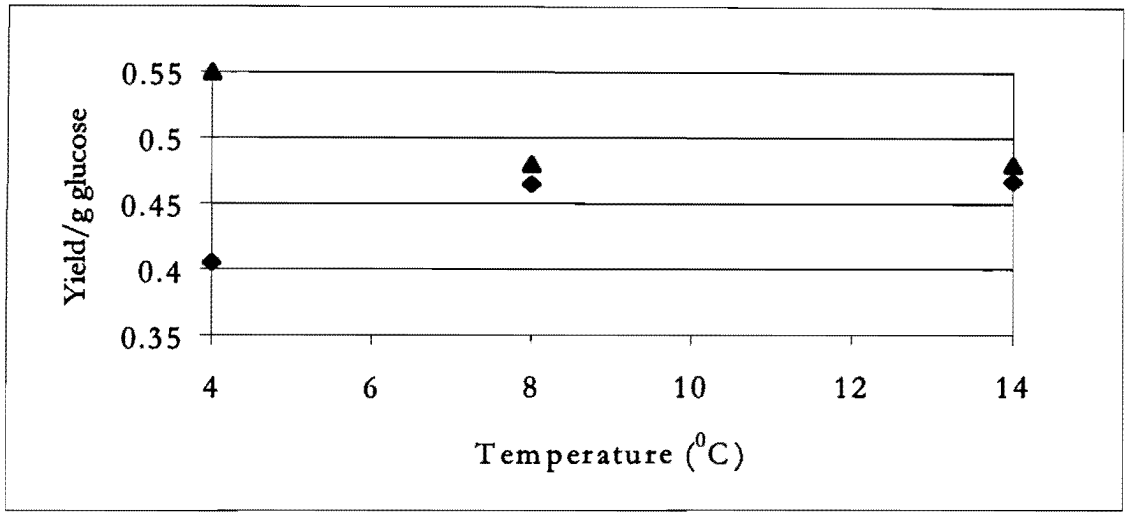


Figure 6.29 Effect final cooling temperature on yeast growth and metabolism. ◆ Biomass yield (cells/g glucose) and ▲ CO_2 yield (g/g glucose).

6.5.3 EFFECT OF COOLING RATE ON YEAST QUALITY

To examine the effect of cooling rate on the extent of cold injury experienced by diluted yeast slurries, an experiment was performed in which yeast at 14 °C was resuspended in 12 °C water and slowly cooled to 4 °C at 2 °C/hr in a jacketed agitated vessel. Another batch of yeast was fast cooled (approximately 3 °C/sec) from 14 °C to 4 °C by resuspension into 4 °C water in a jacketed agitated vessel. Yeast in both systems was held at 4 °C for 4 hours. Further experiments were also conducted to investigate the effect of cooling rates employed in brewery heat exchangers on yeast quality. These experiments were performed both in the laboratory, using laboratory scale cooling coils, and under routine brewery operations at SAB-Newlands brewery. The cooling rates achieved ranged from 1.28 to 4.03 °C/sec.

Results across the experiments suggest increased membrane damage with increasing cooling rate (Figure 6.30). Fargher and Smith (1995) report that the exposure of yeast to a rapid decrease in temperature may cause membrane components to become fixed in a random and disorganised fashion. In this state, the cell membrane may develop leaks resulting in the loss of cellular material. Minor damage to the cell wall (haze) was observed both in brewery and laboratory experiments (Figure 6.31). The amount of haze formed increased with increasing cooling rate (flowrate employed). This haze material was found to have originated from the cell wall and the cytosol. Thus, rapid cooling of yeast in a heat exchanger made the yeast cell envelope more sensitive to hydrodynamic shear damage. Cell wall strength also appeared to diminish with increasing cooling rate

(French Press fragility analysis). Damage to the cell envelope in both laboratory and brewery experiments is amplified at cooling rates in excess of 2 ^{0}C/sec (Figure 6.30 and 6.31).

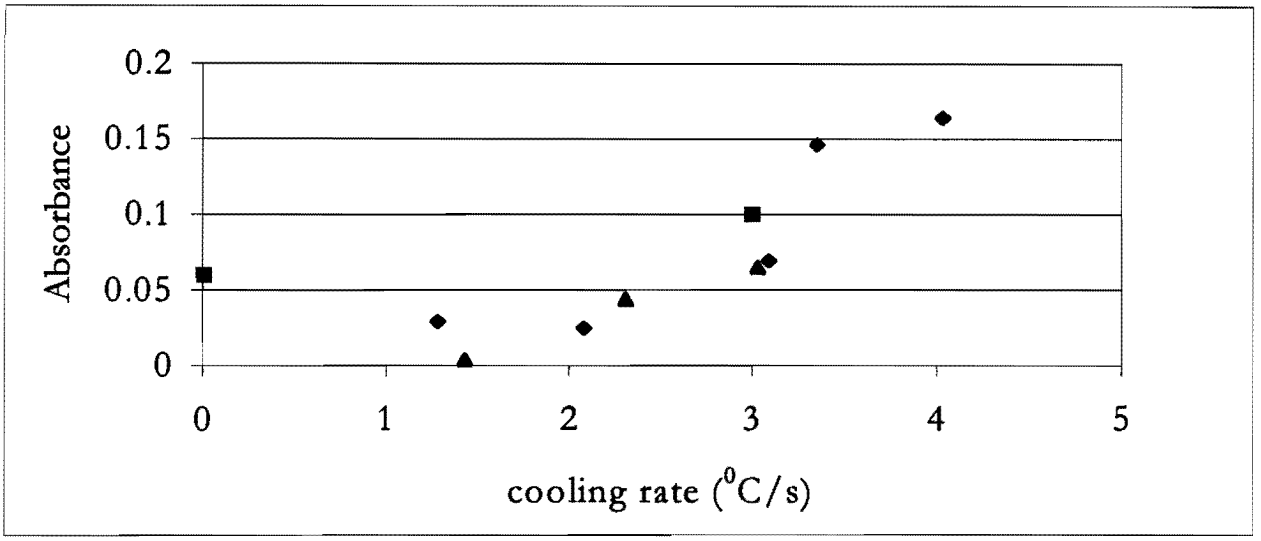


Figure 6.30 Effect of cooling rate on yeast cell membrane integrity. ◆ Yeast cooled in laboratory scale cooling coils, ▲ yeast cooled on-line in the brewery and ■ slow and fast cooling in a jacketed agitated vessel.

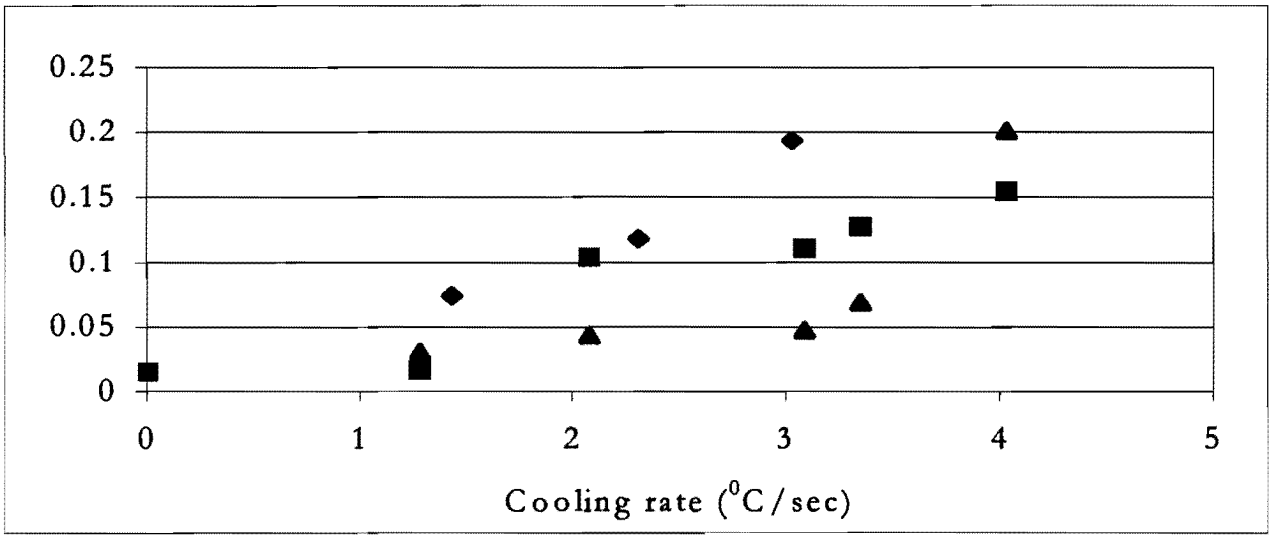


Figure 6.31 Effect of cooling rate on yeast cell wall. ■ Haze (/5) from yeast cooled in laboratory scale cooling coils, ◆ haze (/5) from yeast cooled on-line in the brewery and ▲ protein release from yeast cooled in laboratory scale cooling coils on fragility analysis.

Yeast growth and metabolic activity were also affected by cooling rate. In laboratory and brewery experiments, biomass and CO_2 yield decreased on increasing cooling rates above 2 ^{0}C/sec (Figures 6.32 and 6.33). This may have been due to the increased cell membrane damage found on exceeding this cooling rate (Figure 6.30) and the possible occurrence of lipid phase transitions in membrane bound cytoplasmic organelles (Kaul *et al.*, 1993, Fargher and Smith, 1995). This is postulated have detrimental effects on yeast metabolism. A notable decrease in yeast growth rate was observed in brewery experiments only.

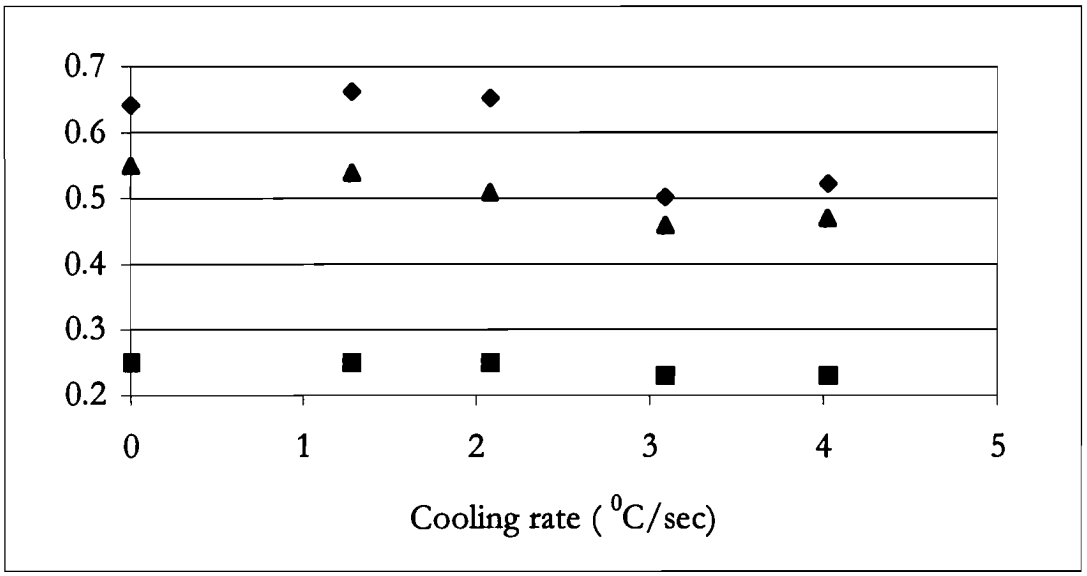


Figure 6.32 Effect of cooling rate on yeast metabolism and growth (Laboratory scale cooling coils). ■ Growth rate (×10), ▲ biomass yield (/10^8) and ◆ CO_2 yield.

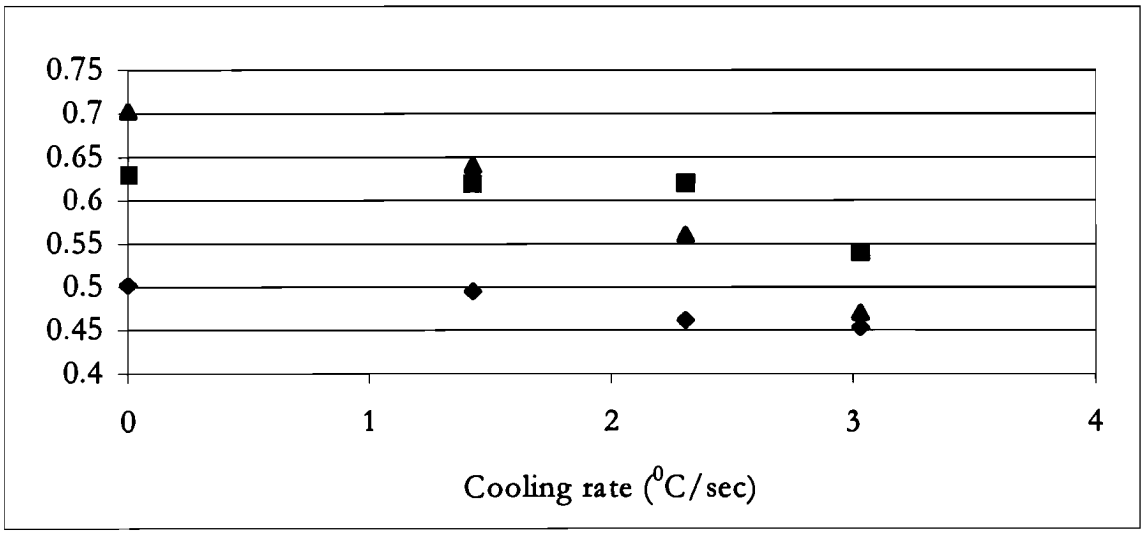


Figure 6.33 Effect of cooling rate on yeast metabolism and growth (Brewery). ■ Growth rate (×10), ▲ biomass yield (/10^8) and ◆ CO_2 yield.

CHAPTER

7

CONCLUSIONS

7.1 CONCLUSIONS

In brewing, it is common practice to cool cropped yeast from a fermentation temperature of approximately 14 ° C to a storage temperature close to 0 ° C prior to re-pitching. The cooling systems employed by brewers may involve rapid heat transfer in a plate and frame heat exchanger or dilution and subsequent slow cooling of yeast in a jacketed agitated vessel. Walker (1998) reports that the exposure of yeast to low positive temperatures may have the following effect on cell physiology (Walker, 1998):

- Cell membrane fatty acids may undergo a phase transition, compromising cell membrane integrity.
- Sterol synthesis is reduced, weakening membranes and thus increasing potential for cell disruption.
- Yeast cells shrink uniformly, inhibiting budding.
- Vacuolar membrane damage may occur leading to changes in metabolic activity.

Further, Fargher and Smith (1995) and Komatsu *et al.* (1990) report that cold injury experienced by yeast at low temperatures is governed by yeast cell condition, cell growth phase, growth temperature, temperature range over which cooling is performed, cooling rates employed, and level of low molecular weight protectants in the yeast. In this research project, the effect of cooling, in terms of both extent of cooling and cooling rate, on stationary phase yeast, was investigated. Experiments were performed to assess the potential for a loss in yeast quality as a result of cooling of diluted yeast slurries in a jacketed agitated vessel and in a heat exchanger. These experiments were carried out in order to ascertain the effects of osmotic shock, agitation, final cooling temperature and rate of cooling on the physiological state of yeast and its ability to withstand subsequent mechanical handling. Critical cooling rates above which extensive cold injury will occur in yeast were also identified.

As the terms "loss of viability" and "loss of vitality", generally used in brewing to characterise a loss of yeast quality, did not provide a detailed description of the nature of a loss of yeast quality (Basson, 1996), a scheme whereby cold stress could affect yeast was hypothesised. Firstly, changes in cell membrane composition may occur leading to the synthesis of low molecular weight cryoprotectants (membrane stress compounds), the levels of which are dependent on the magnitude of the cold stress. Such a response may be detected by changes in the degree of expression of stress compounds such as trehalose and heat shock proteins. Secondly, as cold stress increases, loss of integrity of the cell membrane may occur owing to phase transitions within the membrane. Jones (1987) reports that replicative competence in yeast relies on the functional and structural integrity of the cell membrane. Damage of this sort can be measured through methylene blue viability staining or release of protease enzymes. This cold stress may also affect the ability of the cell wall to withstand hydrodynamic shear. Loss of cell wall strength can be monitored through haze and fragility analyses. More severe cold stress may cause damage to intracellular organelles altering the yeast fermentation performance and in extreme cases causing cell disruption. Overall yeast quality and cytoskeletal function can be monitored by small-scale fermentations (biomass yield, yeast growth rate, substrate utilisation and CO_2 production) and cell disruption can be monitored through microscopic cell counts.

The results obtained from experiments to investigate the effects of cooling on yeast quality demonstrated the following:

- The osmotic stress encountered by cropped yeast on dilution had little or no effect on yeast cell integrity, growth and fermentative ability. Prolonged storage of yeast at 14 ^{0}C in a nutrient depleted media may however lead to a decrease in internal glycogen content of the yeast and an increase in protease externally.
- The agitation employed in the jacketed agitated vessel, in the absence of cooling, had no effect on cell envelope (wall and membrane) integrity. Yeast growth and the ability of the yeast to adapt to the fermentation media were also not affected by the agitation.
- Over the range of final cooling temperatures investigated (14 to 4 ^{0}C) on cooling diluted yeast slurries in a jacketed agitated vessel, there was an increase in the level of expression of membrane stress compounds (trehalose and hsp) in the yeast with decreasing final cooling temperature. Cellular and membrane desiccation (indicated by reduction in cell size and expression of hsp 12) also increased with decreasing final cooling temperature. These findings support the hypothesis that membrane composition is altered at low temperature the extent of which is dependent on the magnitude of the low temperature stress. The exposure of yeast to a temperature stress in the range 8-4 ^{0}C led to cell membrane permeabilisation and a decrease in cell wall strength. Damage to the cell envelope was dependent on the intensity of the cold stress. There was also a decrease in biomass yield as indicated by small-scale fermentations with decreasing final cooling temperature.
- Over the range of cooling rates that could be achieved in the laboratory cooling coils and on-line at SAB-Newlands using a plate and frame heat exchanger (1.03 to 4.03 ^{0}C/sec), the amount of haze generated and protease released increased with increasing cooling rate (flow rate employed). Yeast growth and metabolic activity were similarly affected by increasing cooling rate. In laboratory and brewery experiments, yeast growth rate, biomass and CO_2 yield decreased with increasing cooling rate. Overall, cooling rates above 2 ^{0}C/sec aggravated damage to the cell envelope and showed the most detrimental effect on subsequent small-scale fermentation performance of the yeast.

7.2 RECOMMENDATIONS

From the data collected in this study it has been possible to postulate cooling rates above which cold injury to yeast may impinge on yeast performance. In the laboratory and in the brewery this has been identified as a cooling rate of 2 ^{0}C/sec. As the cooling rate is increased beyond this value, damage to the cell envelope is aggravated and yeast fermentation performance detrimentally affected. Therefore it is recommended cooling rates below 2 ^{0}C/sec be used during re-circulation.

This investigation was limited to cooling rates representative of the brewery yeast handling circuit. Further work should be conducted to identify rates below which cold injury will not occur in yeast.

From this study it has been shown that cold stress makes the cell envelope more susceptible to damage. Further work should be carried out to investigate the effects of agitation rates and duration of agitation on cooled yeast slurries.

Protease release and haze formation has been shown to be assay sensitive to the detection of cold stress generated in the heat exchanger. It is therefore valuable to conduct regular haze and protease analyses after the heat exchanger during routine brewery operations.

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APPENDIX

A

ASSAY METHODS

A.1 EXTRACELLULAR PROTEASE

A.1.1 REAGENTS

Incubation Buffer: Tris-HCl Buffer
(0.2 mol/L Tris-HCl, pH 7.8, 0.02 mol $CaCl_2$)

Stop Reagent:: Trichloroacetic acid
(5% w/v in deionised, distilled water)

Assay Buffer: Tris-HCl
(0.5 mol/L, pH 8.8)

Substrate solution: 15 mg Casein, resorufin-labelled (Boehringer Mannheimer Universal Protease substrate, Catalogue No. 108073)

Preparation: Add 3.75 ml deionised, distilled water to contents. Batch out 50 µL into eppendorf tubes. Store in deep freeze.

A.1.2 METHOD

1. Centrifuge the yeast slurry in an Eppendorf microfuge at 5000 g for 5 minutes
2. Decant supernatant
3. Centrifuge supernatant for 5 minutes
4. Pipette 50 µL substrate into fresh eppendorf tubes(Pre-prepared)
5. Add 50 µL incubation buffer
6. For sample: add 100 µL supernatant
7. For blank: add 100µL deionised water
8. Incubate tubes overnight at 37 ^{0}C
9. Add 480 µL of stop reagent and incubate at 37 ^{0}C for 10 minutes
10. Centrifuge tubes for 5 minutes and decant supernatant into fresh Eppendorf tubes
11. Pipette 400 µL of supernatant into 1mL cuvettes
12. Add 600 µL assay buffer and measure absorbance at 574 nm using a spectrophotometer

The protease absorbance was expressed as

$$\Delta A_{sample} = Absorbance_{sample} - Absorbance_{blank}$$

A.1.3 REPRODUCIBILITY

The reproducibility of the protease assay was tested. The results are presented in TableA.1.

Table A.1 Reproducibility of the protease assay

Sample Number	Absorbance
1	0.263
2	0.255
3	0.275
4	0.264
Average	0.264
Standard deviation	0.008
Coefficient of variance (%)	3.1

A.2 METHYLENE BLUE VIABILITY

A.2.1 REAGENTS AND METHODS

The solution consists of methylene blue (0.25 g/L), NaCl (9 g/L), KCl (0.42 g/L), $CaCl_2$ (0.48 g/L), $NaHCO_3$ (0.2 g/L) and glucose (10 g/L) dissolved in distilled water giving a final methylene blue concentration of 0.025% (w/v). Dilute the yeast in PBS (pH 7.4) to give a concentration of 10^7 cells/mL. Add 0.1 mL of yeast suspension to 0.9 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_{ble}) and the total number of cells(N_{total}) under a light microscope with brightfield illumination at ×400 magnification.

$$\text{Viability} = (N_{total} - N_{blue}/N_{total}) \times 100\%$$

A.2.2 REPRODUCIBILITY

The standard deviation of viability measurements used in this study was calculated to be 0.80 %.

A.3 DIRECT CELL COUNTING

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 400 × 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 (Figure A.1). 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. These can be calculated using Equations A.1 and A.2 respectively.

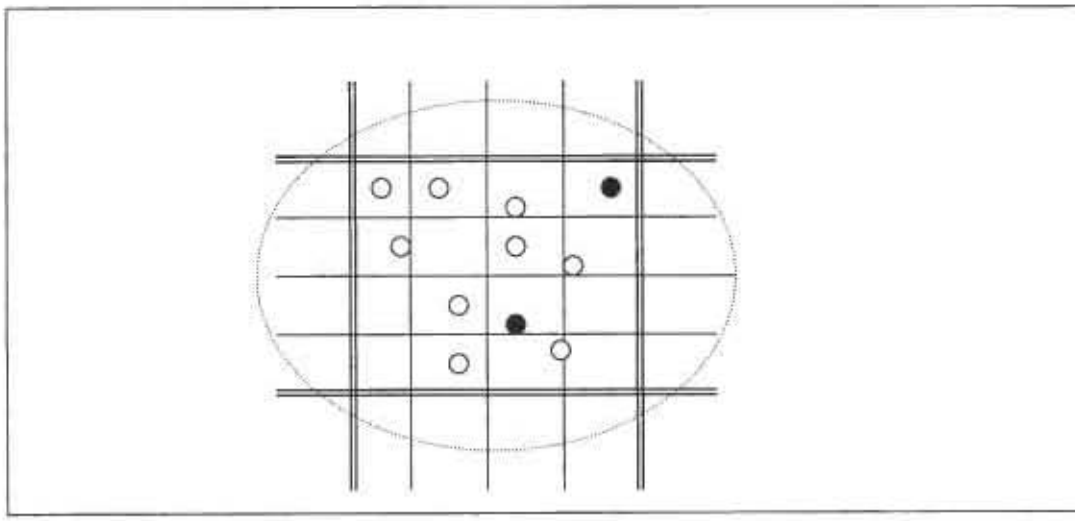

Figure A.1 A Counting block on a haemocytometer.

$$\text{Cell conc. (cells/mL)} = \text{cell count} \times \text{dilution} \times 1000 / (0.0025 \times 0.1) \quad \text{(A.1)}$$

$$\text{Viability (\%)} = [(\text{total cell count} - \text{stained cells}) / (\text{total cell count})] \times 100 \quad \text{(A.2)}$$

A.3.1 REPRODUCIBILITY

The standard deviation of replicate samples was calculated to be 0.8 %.

A.4 HAZE ANALYSIS

Yeast was removed by centrifuging the sample for 5 minutes at 3000 g in a Beckman J-25 Avanti centrifuge. The supernatant was recovered and a portion analysed for haze using a Malvern Mastersizer. This consists mainly of haze material with a small amount of yeast still present. A size analysis of the haze particles was then conducted. The samples were deflocculated in the ultrasound bath at 50 % power, and pumped continually at 50 % of the maximum flow rate through the measuring chamber and size measurements taken. Data of % number as a function of size range was obtained.

A.4.1 REPRODUCIBILITY

The percentage error in the average yeast cell size four replicate samples (Figure A.2) of yeast supernatant was found to be 0.74 %.

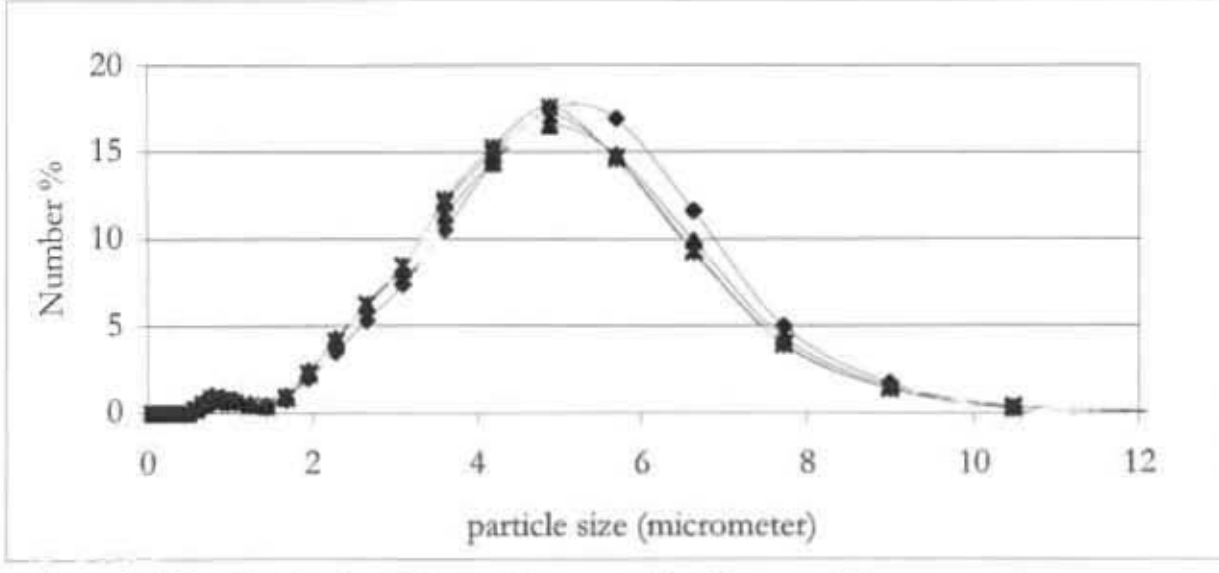

Figure A.2 Size distribution of four replicate samples of yeast and haze suspension.

A.5 FRAGILITY ANALYSIS

A.5.1 METHOD

1. Resuspend yeast in cold buffer solution (10mM Tris and 50 mM NaCl) at a mass ratio of 1:4 i.e. 4 g yeast in 16 ml buffer
2. Vortex to mix
3. Place on ice
4. Assemble piston chamber and fill with yeast suspension
5. Place assembled unit onto french press
6. Eliminate air from collection pod
7. Pump pressure to 1 Mpa, collect zero
8. Pump pressure to 5 Mpa, collect supernatant
9. Take pressure up in steps of 5 Mpa to 30 Mpa
10. Take samples at each pressure and place them on ice
11. Remove yeast from supernatant by centrifuge for 5 minutes in a microfuge
12. Use PBS solution to zero spectrophotometer at 280 nm
13. Measure absorbance at 280 nm to determine protein in supernatant. Note, absorbance should be between zero and 1, so dilute samples down in buffer.
14. Plot absorbance versus pressure

A.5.2 REPRODUCIBILITY

The standard deviation for replicate samples was calculated to be 1.60 %.

A.6 SMALL-SCALE FERMENTATION

Aliquots (5 mL) of yeast were inoculated into 60 mL MYPG media of the following composition: 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone and 10g/L glucose. Each flask was stoppered and fitted with a glass tube containing silica gel to absorb any moisture evaporating from the flask (Figure 4.4). These flasks were incubated in a 30 °C shaker for 24 hours and sampled 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. Samples taken were analysed for glucose concentration, biomass concentration, growth rate and methylene blue viability.

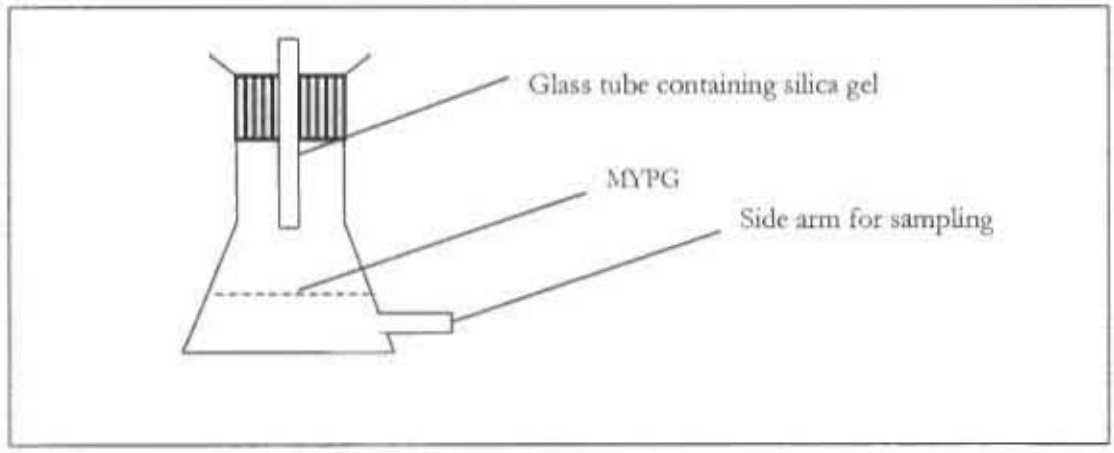

Figure A.3 Apparatus used in small -scale fermentations to assess yeast vitality

A.7 GLUCOSE ANALYSIS

A.7.1 METHOD

Dissolve 0.1 g of dextrose (glucose) in 100 mL distilled water. Pipette 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mL of the above solution into test tubes to prepare the glucose standard curve. Each concentration was done in triplicate. The total volume of all test tubes was brought to 1 mL with distilled water. The GOD-Perid kit (Boehringer Mannheimer GmbH Diagnostica, Catalogue No. 124036) contains the following solutions:

Phosphate buffer	100 mMol/L, pH 7.0
Peroxidase	> 0.8 units/mL
Glucose oxidase	> 10 units/mL
ABTS	1.0 mg/mL

1. Pipette 0.12 mL of each standard glucose solution and each sample into test tube
2. Rapidly add 3.0 mL of the glucose kit solution 2 to each test tube and mix immediately. The glucose kit solution 2 must be at 25 °C.
3. Incubate the test tubes in a dark place at temperature for 25 minutes. The colored complex retains its colour for about 20-25 minutes after incubation.
4. Measure the absorbance of the glucose standards and the samples against the blank (distilled water) at 620 nm.
5. Determine a standard glucose curve from the absorbance data of standard glucose solutions. From this curve, determine the glucose concentrations of samples.

Linear regression

Absorbance = Constant + X coefficient × Glucose conc.

Glucose (g/L) = (Abs-constant)/ X coefficient

A.7.1 REPRODUCIBILITY

The coefficient of variance of samples with an absorbance of 0.7 was 2.8 %.

A.8 TREHALOSE AND GLYCOGEN CONTENT

A.8.1 METHOD

1. Sample about 150 mL slurry into clean centrifuge bottles and keep it on ice.
2. Centrifuge 5 minutes at 5000 rpm to harvest the yeast and discard the supernatant (alternatively this supernatant could also be used for pH, protease and FAN determination of the yeast slurry).
3. Wash the cells i.e. use 150 mL pre-cooled distilled water (4°C) to add to the cells, shake until all the cells are homogeneously distributed in the water and centrifuge as before. Discard the supernatant.
4. Repeat step (3).
5. Spread the washed cells onto a plate (e.g. sideplates obtainable from the Hyperama) with a spatula (about 3-5mm thick). Work as fast as possible and do not delay drying of the cells in the microwave.
6. Place the plates in a microwave and dry (about 6 minutes on a medium-high setting until the yeast slurry is dry and crisp).
7. Scrape the dried cells off onto clean paper using a spatula and decant it into a clean, dry McCartney bottle if not proceeding immediately with step 8).
8. Mill the yeast in a coffee mill until it is a fine powder. Sift it onto a clean piece of paper through a Star Screen Test Sieve (500μm-aperture size) and transfer the powder into a clean, dry McCartney bottle.
9. Read the sample on the NIR for glycogen and trehalose according to the equipment manual (Bran &Luebbe InfraProver II).

A.8.2 REPRODUCIBILITY

From glycogen and trehalose measurements, the percentage error for replicate samples was calculated to be 3.8 % and 4.5 % respectively.

A.9 HEAT SHOCK PROTEIN ANALYSIS

A.9.1 METHOD

1. Collect yeast sample from reactor and centrifuge
2. Decant supernatant and place yeast cell in a sample vial on ice
3. Weigh out 4g of yeast cells into round bottom bottle
4. Add 4 mL of 1 M NaOH (cold) to the yeast. Leave in ice for 30 minutes
5. Centrifuge samples and collect supernatant
6. Place supernatant into Sample Application Buffer (SAB) at 1:1 ratio (by volume) (1:1)
7. Clamp together plates and strips to prepare chamber for gel preparation
8. Seal the base and sides with Agarose, and leave to stand (20min)
9. Make up 20 % separating gel. Note, add TEMED last
   - 25 mL 40 % acrylimide 0.2 % bis
   - 16.7 mL 1.125 M Tris-HCL pH 8.8
   - 0.5 mL 10 % AMPS
   - 50 µL TEMED
10. Place separating gel solution into plate, and fill to ¾ full
11. Make up rest of volume with water
12. Prepare stacking gel
13. Discard the water once the separating gel is set
14. Add stacking gel
    - 2 mL acrylamide
    - 4 mL 0.375 M Tris-HCL pH 6.8
    - 0.3 mL AMPS
    - 20 µL TEMED (add last)
    - Water to 12 mL
15. Insert combs and allow to set

A.9.2 GEL ANALYSIS

16. Take histone standard from fridge

17. Remove bottom clamps and combs from plate
18. Place plate into a bottom support tank containing tank buffer solution
    - 30 g glycine
    - 6 g Tris
    - 2 g SDS
    - Add water to 2 L
19. Attach a top support tank filled with tank buffer solution to the plate in a manner that allows the buffer solution to completely fill the comb imprints
20. Place 20 µl of histone standard into a comb imprint using a Hamilton syringe
21. Place 20 µl sample in other imprints
22. Connect the top and bottom support tanks to a power pack at 40 V, and leave overnight
23. Disconnect power supply and stain gel in coomassie blue stain (3hrs)
    - 1 g R250 coomassie
    - 225 mL water
    - 225 mL methanol
24. Destain with 7 % acetic acid

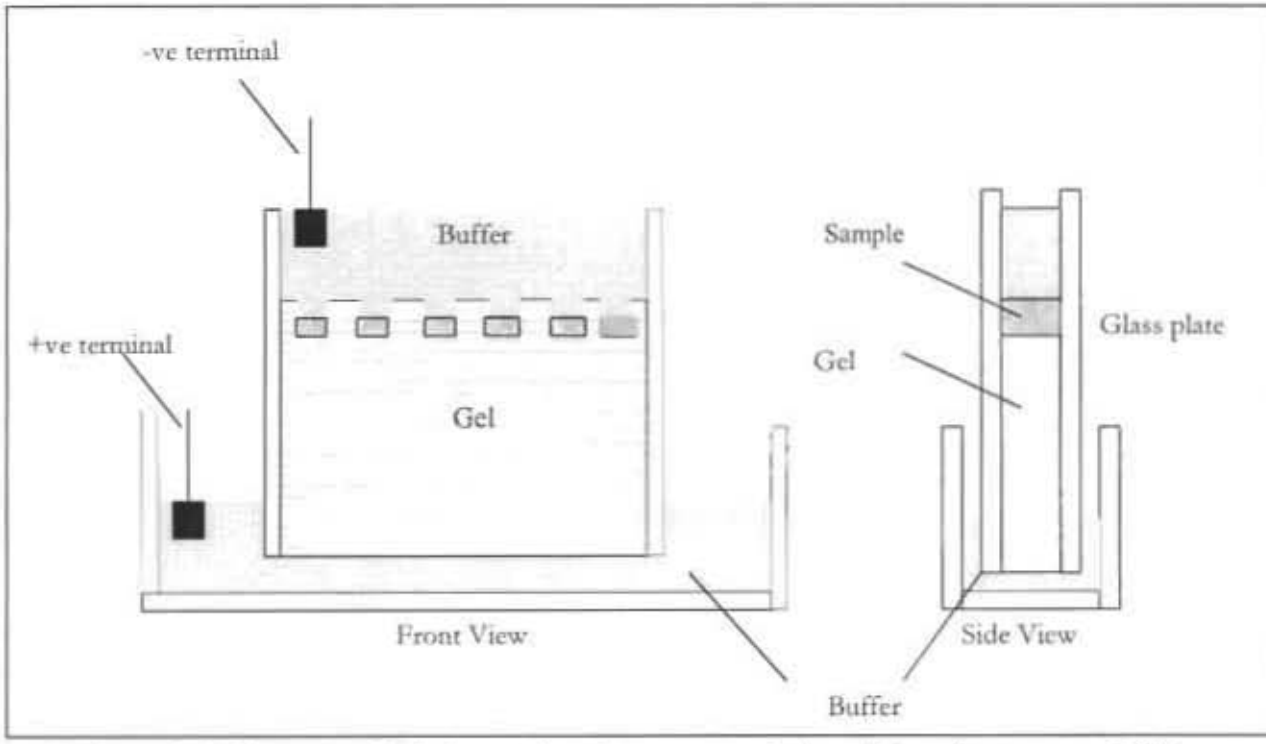

Figure A.4 Schematic of gel-electrophoresis apparatus. Adapted from Prescott *et al.* 1996.

APPENDIX

B

ESTIMATION OF BREWERY COOLING RATES

Estimation of brewery heat exchanger cooling rates

Typical dimensions of a brewery plate and frame heat exchanger are illustrated in Figure B.1 with its specifications listed in Table B.1. The brewery heat exchanger cooling rates were estimated as follows:

Average yeast flowrate employed in brewery heat exchanger = 34×10^2 l/hr

Yeast flowrate = 34×10^2 l/hr =0.94 l/s
= 9.4×10^{-4} m^3/s

Thus

Heat exchanger inlet pipe diameter = 80 mm

Cross sectional area of inlet = 0.00503 m^2

Therefore superficial velocity at inlet = $\frac{9.4 \times 10^{-4}}{0.00503}$
= 0.19 ms^{-1}

Cooling rate

Longest path yeast travels in HE = 0.38 + 0.68 + 0.38 =1.44 m

Time taken = 1.44/0.19 =7.58 sec

Shortest path =0.68 + (0.003 ×4) = 0.70 m (0.003 is distance between plates)

Time taken = 0.70/0.19 =3.68 sec

Average time in HE = (7.58 + 3.68)/2 = 5.63 s

Temperature change = (15 –3) °C = 12 °C

Cooling rate = 12/5.63 = 2.13 °C /s

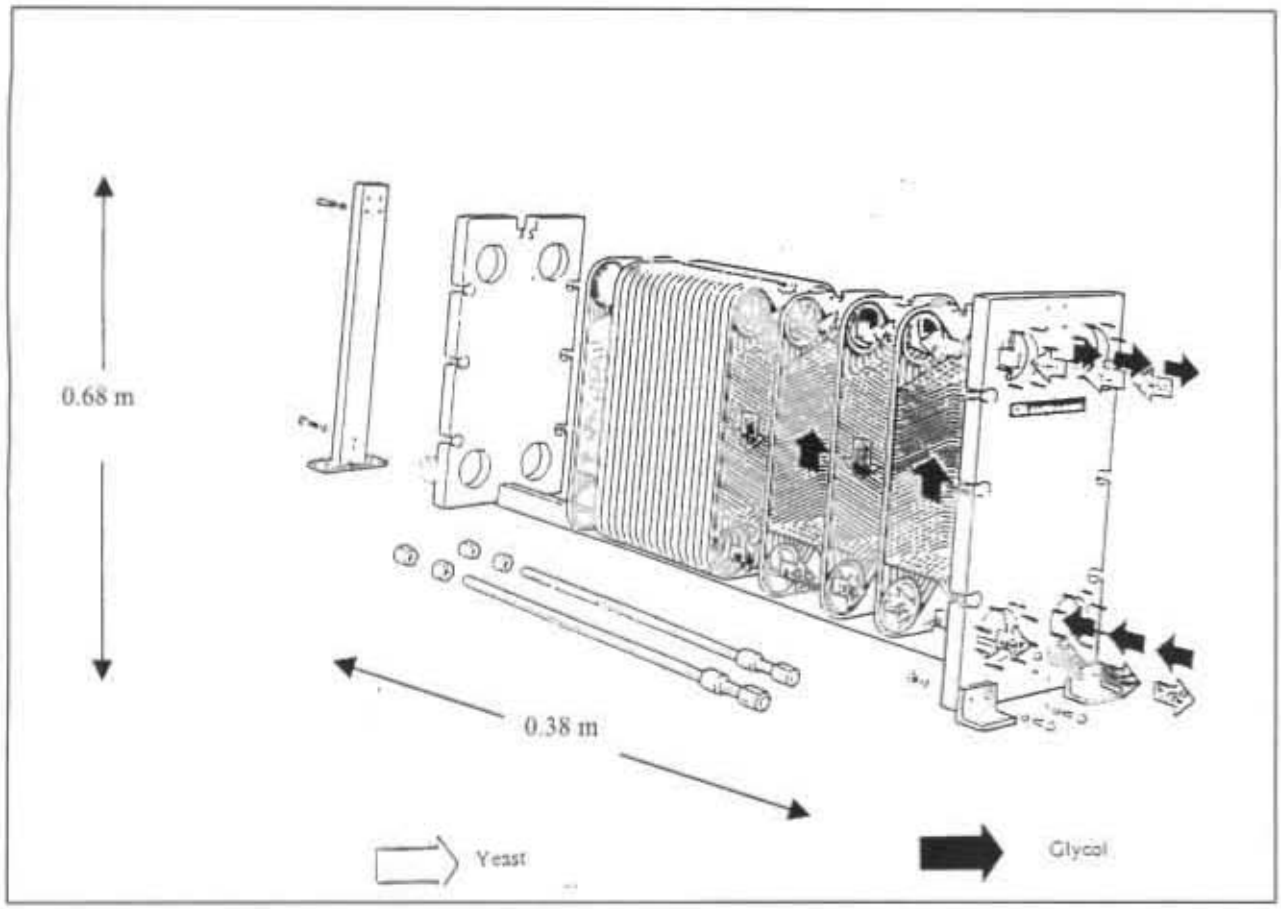

Figure B.1 Schematic of brewery plate and frame heat exchanger

Table B.1 Brewery heat exchanger specifications.

Type	Paraflow Plate heat exchanger
Manufacturer	APV
Material of construction	316 stainless steel
Series	SR 20
Serial number	SA 1076
Number of plates	127
Space between plates (mm)	3
Heat exchange area (m^2)	21.6
Inlet diameter (mm)	80
Coolant	Glycol
Temp. Glycol in	-3
Temp. Glycol out	14
Glycol flowrate (kg/hr)	6300
Substance being cooled	Yeast
Temp. yeast in	15
Temp. yeast out	2.8
Yeast flowrate (m/s)	0.1-0.3
Range of yeast flowrates used(hl/hr)	33-35
Yeast density (g/cm^3)	1.09

Laboratory set – up

The laboratory set-up for rapid cooling experiments is given Figure B.2. The calibration curve for the Marlow S40 pump is given in Figure B.3. The residence time of the yeast in the 2 m long copper coils at each of the flowrates is given in Table B.2. The cooling rate achieved at a particular pump setting was calculated as:

Cooling rate = (Temp. Range/Yeast residence time in copper coils)

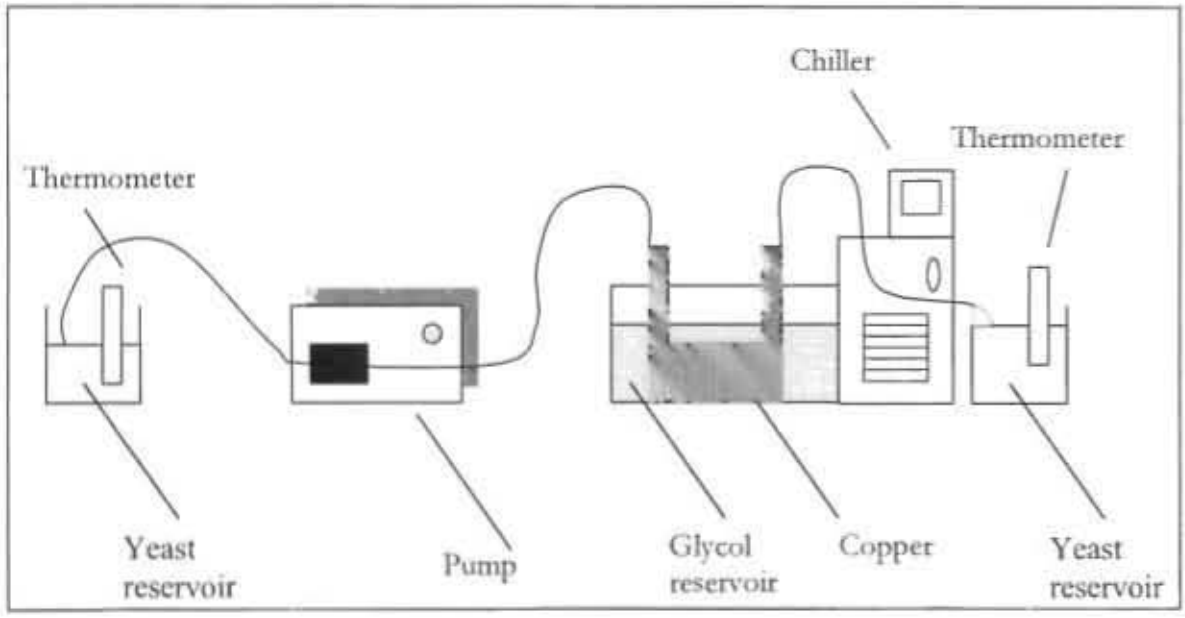

Figure B.2 Experiment set-up used to simulate cooling in a heat exchanger.

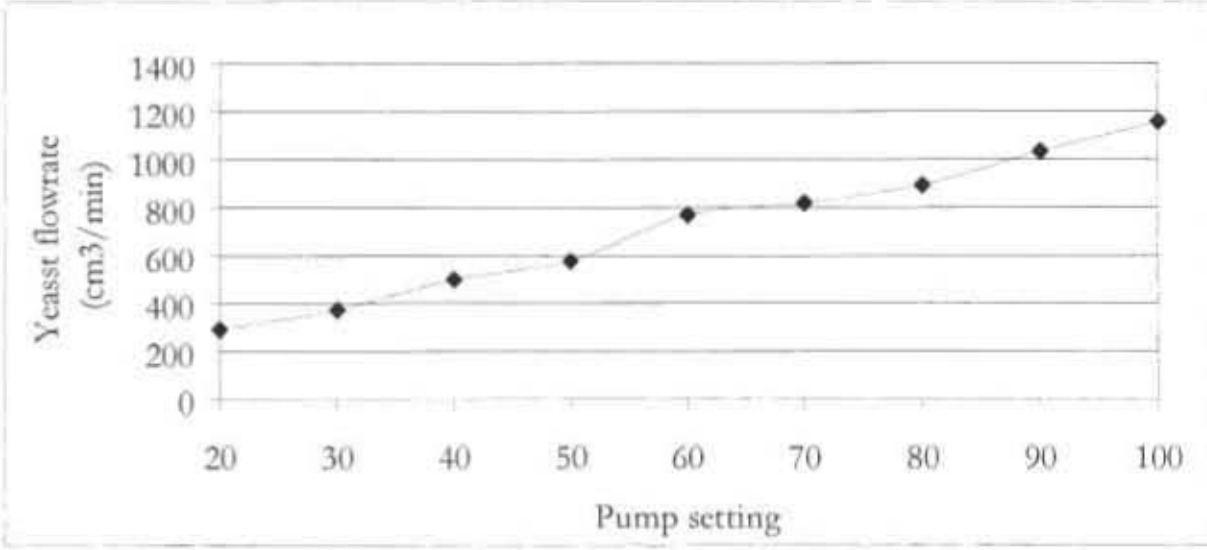

Figure B.3 Marlow S40 calibration curve.

Table B.2 Residence time of yeast in the copper cooling coils at each pump setting.

	Flowrate (cm^3/min)			
Pump setting (%)	1	2	Avg. Flowrate(cm^3/min)	Resd. Time(sec)
20	290	291	290.5	12.8
30	375	370	372.5	10.0
40	500	500	500	7.5
50	579	579	579	6.5
60	760	780	770	4.8
70	820	818	819	4.6
80	898	890	894	4.2
90	1030	1040	1035	3.6
100	1159	1158	1158.5	3.2