OPTIMISATION OF BREWERY YEAST MANAGEMENT: A STUDY INCORPORATING IMAGE ANALYSIS

A thesis presented for the degree of Ph D

by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Gearóid Cahill

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Date: 6/8/99

DEDICATION

My sledge and anvil lie declined

My bellows too have lost their wind

My fire's extinct, my forge decayed,

And in the dust my vice is laid

My coals are spent, my iron's gone

My nails are drove, my work is done

Blacksmith's Epitaph commemorating William Strange d 6 June 1746 (Anon)

Dedicated to the memory of James Cahill, blacksmith, father and role model

Grossbooted draymen rolled barrels dullthudding out of Prince's stores and bumped them up on the brewery float On the brewery float bumped dullthudding barrels rolled by grossbooted draymen out of Prince's stores

Dullthudding Guinness's barrels

Ulysses (James Joyce)

A NOTE ON THESIS FORMAT

The work presented in this thesis consists of a number of detailed studies of key elements of brewery yeast management Image analysis techniques have been developed to provide morphological and physiological information on brewery yeast strains in order to improve these yeast handling processes The introduction to the thesis therefore consists of a brief overview of the entire brewing process and a detailed insight into all aspects of brewery yeast management The fundamentals of image analysis are introduced and its applications discussed with particular reference to yeast

The format of chapters 2 to 6 of the thesis has been chosen as each chapter has been submitted for publication in brewing and non-brewing journals as detailed below

Chapter 2. Cahill, G , Murray, D M , Walsh, P K and Donnelly, D (1999) The effect of the concentration of propagation wort on yeast cell volume and fermentation performance J Am Soc Brew Chem (in press)

Chapter 3 Cahill, G , Walsh P K and Donnelly, D (1999) Improved control of brewery yeast pitching using image analysis J Am Soc Brew Chem , 57 76-78

Chapter 4 Cahill, G , Walsh, P K and Donnelly, D (1999) Determination of yeast glycogen content by individual cell spectroscopy using image analysis Biotechnol Bioeng (submitted July 1999)

Chapter 5 Cahill, G , Walsh, P K and Donnelly, D (1999) A study of thermal gradient development in yeast crops Proc 27th Congr Eur Brew Conv , Cannes, (in press)

Chapter 6. Cahill, G , Walsh, P K and Donnelly, D (1999) A study of the variation in temperature, solids concentration and yeast viability in agitated stored yeast To be submitted to J Inst Brew (September, 1999)

To ensure clarity and uniformity of style, each chapter contains introductory material and experimental methods

CONTENTS

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ABSTRACT

Changes in cell volume occur when yeast is propagated in worts of increasing original gravity (OG) in the range 7 5 to 17 5°P Using image analysis to measure cell volume, the mean individual cell volume of ale and lager yeast increased by up to 30% as OG increased to 17 5°P Propagation of yeast in high-gravity wort (17 5°P) has a deleterious effect on yeast quality during subsequent high-gravity fermentations

The mean cell volume of yeast changes during storage and this has been identified as a contributing factor to over-pitching of fermentations A reduction in mean cell volume of up to 19% for ale yeast and up to 7% for lager yeast has been observed Using image analysis, a new pitching regime was developed which improves fermentation consistency

A rapid technique has been developed to determine the glycogen content of yeast on an individual cell basis using a combination of image analysis technology and staining of yeast cells with an I_2 KI solution Analysis of the distribution of yeast glycogen during fermentation indicates that a fraction of yeast cells do not dissimilate glycogen Therefore, conventional glycogen analysis of yeast used to inoculate fermentations is of limited use, unless information regarding the proportion of cells which utilise glycogen is known

Thermal gradients in yeast crops of 3 5°C were recorded within 5 hours of unmixed storage, using a specially designed cooling rig These gradients increased with increasing yeast metabolic activity to a maximum of 11°C

Using a custom-built yeast storage vessel, it was determined that mechanical agitation achieves better mixing than recirculation of slurry through an external loop In all trials, a dense biomass layer (up to 75% w/w) developed on the slurry surface (due to CO_2 evolution), in which the yeast viability was up to 13% lower than in the remainder of the vessel

NOMENCLATURE

A	Projected area of cell	[μm^2]
B	Cell breadth	[μm]
C	Circularity	[-]
D	Impeller, vessel diameter	[mm, cm, m]
I	Maximum luminence (in grey scale)	[-]
I_0	Grey value of pixel	[-]
L	Cell length	[μm]
V	Cell volume	[μm^3]
t	Time	[hr]

Abbreviations

Aber&IA	Aber instrument and Image Analysis (basis for pitching)
ADY	Active Dried Yeast
CER	Carbon dioxide Evolution Rate
DO	Dissolved Oxygen
EBC	European Brewery Convention
FV	Fermentation Vessel
ICS	Individual Cell Spectroscopy
MB	Methylene Blue (basis for pitching)
MB&IA	Methylene Blue and Image Analysis (basis for pitching)
MV	Maturation Vessel
NIR	Near Infra-Red
OD	Optical Density
OD-L	Optical density of a yeast cell stained with Lugol's solution
OG	Original Gravity
PC	Personal Computer
PCR	Polymerase Chain Reaction
YSV	Yeast Storage Vessel

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1
GENERAL INTRODUCTION

1 1 INTRODUCTION

The brewing of beer is a traditional craft involving many different processes ranging from the growing of barley to the packaging of the finished beer product Although steeped in tradition and craft, there is ongoing development of brewing technology to improve beer quality and plant efficiency The purpose of this chapter is to introduce the fundamentals of brewing and in particular the aspects of brewing associated with the handling of yeast Many detailed descriptions of the entire brewing process are available (Briggs *et al*, 1996, Hardwick, 1995, Kunze, 1996, Hough *et al*, 1995, Pollock, 1979, Pollock, 1981 and Pollock, 1987)

Image analysis has been recognised as a powerful tool for the examination of yeast and other micro-organisms The technology has been applied to some aspects of brewery yeast management throughout this work The fundamentals of image analysis are introduced and discussed in this chapter with particular reference to yeast

1.2 FUNDAMENTALS OF THE BREWING PROCESS

Brewing of alcoholic beverages has been practised by mankind for millennia Beer has been a popular choice throughout the ages and the essential elements of its production include barley, malt, hops, water and yeast A basic outline of the

process of modern beer production is illustrated in Figure 1 1 Barley is the primary ingredient of brewing, although other cereals may be included depending on the desirable flavour characteristics of the beer, for example, rice and wheat The process of malting partially germinates the barley grains through a series of wetting and aeration phases Malting activates a number of hydrolytic enzymes in the grain including amylases and proteases The germination process is arrested by drying (kilning) of the malt The final drying or roasting temperature used in the kilning process influences the colour and flavour of the beer Lager malts are kilned at lower temperatures than ale malts

Mashing is essentially an enzymatic hydrolysis and extraction process in which the starch content of the malt is hydrolysed to fermentable carbohydrates, primarily consisting of the disaccharide, maltose Malt is milled using roller mills in the brewery to produce a grist, which effectively exposes the starchy substrate for hydrolysis The grist can consist of a mixture of different malts and barley as specified in the recipe for the specific beer The grist is then mixed with a supply of hot water (45 – 65°C) The resulting 'porridge' is held in a mash vessel for 1 – 2 hours to allow enzymatic hydrolysis to take place The mash temperature depends on the malt used and the beer type Ale is traditionally mashed at a single temperature of 65°C (infusion mashing), while lager is mashed at a series of progressively warmer temperatures in the range 45 - 70°C by removing a portion of the wort, which is heated to boiling and added back to the mash (decoction mashing) When the mashing process is complete, husks and other non-hydrolysed material are filtered from the mash to produce sweet wort Traditionally, filtration

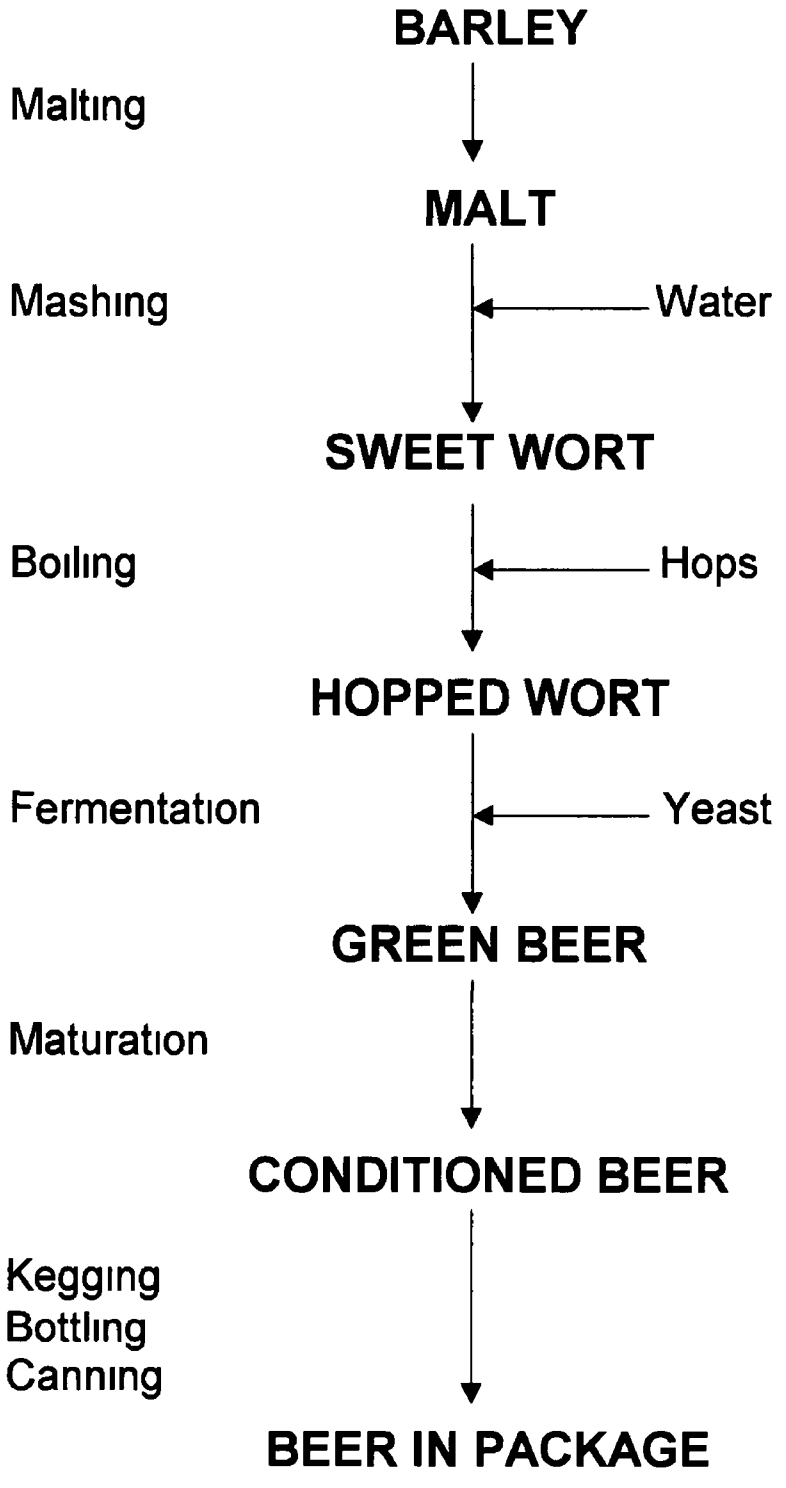

Figure 1.1. Overview of the brewing process

occurs in a lauter tun which has a filter plate in its base The sweet wort drains down through the bed of spent grains and is collected Residual extract is washed from the spent grain by sparging hot water through the bed

Hops are added to sweet wort in the kettle and the wort is typically boiled for 90 minutes A number of important processes occur during wort boiling including sterilisation of wort, extraction of hop bitters (humulones), isomerisation of humulones to iso-humulones (more bitter than humulones), concentration of the wort, precipitation of proteinaceous material (trub), development of colour and removal of unwanted hop volatiles

When boiling is complete, the wort is sent to a whirlpool for removal of trub and spent hops from the hopped wort The clarified wort is then cooled to fermentation temperature en route to the fermentation vessel

Wort is usually aerated and pitched with yeast in-line during filling of the fermentation vessel During the initial lag phase (in which the gravity of the wort does not alter) the dissolved oxygen in the wort is absorbed by the yeast, enabling synthesis of important cell membrane components which can only occur in the presence of molecular oxygen (Aries and Kirsop, 1977) The yeast cells increase in number during early fermentation and thereafter continue to metabolise wort sugars to produce ethanol and CO_2 The fermentation temperature is dependent on

the type of yeast used but is usually in the range 10 – 25°C The duration of primary fermentation can range from 2 to 10 days depending on the beer type

At the end of primary fermentation the green beer contains a number of undesirable compounds including diacetyl All green beer must undergo a conditioning phase after primary fermentation to remove these compounds and develop the final beer flavour Residual yeast in the beer remove the last traces of undesirable compounds Conditioning of lager can take several days at temperatures typically below 5°C, while ales and stouts are conditioned at temperatures close to fermentation temperature A small amount of wort may be added to ale and stout green beer at the start of conditioning to assist in the conditioning process When conditioning is complete, the beer is chilled and finings may be added to clarify the beer

The conditioned beer may be clarified by centrifugation and filtration to remove all yeast, trub and haze particles The resulting bright beer is diluted to trade gravity and stored prior to packaging Bright beer at trade gravity is pasteurised prior to racking into kegs, whereas beer which is packaged into bottles or cans is pasteurised in package after filling The exclusion of oxygen after fermentation is important, as its presence has a deleterious effect on both the flavour and shelf life of the beer

1.3 BREWERY YEAST MANAGEMENT PROCESSES

The 'biocatalyst' used for beer manufacture is yeast and the manner in which it is handled in the brewery plays a central role in determining the quality and consistency of the final beer product This work concentrates on studies of the yeast handling processes used in breweries to ensure that the highest quality fermentations are achieved using yeast of high viability and vitality An overview of the key processes involved in yeast management in the brewery is given below

Yeast management essentially controls all processes in the brewery involving yeast, with the objective of achieving and maintaining the highest quality yeast possible in terms of viability and vitality Effective control is central to the consistent production of beer which meets all of its product specifications A schematic of the yeast handling processes of two breweries within Guinness Ltd is presented in Figure 1 2 to illustrate the range of processes associated with a comprehensive yeast management programme The main yeast handling processes in brewing are propagation, pitching, fermentation, cropping, storage and acid-washing The manner in which yeast is handled in a brewery is based on traditional practices, the plant available and the type of yeast fermentation

The key differences in the yeast handling processes outlined in Figure 1 2 centre on the cropping regime, storage vessels and pitching methods used in each brewery Brewery A centrifuges the beer at fermentation temperature and chills the yeast in a single step after centrifugation In order to minimise the thermal shock to

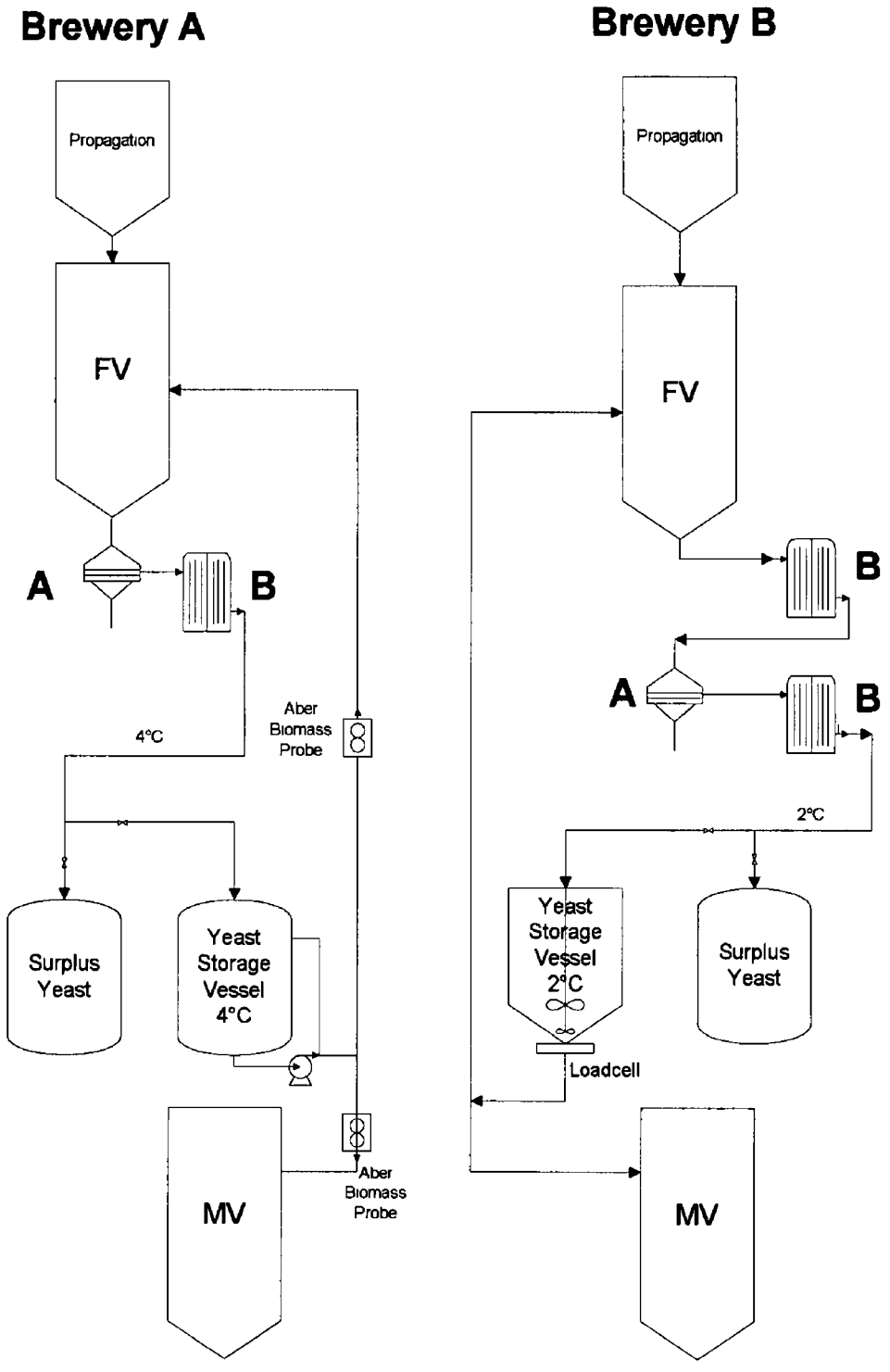

Figure 1 2 Schematic of two brewery yeast handling systems Centrifuge (A), Heat Exchanger (B)

the yeast, brewery B chills the yeast in two stages, one prior to centrifugation and the second en route to the storage vessel The storage temperature of brewery A is 4°C compared to 2°C for brewery B Both temperatures are acceptable for yeast storage The mixing regime used in the yeast storage vessels are radically different Brewery A recirculates the slurry through an external loop to de-carbonate the yeast slurry during storage, while brewery B uses mechanical agitation In-line biomass probes are used for pitching primary and secondary fermentations in brewery A Load cells are used in brewery B to measure the mass of yeast slurry added to fermentations Figure 1 2 is used to illustrate the broad scope of processes involved in a yeast management programme and to outline the diversity of plant and processes used to achieve good yeast handling practices As breweries re-use yeast biomass from one fermentation to the next, each element of the yeast handling process can have a direct impact on other processes Attention to all aspects of yeast management is therefore a prerequisite to good yeast management The key elements of brewery yeast management are discussed in detail in the remainder of this chapter

1.3 1 Brewing yeast cultures

Yeasts are eucaryotic uni-cellular fungi and many species play important roles in industrial fermentation *Saccharomyces cerevisiae* is predominantly used for brewing purposes Brewing yeast consist of top fermenting yeast used for ale and stout production and bottom fermenting yeasts (previously known as *S carlsbergensis*) used for lager production Top fermenting yeast form a yeast head during fermentation, as a result of flotation due to CO_2 evolution in the fermenter The temperature of ale fermentations is typically in the range 18 – 25°C (Hough *et*

al., 1995). Bottom fermenting yeast do not form a yeast head during fermentation and towards the end of fermentation flocculate and sediment into the base of the fermentation vessel to form a dense yeast plug. Typical lager fermentation temperatures are in the range 5 - 12 °C (Hough *et al.*, 1995). There are significant differences in yeast strains used for beer production, each imparting its own characteristic flavour to the beer. It is important that brewers can ensure the integrity of their yeast stocks by eliminating contamination and cross-infection of brewing yeast strains.

Differentiation between top and bottom fermenting yeast can be straightforward. Lager strains of yeast are generally able to metabolise the disaccharide melibiose, (Hough *et al.*, 1995) unlike ale and stout strains. Furthermore lager strains cannot grow at temperatures in excess of 37°C (Hough *et al.*, 1995). Differentiation between different strains of top fermenting yeast and bottom fermenting yeast strains is possible based on classical microbiological analyses including colony morphology, carbohydrate utilisation profile, flocculation characteristics and other analyses (Institute of Brewing Methods of Analysis, 1997). The degree of yeast strain identification is limited using these traditional techniques. Polymerase chain reaction (PCR) techniques have been developed to effectively develop a fingerprint of yeast DNA and allow rapid differentiation of similar yeast strains (Coakley *et al.*, 1996). Detection of contaminants in stocks of brewery yeast is essential to maintain the quality of final beer. The presence of wild yeast and bacteria in pitching yeast (e.g. *Pediococcus*, *Lactobacillus*, *Acetobacter*) can be detected

using a range of selective microbiological media (Institute of Brewing Methods of Analysis, 1997)

1.3.1.1 Yeast viability and vitality

The quality of yeast used to pitch fermentations is of central importance to brewers In the past, brewers have mainly been concerned with the viability of the yeast, i e the percentage of the total yeast cells which are living The are numerous methods employed to determine the viability of yeast including standard procedures such as plate counts, slide culture and vital staining Other techniques have also been used to determine yeast viability, for example, the measurement of the capacitance of viable yeast cells using radio frequency electrical fields (Pateman, 1997) Each method has its own merits and indeed has its own inherent definition of viability Plate counts define viability as the ability of a cell to reproduce, while vital stains define living cells by the activity of intracellular enzymes Methylene blue has been commonly used in breweries to determine yeast viability However, its sensitivity has been questioned in comparison to newly developed methods including fluorescent vital staining and ATP bioluminesence (Lentini, 1993) Currently, methylene blue staining is considered accurate when yeast viabilities are in excess of 90% (O'Connor-Cox *et al*, 1997) This method is still commonly used by breweries, as some newer methods require specialised fluorescent microscope illumination (King *et al*, 1981, McCaig, 1990)

There has been increasing awareness of the limited information obtained from the determination of yeast viability (Imai, 1999) While it is useful to know the

percentage of dead cells in a yeast population, it is equally important to know the condition of the living cells Vitality is a measure of the metabolic activity or fermentation performance of living yeast cells (Lentini, 1993) Methods used to determine yeast vitality include metabolic activity, concentration of cellular components (glycogen, trehalose, sterols), fermentation capacity, acidification power, CO_2 evolution rate and oxygen uptake rate The methods used to determine yeast viability and vitality have been reviewed (Imai, 1999, Lentini, 1993) and new methods continue to be developed (Hodgson *et al*, 1994, Smart *et al*, 1999) The environmental conditions of a brewery can subject yeast to a range of physical stresses including temperature, osmotic, pressure and shear Chemical stress from ethanol or oxygen can also affect yeast physiology All of these stresses can reduce yeast viability and vitality (Walker, 1998) With increased demands placed on yeast in high-gravity brewing (Stewart *et al*, 1997), information regarding the vitality of yeast can prove invaluable in determining pitching rates for fermentation and in establishing specifications for the quality of stored yeast

1 3 1 2 The importance of glycogen in brewing yeast

Glycogen is a polymer of glucose which is an intracellular storage carbohydrate of yeast and other organisms Dissolved oxygen present in wort in the initial stages of fermentation acts as a trigger for the dissimilation of cellular reserves of glycogen (O'Connor-Cox, 1998a) Yeast cells utilise molecular oxygen to synthesise sterols and fatty acids which are essential cell membrane components (Aries and Kirsop, 1977) These compounds cannot be synthesised later in fermentation due to the absence of oxygen Therefore, the quantity of sterols synthesised in the initial

stages of fermentation must be sufficient to maintain the integrity of the pitching yeast cells and their progeny (Aries and Kirsop, 1977) The degree of wort oxygenation and glycogen content of the pitching yeast has a direct correlation with the quantity of sterols synthesised in the initial stages of fermentation (Quain and Tubb, 1982)

The glycogen reserves in yeast decrease dramatically in the first 24 hours of fermentation as glycogen is used as an energy source and a source of metabolic intermediates Reserves of this carbohydrate accumulate in the yeast cells later in fermentation as the sugar concentration of the wort decreases (Murray *et al*, 1984) This carbohydrate reserve is used by yeast under nutrient-limiting conditions as a source of energy and metabolite intermediaries Glycogen dissimilation occurs during the latter stages of fermentation and during storage of yeast The amount of cellular glycogen present in pitching yeast has a direct impact on fermentation performance Pitching yeast which are glycogen-replete ferment at a faster rate than glycogen-deficient yeast (O'Connor-Cox *et al*, 1996)

A novel pitching technique based on the glycogen content of the pitching yeast has resulted in significant improvements in fermentation consistency and quality (Quain and Tubb, 1982) The attainment of pitching yeast with elevated glycogen reserves is therefore a combination of the selection of a suitable cropping time (prior to a significant decrease in cellular glycogen reserves) and storage of yeast without significant loss of glycogen prior to pitching Cellular glycogen concentrations have been used as a monitoring tool of the effectiveness of yeast handling regimes

in major breweries, highlighting processes which are inappropriate or ineffective and result in unacceptable losses in cellular glycogen (O'Connor-Cox, 1998b)

Conventional analysis of the glycogen content of yeast by cell disruption and enzymatic hydrolysis of glycogen to glucose is laborious and time consuming (Parrou and Francois, 1997, Quain, 1981) Such methods do not lend themselves to routine analysis of yeast samples as part a yeast management programme in the brewery Rapid simplified procedures have been developed to measure the glycogen content of yeast cells, for example, near infra-red (NIR) spectroscopy (Mochaba *et al*, 1994) Quain and Tubb (1983) reported the development of a simple and rapid technique for the measurement of glycogen in yeast cells Yeast suspensions were stained with an iodine solution and the optical density at 660 nm was measured using a spectrophotometer Using an unstained yeast suspension as a blank, the optical density of the suspension with iodine solution correlated with the glycogen content of the yeast suspension Using this method, it was possible to visually distinguish between yeast suspensions containing high and low amounts of glycogen As a result, this method has been recommended as a simple and rapid test for brewers to modify yeast pitching rates based on their glycogen content in order to achieve improved fermentation consistency (Quain and Tubb, 1983)

1 3 2 Propagation

Brewers routinely re-use yeast cropped from one fermentation to pitch subsequent fermentations This process cannot continue indefinitely due to the spontaneous occurrence of respiratory-deficient mutants and increased risk of contamination

(Jones, 1997) Therefore, brewers propagate fresh yeast at regular intervals to minimise these occurrences Freshly propagated yeast is usually introduced into the brewery after 8 – 20 fermentation cycles (Boulton, 1996, Smart and Whisker, 1996) Fermentations pitched with freshly propagated yeast usually have an atypical flavour profile compared to fermentations pitched with cropped yeast (Jones, 1997) Blending of this beer with other batches of beer is regularly required to meet the final beer specifications

1.3.2.1 Wort

Good quality brewer's wort is generally replete with nutrients suitable for yeast growth and metabolism These nutrients include carbohydrates, amino acids, trace elements, vitamins and minerals The balance of nutrients in wort (as opposed to synthetic nutrient media) is biased towards a high carbohydrate content (mainly maltose) This is the essence of beer production, as the carbohydrate content of the wort dictates the ethanol content of the final beer Use of wort as a growth medium for the propagation of yeast is necessary as both the fermentation liquor and the newly propagated yeast biomass are added to the first fermentation Therefore, to maintain the flavour integrity of the beer, the same wort must be used

The original gravity of wort used for propagation is not widely reported However, for normal gravity brewing, the propagation gravity is similar to the fermentation gravity (Kunze, 1996) This situation is acceptable for normal gravity brewing (up to 12°P) but may not be acceptable for high-gravity brewing, where gravities up to

25°P may be used Propagation in wort with a high sugar concentration does not allow full respirative yeast metabolism (Jones, 1997) In contrast, fed-batch systems used for baker's yeast production maintain the sugar concentration in the growth medium at a low concentration to ensure that the yeast are maintained in respirative metabolism (Kristiansen, 1993) Fed-batch systems for brewery yeast propagation have been studied on laboratory scale (Masschelein *et al*, 1994) Fermentative metabolism is even more likely with high-gravity worts due to the increased sugar content of the wort The ethanol concentration at the end of propagation increases with increasing wort original gravity and therefore puts freshly propagated yeast under additional stress The tradition of batch yeast propagation in wort emphasises the necessity for the production of yeast capable of fermenting beer with specific flavour attributes, as opposed to producing maximal quantities of biomass

1 3 2 2 Multi-vessel propagation

Traditional yeast propagation regimes consist of a gradual scale-up from laboratory agar slopes to shake flasks, to 25 L Carlsberg flasks and thereafter a 1 5 or 1 10 stepchange in volume using two-stage and three-stage propagation vessels (Schmidt, 1995) The low scale-up factor between propagation vessels is to minimise the risk of contamination of the fresh yeast Aeration is usually intermittent or once-off to minimise foam production and to maintain the yeast in a metabolic and physiological state which will produce beer close to the required specification on its first fermentation Brewery propagations are generally oxygen-

limited due to plant design and the use of intermittent aeration (Jones, 1997), resulting in low cell counts at the end of propagation

1 3 2 3 Single vessel propagation

Improvements in the design of propagation vessels have resulted in the development of single vessel propagation systems Efficient aeration systems have been reported using an aeration lance positioned in the wort with perforations to generate small air bubbles (Munday and Dymond, 1998) Alternatively, wort aeration can be achieved using air injection via a recirculation loop on the propagation vessel (Geiger, 1993) Both off-centre and centrally-mounted agitation systems have been developed which improve the degree of wort oxygenation and mixing during propagation (Cholerton, 1995, Munday and Dymond, 1998)

The scale-up factor for single vessel propagators is significantly greater than for traditional propagation vessels due to improved aeration and mixing Scale-up factors can be as high as 1 300 (Wackerbauer *et al* , 1999) Significant increases in cell counts with single vessel systems have been achieved, for example 1 5 x 10^8 cells/ml for lager yeast and 2 5 x 10^8 cells/ml for ale yeast (Andersen, 1998) Faster yeast propagation rates have been achieved by increasing the temperature of propagation Lager yeast have been propagated at 20°C for subsequent fermentations at 15°C, with no negative impact on fermentation performance or beer flavour (Schmidt, 1995) High final cell counts coupled with rapid propagation times reduce the volume of propagation vessel required by the

brewery. These factors, in conjunction with a reduction in the number of stages required for propagation, result in significant cost savings for the brewery.

1.3.2.4 Continuous propagation

Continuous yeast propagation systems are, in effect, repeated batch propagations. Initially, a propagation vessel is inoculated with a Carlsberg flask as in the traditional propagation system. When propagation is complete, typically 90% of the propagation vessel contents is removed to pitch a fermentation (Schmidt, 1995). The remaining 10% of the propagation serves as a seed for the next propagation. The propagation vessel is topped up with fresh wort and propagation continues until the required cell numbers are achieved to pitch the next fermentation. Therefore, a fresh propagation is available for pitching into a fermentation at regular intervals. Propagation times of 48 hours for lager yeast at 10°C (Brandl, 1996) and 24 hours at 25°C have been reported (Schmidt, 1995). This so-called 'pitch and ditch' system uses freshly propagated yeast to pitch each fermentation and no yeast is cropped at the end of fermentation. These systems claim reduced propagation times compared to conventional propagation regimes and production of beer with a normal taste profile after the first fermentation (Geiger, 1993).

1.3.3 Fermentation

The success of brewery fermentations is dependent on many variables including wort quality and original gravity, yeast quality, pitching rate, aeration rate,

fermentation temperature and reactor configuration In the past, a wide range of fermentation vessel configurations has been used which played an integral part in determining the final flavour of the beer, for example, Yorkshire stone squares and Burton Union fermentation systems (Hough *et al*, 1995) Nowadays, cylindro-conical fermentation vessels are used widely throughout the brewing industry due to their efficient mixing during fermentation and ease of cropping of yeast from the conical base of the fermenter (Nathan, 1930, Shardlow, 1972, Ulenberg *et al*, 1972)

1.3 3 1 Pitching

In the past, the addition of yeast to fresh wort at the start of fermentation was achieved by 'pitching' a yeast wreath into an open fermentation vessel (Andersen, 1998) This term is still used to describe the addition of yeast to wort, although the mechanism of dosing is radically altered In simple terms, pitching of wort involves the addition of a quantity of yeast to fresh wort However, several important questions revolve around pitching, which can have a direct impact on the success of a fermentation The pitching rate used for fermentation is dependent on the original gravity of the wort A pitching rate of one million viable cells per degree Plato per ml of wort is used by many brewers as a guideline (Casey *et al*, 1984, Casey and Ingledew, 1983, O'Connor-Cox and Ingledew, 1990) The actual pitching rate used by the brewer can be strain-dependent In practical terms, the addition of biomass to wort is achieved with pumps, flowmeters, load cells or in-line biomass probes Therefore, calculated volumes or weights of yeast slurry are added to the wort Yeast injection usually occurs in-line as the wort is filling into

the fermenter to ensure adequate re-suspension of the biomass The correlation between volume or weight of slurry with cell numbers is dependent on the mean cell size of the yeast and on the quantity of trub present in the yeast slurry Actual pitching rates can vary due to the presence of trub and due to the non-uniformity of yeast slurry resulting from poor storage conditions (O'Connor-Cox, 1998a)

1 3 3 1 1 Proportional pitching

An alternative to pitching of fermentations using propagated or cropped yeast is proportional pitching or gyling Typically, 10% of the fermenter contents is removed after 24 hours fermentation to pitch a new fermentation (Donnelly and Hurley, 1996) The yeast are actively growing and the lag phase of conventionally-pitched fermentations is effectively eliminated Gyling is routinely used in the production of high-gravity stout (18°P) If the actively fermenting liquor is not required immediately for pitching, it must be stored at 4°C

1 3 3 1 2 Active dried yeast

The manufacturing process for active dried yeast (ADY) involves propagation of the yeast in highly aerated fermentation vessels, followed by harvesting and drying of the biomass using centrifugation and fluidised-bed driers (McLaren, 1991) ADY has a shelf life of years and is used world-wide in the manufacture of bread and wine The advantages of using ADY in brewing are significant The use of ADY to pitch fermentations eliminates the need for a propagation plant in the brewery Furthermore, production of a wide range of beers (for example, seasonal

beers) using a number of different strains is considerably simplified The problems and costs associated with perishability, transportation and storage of yeast are also reduced Reported use of ADY for beer production demonstrated typical fermentation profiles and a final beer which was comparable to fresh yeast control beer (Lawrence, 1986)

1.3 3.2 Aeration

Wort is aerated prior to pitching to provide the yeast with sufficient oxygen to synthesise essential membrane components, i e sterols and fatty acids Dissolved oxygen in aerated wort stimulates glycogen dissimilation and sterol and fatty acid synthesis (O'Connor-Cox *et al* , 1996) Sufficient oxygen must be added to ensure the synthesis of adequate amounts of these compounds for the pitching yeast and their subsequent progeny (buds) These compounds cannot be synthesised later in fermentation due to the absence of molecular oxygen (Aries and Kirsop, 1977) Under-oxygenation of wort can result in lower cell counts in fermenter, slower fermentation rates, possible tailing of fermentation and reduced cell viability in the yeast crop (Noble, 1997) On the contrary, over-oxygenation can result in increased biomass yield, faster fermentation rates, excessive fobbing, altered flavour profile of the beer and excessive beer losses on centrifugation (Edelen *et al* , 1996)

The extent of wort aeration is dependent on the wort gravity, pitching rate and the specific oxygen requirements of the yeast strain The pitching rate for high-gravity fermentations increases on a pro-rata basis according to the aforementioned rule of

thumb for pitching used by brewers Therefore, the degree of wort aeration increases accordingly The maximum concentration of dissolved oxygen attainable in wort using air as the aeration gas is approximately 8 ppm If increased concentrations of dissolved oxygen are required then pure oxygen or oxygen enriched air is required to aerate the wort Over or under-aeration of the wort can obviously occur due to incorrect injection rates of air into the wort stream However, if the aeration rate is correct and the wort is underpitched, then the specific oxygen supply to each yeast cell is increased, leading to increased cell growth and possible flavour alterations In a similar manner, over-pitching coupled with normal aeration can result in each cell being under-aerated, which can lead to reduced cell growth and reduced viability of yeast crops

1.3.3.3 Original gravity

Normal gravity brewing refers to the fermentation and conditioning of beer at the gravity (and alcohol concentration) at which the beer is sold to the consumer (typically 12°P) However, high-gravity fermentation systems have been developed which involve fermentation at gravites in excess of trade gravity followed by dilution with de-aerated liquor prior to packaging High-gravity brewing processes have been in operation for the past 25 years and currently more beer in the USA is produced by high-gravity brewing than by so-called conventional brewing (Stewart *et al*, 1997) Both ale and lager yeast have been utilised in high-gravity fermentations However, lager yeast in general are more robust compared to ale yeast strains (Borthwick *et al*, 1997) The benefits of high-gravity brewing include increased capacity, decreased energy, labour and cleaning costs, improved flavour

stability, increased ethanol yield per degree Plato, smoother beer flavour and the possibility of higher adjunct addition rates There are also a number of disadvantages to high-gravity brewing including decreased brewhouse efficiency, decreased foam stability, reduced hop utilisation rates and decreased yeast viability and vitality (Stewart *et al*, 1997) High-gravity brewing can have deleterious effects on yeast quality Increased wort gravity in fermentation results in significant losses in yeast viability early in fermentation (Casey and Ingledew, 1983) Acid washing in conjunction with high-gravity brewing also reduces yeast fermentation performance compared to conventional gravity brewing (Cunningham and Stewart, 1998) The benefits of high-gravity brewing often outweigh any of the associated problems Therefore, the main objective of the brewer is to ensure satisfactory flavour matching of the beer to that of normal gravity brewing

1.3.4 Cropping

Cropping is the term which applies to the harvesting of yeast from a completed fermentation for storage and subsequent pitching of fermentations The type of cropping regime used in a brewery is dependent on the type of yeast used in the fermentation (top or bottom fermenting yeast) and on the fermenter configuration (e g open-topped vessel, cylindro-conical)

The timing of cropping can have a direct impact on the quality of yeast harvested The quality of yeast cropped from a fermentation which has reached its attenuation limit rapidly deteriorates with time Furthermore, yeast starts to sediment into the cone of a fermenter before the attenuation limit is reached, resulting in the

commencement of 'yeast storage' before the fermentation is complete The yeast in such an environment can rapidly deteriorate due to severe nutrient limitations, high ethanol concentration and increased local temperatures due to metabolic heat generation (O'Connor-Cox, 1998a) Early cropping involves the removal of yeast plug before the fermentation has reached its attenuation limit Early cropping regimes have been implemented in major breweries to overcome this phenomenon and have resulted in improved fermentation performance (Loveridge *et al*, 1997, O'Connor-Cox, 1998a)

1.3 4 1 Top fermenting yeast

Traditional ale fermentations are conducted in shallow vessels open to the environment Such configurations allow the development of a yeast head on the surface of the fermentation liquor during fermentation The yeast head (in the form of a dense yeast foam) is removed at various stages during the fermentation by skimming the yeast head into a small yeast vessel or wagon (Hough *et al*, 1995) The yeast is typically stored without agitation in a refrigeration unit until required for pitching The traditional difference in cropping regimes between top and bottom fermenting yeast is disappearing with the widespread use of cylindro-conical vessels, as both yeast types are cropped from the base of the vessel (Donnelly and Hurley, 1996) Centrifugation of the yeast from the fermentation liquor is usually associated with cylindro-conical vessels

1 3 4 2 Bottom fermenting yeast

The onset of yeast flocculation occurs in fermentation as the sugar concentration decreases and the ethanol concentration increases Cylindro-conical vessels are ideally suited to the development of a yeast plug in the base of the fermenter (Nathan, 1930) Such a plug can be removed by gravity into a yeast collection vessel for storage

1 3.5 Storage

Storage of brewer's yeast is a routine practice in breweries, as yeast is cropped at the end of one fermentation and stored chilled prior to pitching subsequent fermentations Yeast quality should be maintained during storage to ensure that pitching is achieved with yeast of high viability and vitality In the past, yeast has been stored as a pressed yeast cake or in a slurry However, pressed yeast is prone to contamination, is difficult to re-suspend in fresh wort and is difficult to maintain at a uniform temperature throughout (Pollock, 1981) The preferred method of storage is in the form of a yeast slurry in beer or beer and water mixture (Pollock, 1981) The key aspects of yeast storage include the duration of storage, temperature, metabolic heat generation and the effectiveness of the mixing regime These topics will be discussed in detail as each plays an important role in successful yeast storage

1 3 5 1 Duration

Ideally, yeast should be stored for as short a period as possible, although storage times of up to 7 days have been reported (Pagh-Rasmussen, 1978) Yeast quality deteriorates during storage as a result of starvation conditions and due to the presence of ethanol and dissolved carbon dioxide Glycogen reserves of yeast decrease significantly during storage and this results in poor fermentation performance of the yeast (Quain and Tubb, 1982) A number of major breweries have improved the fermentation performance of their yeast by imposing maximum time limits for yeast storage of 48 hours (Loveridge *et al*, 1997) and 24 hours (O'Connor-Cox, 1998a)

1 3.5 2 Temperature

Stored yeast is in a catabolic state and therefore increased storage temperature increases the rate of metabolism which results in glycogen dissimilation and autofermentation (McCaig and Bendiak, 1985a) The recommended storage temperature for yeast slurry is in the range 2 - 5°C (Lenoel *et al*, 1987, Martens *et al*, 1986) Storage temperatures in excess of 10°C result in an increased rate of yeast deterioration compared to 5°C or below (McCaig and Bendiak, 1985a) Storage temperatures below 0°C may result in ice-damage of the yeast cell membranes It is important that the slurry maintains a uniform temperature throughout It is common practice to control the temperature of yeast slurry in a vessel using the output from a single temperature probe close to the vessel wall The temperature readout for the vessel therefore represents the temperature of yeast slurry close to the vessel wall (and cooling jacket) It is important that the

temperature of the entire volume of stored yeast slurry be maintained close to the temperature setpoint of the controller Therefore the storage temperature is important in terms of the setpoint used and also in terms of the uniformity of temperature achieved throughout the yeast crop It has been recognised that storage of pitching yeast in insulated unmixed vessels in a refrigerated coldroom can lead to the development of hotspots in the yeast slurry due to metabolic heat generation (O'Connor-Cox, 1998a) Temperature uniformity can be improved using an appropriate agitation system

1.3.5.3 Metabolic heat generation

At the end of fermentation, brewer's yeast contains significant reserves of the storage carbohydrate glycogen (Murray *et al*, 1984) This reserve serves as an energy source for maintenance metabolism during storage Autofermentation by yeast results in the depletion of internal glycogen reserves, increased ethanol concentration and the generation of heat (O'Connor-Cox, 1998a) Being in a catabolic state during storage, yeast are strongly influenced by local environmental temperatures Therefore, if this metabolic heat is not removed during storage, the slurry temperature will increase accordingly, resulting in an increased rate of glycogen dissimilation and further heat generation Adequate mixing and cooling of the slurry is central to maintaining a low rate of cell metabolism and sufficient glycogen reserves in the pitching yeast for fermentation

1.3.5.4 Agitation

Brewery pitching yeast is typically stored as a slurry of approximately 40% (w/w) solids Such slurries are viscous and pseudoplastic (shear-thinning) Mixing of yeast slurries during storage is essential to ensure the uniformity of solids concentration and temperature throughout the slurry There is a diverse range of agitators used during storage of brewer's yeast including small diameter impellers (Cholerton, 1995, Murray *et al*, 1984), off-centre impellers (Munday and Dymond, 1998), slow speed, large diameter paddles (Kawamura *et al*, 1999) and mixing by recirculation of yeast slurry from the base of the vessel to the top via an external pumping loop (O'Connor-Cox, 1998a)

1.3.6 Acid washing

Pitching yeast is potentially the greatest source of contamination in a brewery (Simpson, 1987) Ideally, contaminated pitching yeast should be discarded and replaced with a contaminant-free yeast crop or freshly propagated yeast As this is not always possible in a brewery, reduction of bacterial contamination of brewer's pitching yeast can be achieved by treating the slurry with a concentrated acid solution, for example, tartaric, phosphoric or sulphuric acids (Hough *et al*, 1995) Acid washing is effective in the pH range 2 2 – 3 0 but at a pH value of 2 0 the pitching yeast is adversely affected (Fernandez *et al*, 1993) Most bacteria present in the yeast slurry are inactivated at the low pH values attained However, some lactic acid bacteria are resistant to such low pH values (Cunningham and Stewart, 1998) Acid washing can have a deleterious effect on yeast viability and vitality if the temperature during washing exceeds 5°C or if the yeast is in poor physiological

condition prior to washing (Simpson and Hammond, 1989) Furthermore, it has little effect on wild yeast or other cross-contaminant yeast strains Therefore, acid washing should not form a routine part of a brewery's yeast handling process, but can be implemented as required to control bacterial contamination of the pitching yeast

1.4 FUNDAMENTALS OF IMAGE ANALYSIS

Vision plays a central role in the lifestyle and understanding of mankind Many people will tolerate significant hearing loss before opting to use a hearing aid, while few will go without corrective lenses for relatively minor defects in sight Expansion of the range of human vision was achieved through the invention of the first microscope in the 17^{th} century by Antonie van Leeuwenhoek The magnification of 200 to 300 times was sufficient to observe a wide variety of microorganisms (VanDemark and Batzing, 1987) The invention of the microscope has played a significant part in the advancement of our understanding of the principles of microbiology Photographic images of microorganisms have been in existence for over a century A photograph of Guinness ale yeast strain 1164 is presented in Figure 1 3 The quality of the image is comparable to images produced by modern microscopes However, this image dates from 1897 The original photograph on a glass plate was scanned to produce a digitised image consisting of hundreds of thousands of picture elements or pixels Enlargement of the image reveals the presence of these pixels as shown in Figure 1 3 Although images of high quality have been available for over 100 years, the development of

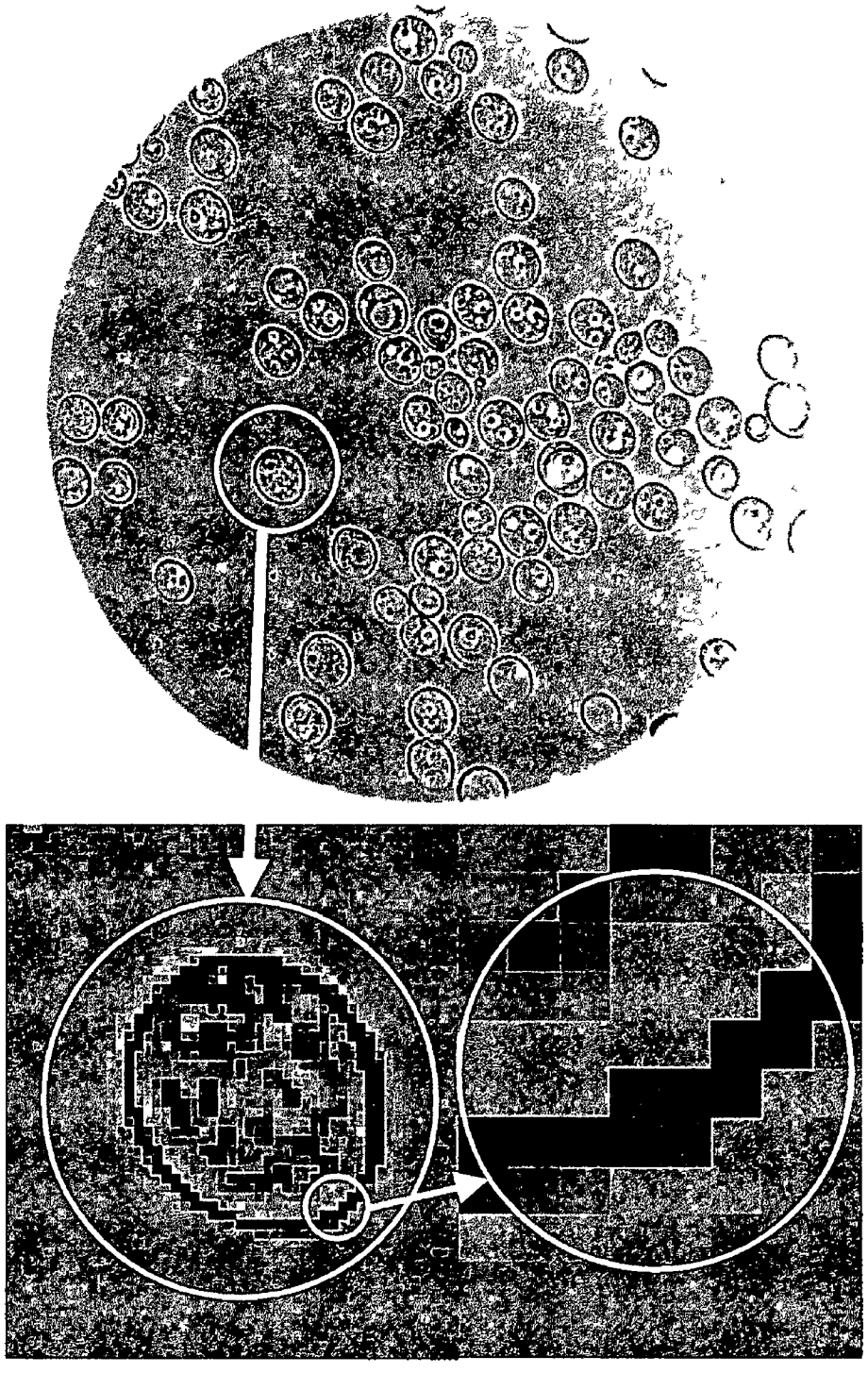

Figure 1.3. A digitised image of a photograph of Guinness ale yeast strain 1164 taken over 100 years ago (30/1/1897)

image analysis has been totally dependent on the evolution of other technologies. The advance of camera, video and computer technology in the past decades has led to the development of image analysis and expanded its applications and methodologies considerably (Vecht-Lifshitz and Ison, 1992). Image analysis refers to the digitisation of an image into a grid of pixels which enables the measurement of the light intensity at each pixel. Eight-bit black and white images are recorded as an array of pixels with a range of light intensity values ranging from black (0) to white (255). These digitised images consist of 256 (2^8) grey levels which surpasses the sensitivity of the human eye which can only distinguish 30 grey levels in monochrome images (Russ, 1995). Colour images consist of three separate colour bands (red, green and blue), each with an intensity range from 0 to 255. Colour images require even greater resolution to compare to the sensitivity of the human eye, for example, 12 bit (2^{12} levels of colour) and 16 bit (2^{16} levels of colour) (Russ, 1995). Image analysis systems can process grey images, colour images or single bands of colour images as required by the application.

1.4.1 Elements of an image analysis system

An image analysis system consists of five main components, a personal computer (PC), a framegrabber board, a camera, appropriate magnification lenses and image analysis software (See Figure 1.4). Depending on the application, the video camera can be attached to a microscope for the examination of microscopic features, a macro lens for the observation of larger features or a telescope for the observation of distant objects. The selection of the type of video camera also depends on the

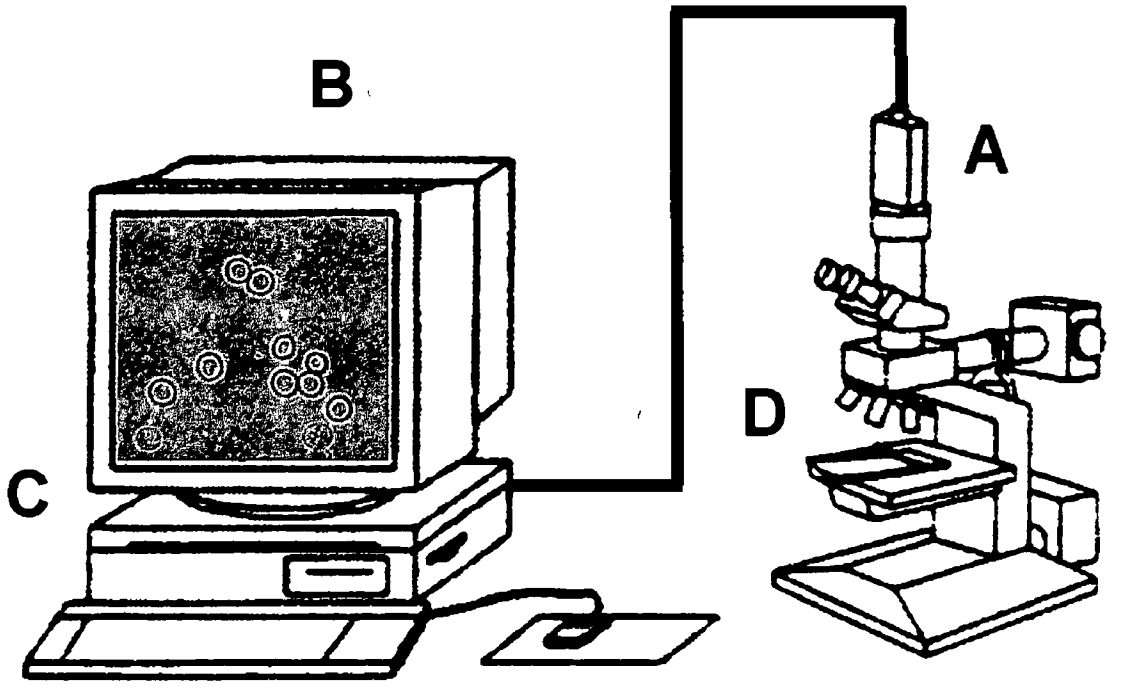

Figure 1.4. A schematic of a typical image analysis system incorporating a video camera (A), PC monitor (B), PC processor and framegrabber board (C) and microscope (D)

application required The array of video cameras is vast and ranges from monochrome cameras to high-speed colour cameras The framegrabber board installed in the PC converts the signal from the video camera into a digitised format consisting of hundreds of thousands of pixels Once digitised, the images can be processed using the image analysis software The processing power of the PC is dependent on the complexity of image processing required for the application Modern PC's are extremely powerful and capable of high speed processing of images

The main elements of image analysis are as follows image acquisition, image enhancement, identification of features of interest, measurement of selected

features and output of data to an appropriate spreadsheet Each of these elements is discussed in further detail in the following sections

1.4.2 Image acquisition

Image analysis can be applied to the examination of images from a wide range of sources, for example, x-rays, telescopic images, microscopic images, hand-held cameras, digital scanners and video cameras The image in question must be in a digitised format consisting of an array of pixels Video cameras and scanners are ideally suited to this purpose The data is relayed to a computer via a framegrabber board Once stored in the memory of the computer as an image file, the appropriate image analysis software can be used to process the image

1 4 3 Image enhancement

In many instances, captured images lack sufficient resolution due to camera vibration, inadequate illumination, electrical interference and the presence of dirt or contamination on the camera lens Image analysis techniques have been developed to enhance image quality by eliminating or reducing the defects mentioned above Electronic noise in the image, which appears as bright spots throughout an image, can be reduced in intensity by using an averaging filter A 3 x 3 filter, for example, replaces the intensity of a pixel with the mean of the surrounding 9 pixels (a 3 x 3 square of pixels with the pixel of interest in the centre) Larger filter sizes can be applied also (5 x 5, 7 x 7, etc) Averaging filters reduce noise but also result in blurring of edges Median filters are particularly effective in removing extreme noise from an image, i e black pixels in a white region and vice versa In this case,

the median value of the surrounding pixels replaces the intensity of the centre pixel Blurring of images is less likely to occur with median filters

Depending on the application, sharp edges may be required in order to identify the features of interest An edge can be defined as a number of pixels which differ in intensity from the surrounding pixels Application of a filter which replaces each pixel value with either the maximum or minimum value in its neighbourhood (posterisation) can successfully detect edges in an image

Adjustment of contrast for poorly illuminated images is effective in highlighting detail otherwise lost in the image Dark images consist of pixel intensities at the lower end of the range 0 to 255 Assume the intensity range in an image is, for example, 0 to 100 Expansion of this intensity range to the entire range 0 to 255 will result in improved resolution within the image The same technique can be applied to light-saturated images

There are many different filters available to enhance image data and it is beyond the scope of this chapter to explore these in detail However, many of these techniques are discussed in detail by Russ (1995) These filters are most effective when used in conjunction with other filters to enhance the images so as to enable detection of the features of interest

1.4 4 Feature identification

A fundamental element of image analysis is the identification of the features of interest by distinguishing them from the image background and from other objects

which may be present The most widely used method of feature detection is thresholding This method is based on the assumption that the features of interest consist of, or are bounded by, pixels which are significantly different from the image background by being darker or brighter By setting an appropriate threshold value, the operator selects all pixels in the image with a specific range of intensity values Once selected, the image is converted to a binary image which consists of black pixels and white pixels only as determined by the threshold value Features are then identified as a connected group of black pixels in a binary However, it is likely that other objects in the image have been detected which are not of interest Classification of all detected objects allows the filtering out of all unwanted objects Objects can be classified based on size and shape so as to capture all features of interest and eliminate all unwanted objects The features of interest can then be measured and the data output sent to a spreadsheet for analysis Basic measurement of features include length, breadth, cross-sectional area, perimeter, circularity, centroid of object and equivalent mean diameter A plethora of measurements can be conducted by superimposing the outline of the detected features back onto the original image, including mean grey value, colour (as intensities of red, green and blue), maximum grey (or colour band) values, minimum grey (or colour) values and optical density

1 5 APPLICATIONS OF IMAGE ANALYSIS

Image analysis has been used for a wide range of microbiological applications including colony counting on agar plates (Caldwell and Germida, 1985), determination of minimum inhibitory concentrations of antibiotics using plate assays (Hammonds and Adenwala, 1990) and the observation of chemotaxis in

amoebae (Fischer *et al.*, 1989). Many reported biotechnological applications of image analysis concern the morphological and physiological characterisation of industrially-important microorganisms.

1.5.1 Filamentous organisms

Cultivation of filamentous organisms in submerged fermentations is an essential part of the production of many industrially-important products including antibiotics, alcohols and organic acids (Cox and Thomas, 1992). It is acknowledged that physiological control is of prime importance for secondary metabolite production by filamentous microorganisms. Cell morphology in many instances relates to the physiology of the microorganism. Characterisation of the morphology of filamentous organisms during fermentation is difficult due to the diversity of forms in which biomass can occur, ranging from filaments to pellets. Initial characterisation of filaments involved the use of a digitising table with which the key points of the filaments in an image were manually digitised for further processing (Metz *et al.*, 1981). This type of approach to morphological characterisation was extremely labour intensive with an estimated processing time of 83 hr for each sample of 1000 filamentous cells of *Streptomyces clavuligerus* (Adams and Thomas, 1988). Image analysis has superseded this technology and is capable of rapid processing of images. The degree of complexity of image processing has a direct impact on the processing time required. However, improvements in the speed of computer processors has resulted in the decrease in processing times from 17 hr (Adams and Thomas, 1988) to 4 – 7 hr (Packer *et al.*, 1992) to 89 min. (O'Shea and Walsh, 1996). With current processing speeds

available, the realisation of real-time or near-real-time morphological analysis of filamentous microorganisms is imminent.

Morphological examination of filamentous organisms using image analysis is capable of characterising the microbial population into a series of classes including mycelia, mycelial aggregates, smooth pellets and rough (or hairy) pellets (Treskatis *et al.*, 1997). A major advantage of using image analysis in morphological studies is the ability to measure the mean size of various classes and also the frequency distribution of classes (for example, hyphal length, pellet density, pellet size). Cultivation of *Streptomyces tendae* under different growth conditions results in either a normal distribution or bimodal distribution of pellet size in the population (Reichl *et al.*, 1992).

The combination of image analysis technology with classical and modern staining techniques can provide a valuable insight into the physiology of filamentous microorganisms. Colour image processing, combined with differential staining techniques using a combination of methylene blue and Ziehl fuschin, have identified 6 physiological states in *Penicillium chrysogenum* during fermentation (Vanhoutte *et al.*, 1995). Grey level processing was shown to be less sensitive and had increased incidence of mis-classification compared to colour processing. Image analysis techniques have been developed to study the physiological state of filamentous organisms by measuring the degree of vacuolisation which occurs during fermentation (Packer *et al.*, 1992; Paul *et al.*, 1992).

1.5.2 Dimorphic organisms

The morphology of dimorphic yeast strains has been shown to vary from ellipsoidal yeast cells to branched filaments as a result of changes in environmental conditions (Walker and O'Neill, 1990) Image analysis techniques have been developed to enable the classification of dimorphic yeast into 6 morphological groups including yeast-like cells, elongated cells, double yeasts, filaments, double filaments and mycelia (O'Shea and Walsh, 1996) Such techniques have been used to monitor the distribution of cell morphology during fermentation Measurement of the distribution of various cell morphologies in fermentation broths using image analysis has also been successfully used to predict filtration characteristics (McCarthy *et al*, 1998)

1.5.3 Yeast

The image analysis techniques which have been applied to the study of yeast morphology have mainly related to the dimensions and shape of individual cells, double cells, clusters and flocs The morphology of yeast cells can be related to its physiological status For example, it is known that some yeast strains elongate when exposed to stress (Shimozaka *et al*, 1991) Measurement of the size distribution of yeast flocs enables calculation of the mass transfer limitations which are likely to occur during fermentation (Vicente *et al*, 1996) Analysis of the geometric parameters of individual yeast during fermentation provides near-real-time information about the frequency distribution of yeast cell size and the proportion of the population which exist as single cells, budding cells and clusters (Pons *et al*, 1993, Zalewski and Buchholz, 1996) Automatic sampling and dilution systems have been developed using flow-through cells attached to a

camera system, which allow continuous automatic monitoring of cell counts and morphology during fermentation (Zalewski and Buchholz, 1996)

Techniques have been developed to determine the actual physiological state of yeast (as opposed to its morphology) using a combination of staining techniques and image analysis Cell viability has been measured using a combination of image analysis and methylene blue staining (Pons *et al*, 1993) Once identified, the yeast cells are separately classified as living or non-living based on the mean grey value of the cells A non-destructive method involving modern fluorescent staining techniques combined with image analysis have been used to measure the intracellular pH of intact yeast cells (Imai and Ohno, 1995) Using a fluorescent microscope, yeast cells stained with 5 (and 6)-carboxyfluoroscein are exposed to excitation wavelengths of 441 nm and 488 nm The ratio of emitted light (at a wavelength of 518 nm) from each source is proportional to the intracellular pH of the yeast cells This technique illustrates the evolutionary direction of image analysis Early methods involved the processing of monochrome images to yield physical measurements of cells (Pons *et al*, 1993) Later methods combined staining techniques to identify cells or regions of interest in images based on staining or non-staining Currently, imaging techniques have become more sophisticated, and are capable of estimating physiological parameters such as intracellular pH based on the colour intensity measured in yeast cells

1 6 OBJECTIVES OF THIS WORK

Yeast management encompasses a broad range of processes within the brewery With increasing pressure on breweries production capacity and the prevalence of high-gravity brewing, there are increased demands placed on brewery yeast Increased automation and improved fermentation plant go some of the way towards meeting the demands placed on breweries However, yeast management cannot remain unaltered if the goals of maintaining yeast quality and fermentation performance are to be met in an ever-changing brewery environment The objectives of this work are to study some of the key aspects of yeast management in order to optimise yeast handling regimes and to gain greater insight into the effect of handling processes on yeast physiology Image analysis techniques will be developed and applied to the measurement of yeast geometric parameters and intracellular components The objectives of this work are detailed below

Yeast propagation

- To investigate the effect of propagation wort gravity on yeast morphology using image analysis
- To determine the optimal gravity in which yeast can be successfully propagated
- To determine the impact of propagation gravity on subsequent fermentation performance

Pitching of fermentations using stored yeast

- To measure changes in cell volume during storage of yeast using image analysis technology
- To optimise pitching regimes for fermentations

Glycogen content of yeast

- To develop a near-real-time image analysis technique to determine the mean glycogen content of yeast
- To use this technique to indicate the distribution of cellular glycogen throughout a yeast population

Temperature control in unmixed yeast crops

- To measure the extent of thermal gradient development in un-mixed yeast crops
- To determine the contribution of metabolic heat generation to the magnitude of thermal gradients

Temperature control in mixed stored yeast

- To design a 10 hl yeast storage vessel to examine the effect of different agitation regimes on the distribution of temperature, cell viability and solids concentration in stored yeast slurry
- To determine the best agitation regime for storage of yeast slurry

CHAPTER 2

THE EFFECT OF THE CONCENTRATION OF PROPAGATION WORT ON YEAST CELL VOLUME AND FERMENTATION PERFORMANCE

2 1 INTRODUCTION

Propagation of yeast is an integral part of brewery operations and is central to the maintenance of quality and consistency of fermentations However, the propagated yeast must be in an appropriate physiological state and be capable of producing standard beer from its first fermentation Brewery yeast propagation is unlike the biomass propagation systems used in other fermentation industries Baker's yeast, for example, has been the focus of much research and is normally produced in highly aerated fed-batch systems, maximising biomass yield and minimising ethanol production (Pirt, 1975, Rose and Vijayalakshmi, 1993, Van Hoek *et al*, 1998) This process eliminates fermentative metabolism in *Saccharomyces cerevisiae* Brewer's yeast is, however, propagated in standard brewery wort with a variety of aeration regimes (once-off, intermittent or continuous) The degree of aeration and mixing used in brewery propagations is likely to lead to oxygen-limited growth (Jones, 1997) Likewise, the growth medium itself is high in sugars and due to the limited oxidative capacity of *S cerevisiae*, significant quantities of wort sugars are converted to metabolic end-products by oxido-reductive metabolism (Nielsen and Villadsen, 1994) To overcome these difficulties some smaller breweries import yeast from larger breweries as an alternative to yeast propagation (Litzenburger, 1988)

There are numerous developments reported on efficient propagation systems using some of the techniques normally employed outside of the brewing industry, including single-vessel propagators (Andersen, 1998, Cholerton, 1995), continuous propagation systems (Geiger, 1993, Kunze, 1996) and fed-batch systems (Masschelein *et al*, 1994) Single vessel and fed-batch systems produce yeast of improved quality in reduced times compared to conventional multi-vessel systems, with standard beer quality resulting from the first fermentation using propagated yeast (Masschelein *et al*, 1994, Wackerbauer *et al*, 1996) The gravity of wort in which brewer's yeast is propagated is an important process parameter and, in some instances, standard casting wort is used (Kunze, 1996) However, the wort OG used for brewer's yeast propagation is not widely reported, and as high-gravity brewing continues to become widespread in the brewing industry (O'Connor-Cox, 1998b), it is likely that high-gravity propagation will become a normal feature of brewery operations The use of standard brewhouse wort eliminates the need for special propagation wort brews and the possible 'dilution' of high-gravity fermentations with low gravity propagation wort High-gravity propagation eliminates the need for sterile liquor addition or re-sterilisation of brewhouse wort after liquor addition in the propagation vessel However, high-gravity brewing puts brewer's yeast under additional stress compared to conventional brewing, for example, osmotic stress, ethanol stress and reduced resistance to acid washing (Borthwick *et al*, 1997, Casey and Ingledew, 1983, Schmidt, 1995, Stewart *et al*, 1997) It is likely that high-gravity propagation will also impose increased stress on yeast

This chapter examines the effect of wort OG on the propagation of ale and lager yeast strains Propagation of these yeasts in increasing wort gravity alters yeast cell volume The effects of propagation wort OG on yeast cell volume and on fermentation performance are examined in order to optimise the wort gravity used for yeast propagations

2.2 EXPERIMENTAL

2.2.1 Yeast strains

Ale and lager fermentations were conducted using three Guinness brewery strains of *Saccharomyces cerevisiae* The yeast strains are identified as ale yeast 1164, ale yeast 662 and lager yeast 7012

2.2.2 Wort

A batch of standard brewery ale and lager wort was diluted with deaerated brewing liquor as required to give wort gravity values in the range 7 5 to 17 5°P for yeast propagations One litre aliquots of diluted wort were dispensed into 2 L Erlenmeyer flasks and sterilised in a steam cabinet at 100°C for 2 hours For each fermentation trial, a single batch of standard brewery ale (17 5°P) and lager wort (12 5 and 17 5°P) was used Each batch of wort was dispensed into 12 L aliquots and sterilised as above This procedure results in microbiologically stable worts The original gravity was determined after steaming

2.2.3 Yeast propagation

All propagations were carried out using 1 L of wort in 2 L Erlenmeyer flasks and incubated in an orbital shaker (Gallenkamp, model INR 250 010J, Gallenkamp UK) at 24°C and 120 rpm The standard propagation times were 48 hr for both ale yeasts and 72 hr for lager yeast The wort OG used for propagation ranged from 7 5°P to 17 5°P as indicated in Table 2 1

2.2.4 Pitching regime

Fermentations were undertaken using a top fermenting (ale 1164) yeast and a bottom fermenting (lager 7012) yeast For all fermentations, the propagated yeast was centrifuged and pressed to produce a yeast cake in order to avoid dilution of the fermentation gravity with low gravity propagation wort Fermentations were pitched either on a weight per volume basis or on a cell number basis in order to examine the effect of different cell sizes on pitching rate When pitching on a weight per volume basis, the appropriate weight of yeast cake was re-suspended in

Table 2 1 Propagation conditions for yeast strains

Yeast Strain	Temperature (°C)	Duration (hr)	Wort OG (°P)
Ale Yeast 1164	24	48	7 5, 10, 12 5, 15, 17 5
Ale Yeast 662	24	48	7 5, 10, 12 5, 15
Lager Yeast 7012	24	72	7 5, 12 5, 17 5

100 ml of wort and then added to the fermentation vessel The pitching rate was 2 5 g/L for top fermenting yeast and 3 75 g/L for bottom fermenting yeast The percentage viability of the stored yeast was taken into account when used for pitching, therefore, 2 5 g/L and 3 75 g/L of viable yeast were added to each fermentation When pitching by cell number, the cell count on the pressed yeast was performed and the weight of yeast required was calculated to ensure a specific yeast cell number for pitching The pitching rates for top fermenting yeast and bottom fermenting yeast were 1 x 10^7 and 1 5 x 10^7 viable cells per ml respectively

2.2.5 Fermentation conditions

All fermentations were conducted in duplicate in 2 L European Brewery Convention (EBC) tall-tube glass fermenters The fermentation conditions are outlined in Table 2 2 The dissolved oxygen concentration was measured prior to pitching using an Orbisphere DO meter (Model 26131, Orbisphere Laboratories, Geneva Switzerland)

2.2 6 Determination of specific gravity

10 ml samples of fermenting broth were centrifuged to remove yeast using a bench centrifuge at 2,500 rpm (600 g) (Baird & Tatlock Auto Bench Centrifuge Mark IV, Baird & Tatlock Ltd UK) The supernatant was degassed by shaking in a 50 ml conical flask and analysed for present gravity using a Paar Density meter (Model DMA46, Anton Paar KG, Austria)

Table 2 2. Fermentation operating parameters

	Ale Trial	Lager Trial
Wort	Ale	Lager
OG (°P)	17 5	12 5 or 17 5
Temperature (°C)	24	15
Pitching Rate	2 5 g/L or 1 0 x 10^7 / ml	3 75 g/L or 1 5 x 10^7 / ml
Initial DO (ppm)	8	8

OG Original Gravity
DO Dissolved Oxygen

2 2 7 Yeast enumeration and measurement of viability

Cell counts were performed in duplicate on all samples using a Thoma counting chamber In all cases, the viability of yeast was measured using the methylene blue staining technique (Pierce, 1970)

2.2.8 Wet weight determination

Duplicate 10 ml samples were centrifuged and washed twice in 5M NH_4OH to remove trub (Enari, 1977) Trub removal was required in order to minimise errors in wet weight analysis for the range of wort gravities used The washed yeast suspension was then filtered through a pre-weighed 0 45 µm membrane filter (Gelman Sciences, Michigan USA) and washed with distilled water The wet

weight was expressed as g/L A correlation between wet weight and dry weight yielded an r^2 value = 0 98

2.2 9 Image analysis

Microscopic images of the yeast samples were recorded using a JVC KY-F55B colour video camera (Victor Company of Japan Ltd , Japan) attached to a Nikon Optiphot microscope (Nikon Corp , Tokyo) at 400X magnification The images were stored and processed with Optimas 6 1 image analysis software (Media Cybernetics, Washington, USA) using a Dell Optiplex GX1 (300 Mhz) PC The system was calibrated using a stage reticle and the resolution obtained was 0 37 x 0 37 μm per image pixel The image analyser produced a digitised image of yeast samples (768 x 572 square pixels) with intensity values assigned to each pixel (0 to 255) To achieve a mean cell volume accuracy of ±3%, a minimum sample size of 600 yeast cells was used for all measurements (Cahill *et al*, 1999) Twenty microscope fields were recorded for each sample (approximately 1200 cells)

An algorithm was developed to process the colour images and measure geometric parameters of individual yeast cells (Figure 2 1) An image is initially converted from colour to monochrome (Figure 2 2a) Using operator intervention, the image contrast is adjusted and the optimum grey level threshold is selected to detect yeast cells (Figure 2 2b) Using these values, each of the 20 sample images is automatically processed and converted to a binary image, where the outline of the yeast is detected (Fig 2 2c) The binary outlines of the yeast are then filled (Figure

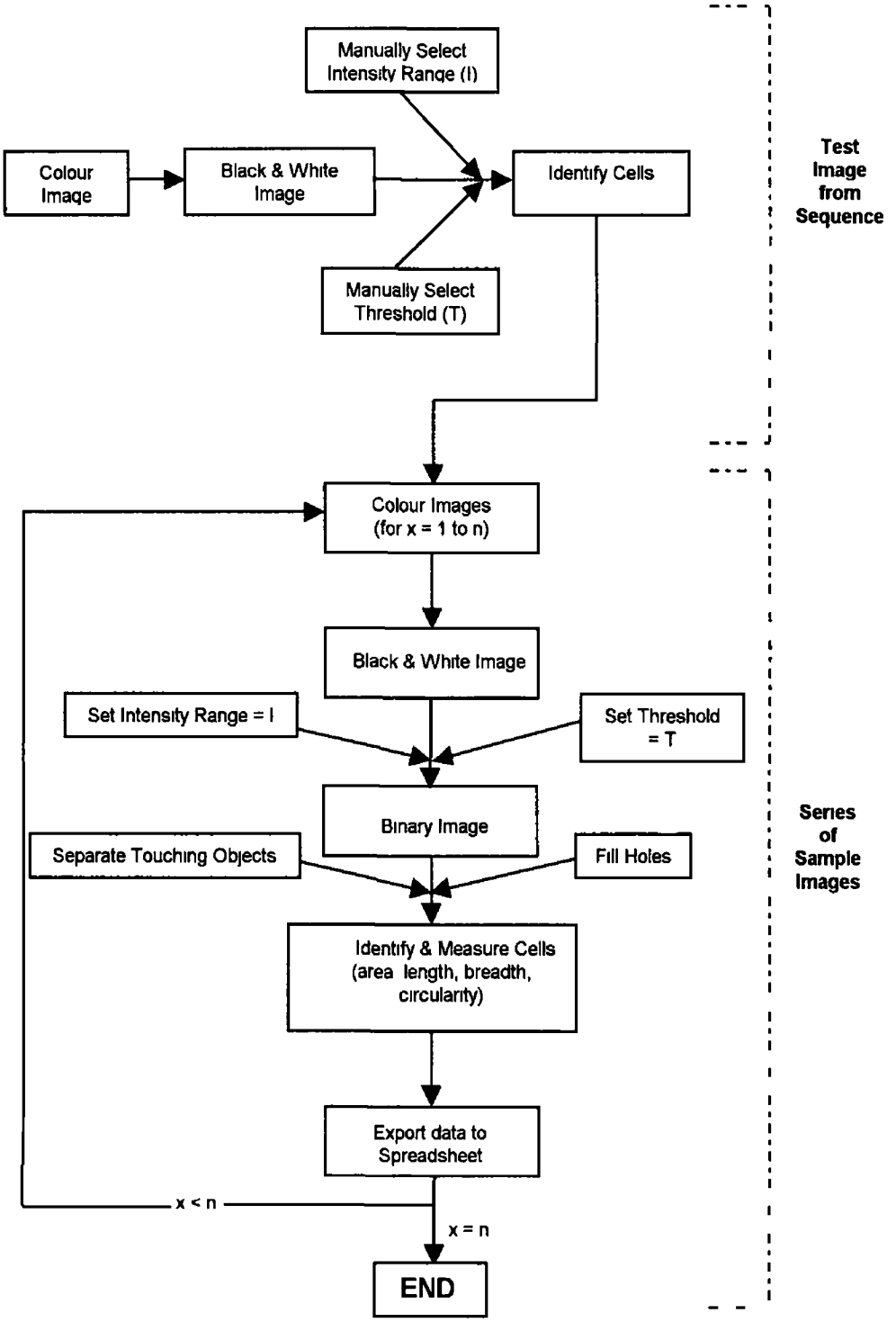

Figure 2.1. Image analysis algorithm developed to identify and measure yeast cells

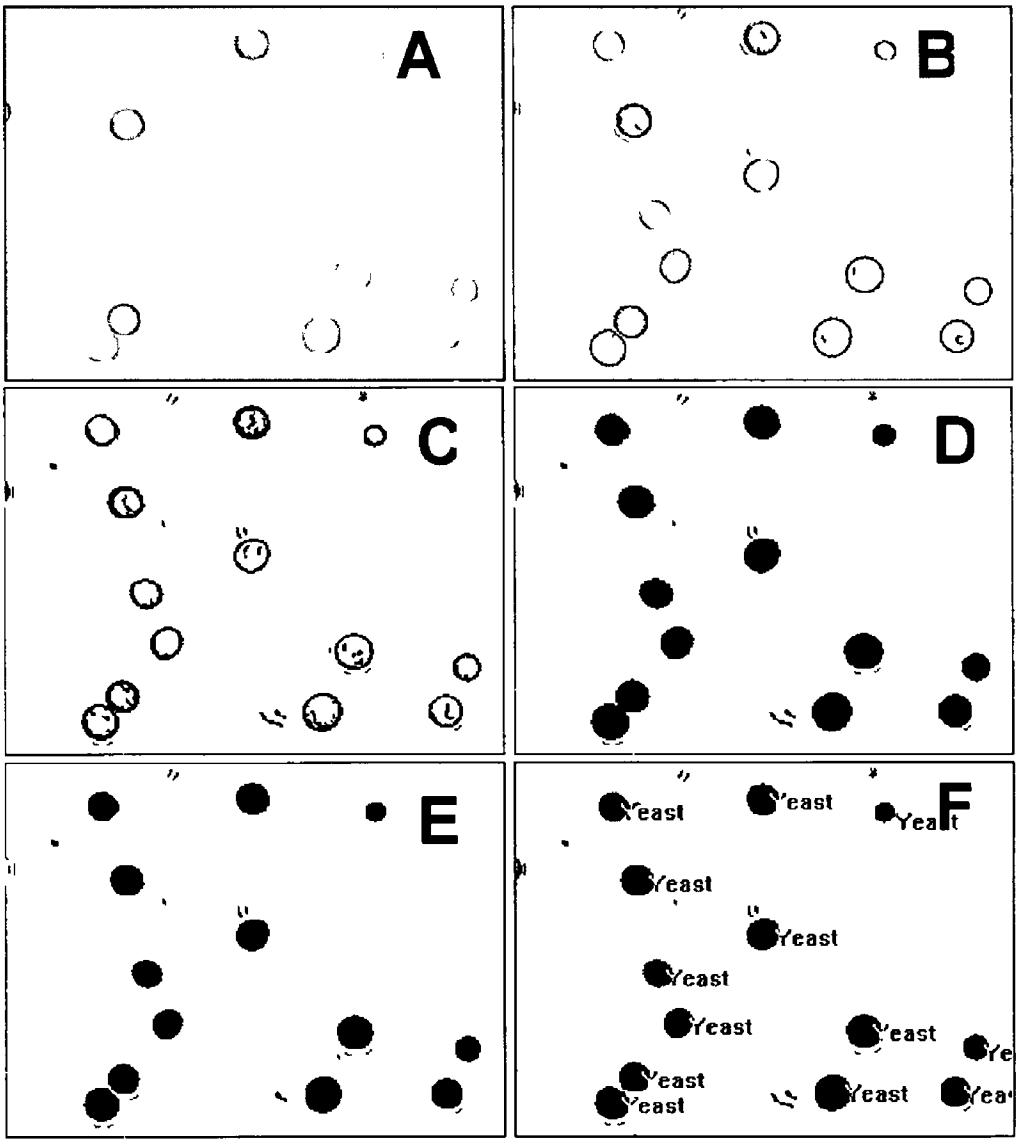

Figure 2 2 Outline of the stages involved in processing of images to identify yeast cells Microscopic image of yeast (A), adjustment of intensity range to highlight outer edge of yeast cells (B), binary image based on grey level threshold (C), binary image after hole filling (D), separation of touching yeast cells (E) and identification of yeast cells using classifying criteria (F)

2 2d) and touching cells separated (Figure 2 2e) All objects are then assessed using classifiers to identify each as yeast or as non-yeast (e g trub or debris) The classification criteria for yeast are

Projected area (A)	$16\ \mu m^2 < A < 100 \mu m^2$
Circularity (C)	< 18

where C = (perimeter length)^2 / Projected Area

Note the circularity of a circle is 4 π

The identified single yeast cells in the processed binary images are measured for individual cell length, breadth and projected area (Figure 2 2f) The processing time for each sample of approximately 1200 cells is typically 80 seconds The volume of each yeast cell is calculated based on the assumption that *S cerevisiae* generally conform to the shape of a prolate ellipsoid (Lord and Wheals, 1981)

Cell volume V (μm^3) is defined as

$$V = L B^2 \pi/6$$

where L and B are cell length (μm) and breadth (μm) respectively

2 2.10 Osmotic pressure effects on mean cell volume

Use of worts of increasing OG expose yeast cells to increased osmotic pressure in the initial stages of propagation due to the presence of high concentrations of sugars (Stewart *et al*, 1997) In addition, increased wort OG leads to higher ethanol concentrations at the end of propagation In studying the effect of wort OG on yeast cell volume it is imperative that these parameters are examined The effect of changes in osmotic pressure on the size of ale yeast 1164 was examined using ethanol and NaCl Wort sugars were not used to study these effects as this would inevitably lead to the development of yeast buds, therefore altering the mean cell volume NaCl was used as an alternative solute A series of 250 ml Erlenmeyer flasks was prepared with 100 ml of distilled water containing the following ethanol concentrations 0, 2, 4, 6 and 8% (v/v) A batch of freshly cropped ale yeast 1164 was pressed and added at a rate of 2 5 g/L to each flask The flasks were incubated at 24°C for 24 hr at 120 rpm Samples were withdrawn at t = 0 5 hr and 24 hr for measurement of mean cell volume using image analysis as described above A second batch of freshly cropped ale yeast 1164 was studied using NaCl concentrations of 0, 4, 9, 13, 18 and 36 g/L The flasks were incubated at 24°C for 24 hr at 120 rpm

2.3 RESULTS & DISCUSSION

2.3.1 Propagation

A study of yeast propagation was undertaken for ale and lager yeast using a range of wort gravities In all cases, the biomass yield expressed as wet weight (g/L)

increased with increasing wort OG (Figure 2 3) This is not attributable to the presence of higher levels of trub in high-gravity worts, as the method of wet weight determination involved dissolution of trub prior to filtration of samples Typical results for ale yeast 1164 propagated in worts of increasing OG, indicate corresponding increases in biomass yield up to 48 hr (Figure 2 3) This data agrees with other reports of increased biomass yields with increasing wort strength (Schmidt, 1995) However, in contrast to these findings, the cell count data for the ale yeast 1164 propagations (Figure 2 4) do not increase in magnitude to the same extent as the corresponding wet weigh biomass data (Figure 2 3) Propagations using each yeast strain showed no definite relationship between final yeast cell counts and wort strength in the range 7 5 to 17 5°P These seemingly contradictory sets of data suggest that cell size alters depending on the propagation OG chosen

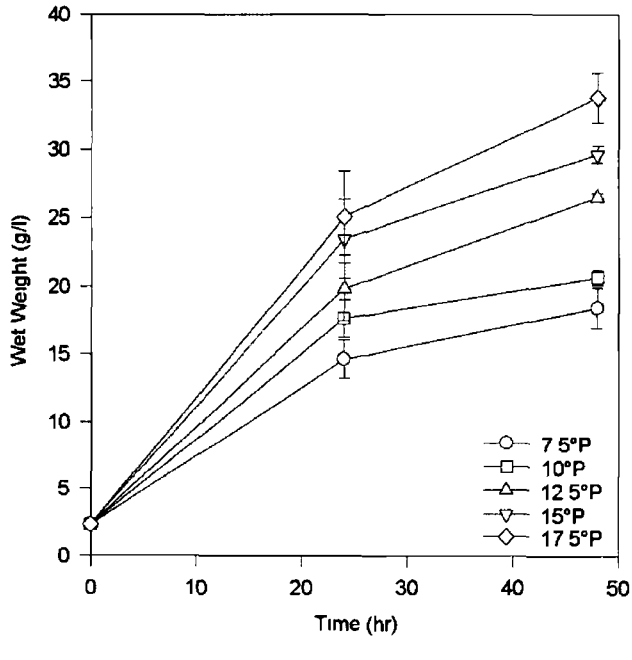

Figure 2.3. The effect of wort OG on biomass concentration during propagation of ale yeast 1164

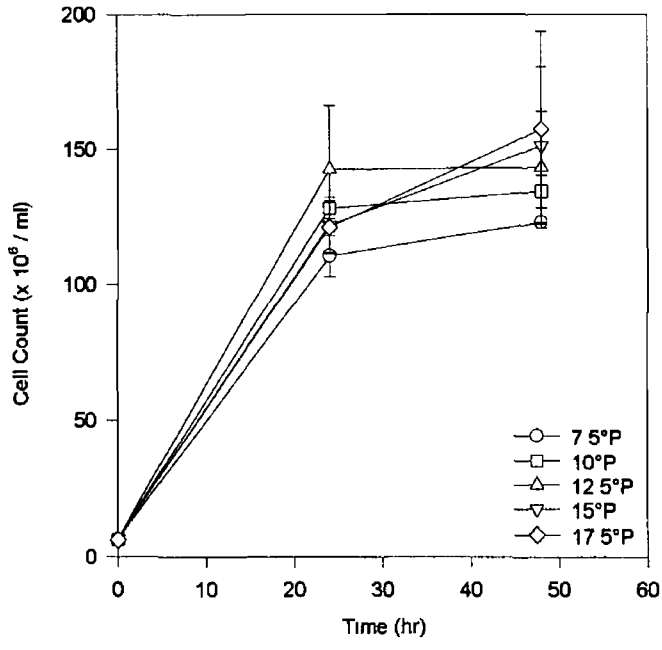

Figure 2 4 The effect of wort OG on yeast cell numbers during propagation of ale yeast 1164

This is clearly demonstrated for propagations (ale yeast 1164) using mean cell volume data obtained from image analysis measurements (Figure 2 5) The results indicate that yeast mean cell volume increases during propagation with increasing wort OG This trend was also observed for ale yeast 662 and lager yeast 7012 Mean cell volume data of yeast sampled at the end of propagation for ale yeast 662 and lager yeast 7012 are summarised in Table 2 3 The results indicate that mean cell volume increases with increasing wort gravity as found for ale yeast 1164 Although the cell volume of yeast is significantly altered with increasing wort gravity, the yeast viability for all propagations remained above 98% Worts of increasing gravity have different characteristics such as higher osmotic pressure early in propagation due to the presence of increased concentrations of wort

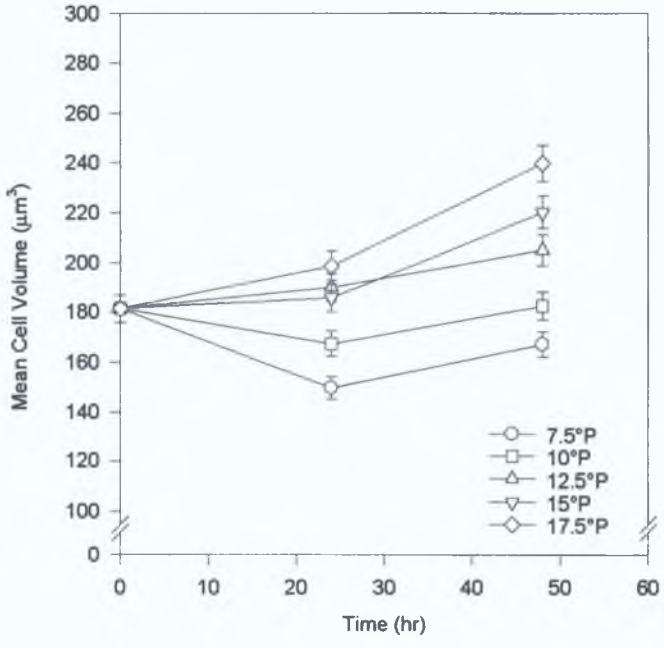

Figure 2.5. The effect of wort OG on yeast mean cell volume during propagation of ale yeast 1164.

Table 2.3. Summary of data for ale yeast 662 and lager yeast 7012 at the end of propagation.

	Wort OG (°P)	Cell Count[1] (10^6/ml)	Final Wet Weight[1] (g/L)	Final Mean Cell Volume[2] (μm^3)
Ale Yeast 662	7.5	131	29.3	212
	10	134	33.0	261
	12.5	129	37.1	267
	15	137	41.2	270
Lager Yeast 7012	7.5	180	25.1	171
	12.5	239	35.1	187
	17.5	249	43.7	195

1 Mean of duplicate analysis

2 An average of 1000 yeast cells analysed resulting in an accuracy of ± 3% of the mean value quoted.

sugars In addition, the alcohol concentrations attained at the end of propagation increase with increasing wort OG despite wort aeration This is due to the limited oxidative capacity of *S cerevisiae* and results in oxido-reductive metabolism of wort sugars in the presence of high concentrations of glucose (and other wort sugars) irrespective of the degree of wort aeration (Masschelein *et al*, 1994) These environmental differences may alter the mean cell volume of yeast The effect of various osmotic pressures on the mean cell volume of ale yeast 1164 was examined using a range of ethanol and NaCl concentrations The corresponding mean cell volume measurements at t = 0 5 hr and 24 hr are detailed in Table 2 4 The data indicate that both increasing osmotic pressure using NaCl and increasing

Table 2.4. Effect of ethanol and osmotic changes on the mean cell volume of ale yeast 1164

Saline Concentration g/L	Mean Cell Volume[1] (μm^3)		Ethanol Concentration (%v/v)	Mean Cell Volume[1] (μm^3)	
	t = 0 5hr	t = 24hr		t = 0 5hr	t = 24hr
0	370	337	0	356	300
4	340	325	2	346	258
9	330	292	4	338	249
13	297	270	6	337	244
18	283	246	8	333	241
36	250	216			

1 An average of 1000 yeast cells analysed resulting in an accuracy of ± 3% of the mean value quoted

alcohol concentration actually decrease mean cell volume However, the trends which are observed during propagation in increasing wort gravity indicate the opposite effect This suggests that the changes in yeast mean cell volume are not a result of osmotic effects on the yeast, but rather a physiological response by the yeast to environmental conditions during propagation brought about by increased wort OG

For all yeast strains studied, the data suggest that the wort gravity used in propagation has a profound effect on yeast cell volume The reasons for such significant changes in mean cell volume are not known However, it is likely that increased wort gravity increases the stress which the yeast endures and consequently affects cell size Changes in yeast cell volume have been attributed to stresses imposed on yeast, for example, increased CO_2 pressure in fermenter during fermentation leads to increased yeast cell volume (Knatchbull and Slaughter, 1987) and temperature stress on yeast can lead to increased cell volume (Walker, 1998) while ethanol stress can reduce cell volume (Walker, 1998)

Biomass measurement techniques such as wet weights, dry weights, packed volume and turbidity are routinely used in breweries (Hough *et al*, 1995) These methods overestimate propagation cell numbers as wort gravity increases due to a corresponding decrease in mean cell volume This in turn can lead to altered pitching rates Overpitching can lead to off-flavours, poor fermenter utilisation and poor yeast crop viability (Edelen *et al*, 1996), whereas under-pitching can lead to tailing fermentations (Noble, 1997) The effect of these cellular changes on

fermentation performance was examined for top fermenting (ale yeast 662) and bottom fermenting (lager 7012) yeast strains Such changes may contribute to atypical fermentations pitched with propagated yeast

2.3 2 Fermentation· top fermenting yeast

In order to examine the effect of propagation gravity on fermentation performance it is important that changes in mean cell volume are taken into account The issue of pitching was therefore approached in two separate ways Pitching of top fermenting (ale) and bottom fermenting (lager) fermentations was carried out on a viable cell number basis (10 and 15 x 10^6 cells per ml respectively) and on a weight per volume basis (2 5 and 3 75 g/L respectively) There can be no direct relationship between cell numbers and wet weights when changes in mean cell volume occur (as observed for yeast propagated in different wort strengths) An example of the difference that can occur in pitching rates for ale and lager fermentation is illustrated in Table 2 5 The data clearly demonstrate that pitching on a weight per volume basis results in significantly different viable cell numbers at the start of fermentation Similar findings have been reported for weight per volume pitching of fermentations using stored yeast (Cahill *et al*, 1999) Conversely, significantly different weights of yeast cake must be pitched to ensure a constant number of viable yeast cells at the start of fermentation The specific gravity profile of a series of ale fermentations (at 17 5°P) pitched on a cell number basis with yeast propagated at different wort gravities (7 5, 12 5 and 17 5°P) shows consistency between fermentations despite the apparent physiological

Table 2.5. Pitching details for ale (1164) and lager (7012) fermentations

Propagation Wort OG °P	Pitch Rate = 1 0x10^7/ml (Ale) = 1 5x10^7/ml (Lager)		Pitch Rate = 2 5g/l (Ale) = 3 75g/l (Lager)	
	Weight added (g/L)	Calculated Count 10^6/ml	Weight added (g/L)	Calculated Count 10^6/ml
7 5 (Ale)	1 35	10	2 5	18 5
12 5 (Ale)	1 90	10	2 5	13 1
17 5 (Ale)	2 25	10	2 5	11 1
7 5 (Lager)	2 09	15	3 75	26 8
12 5 (Lager)	2 20	15	3 75	25 5
17 5 (Lager)	2 63	15	3 75	21 4

differences in the yeast (Figure 2 6) However, the profile of fermentations pitched on a weight per volume basis indicates a difference in fermentation rates (Figure 2 7) The fermentation profiles for the 7 5°P and 12 5°P propagated yeast are similar, while the yeast propagated at 17 5°P performs poorly in fermentation and trails at least 15 hours behind the other fermentations This fermentation was pitched with approximately half the number of viable yeast cells used to pitch the other fermentations and ferments slowly from the start of fermentation The viability profile of ale yeast during fermentation at 17 5°P for both constant number and constant weight per volume pitching showed similar trends The measurements during fermentation indicate that cell viability is dramatically affected by the wort gravity used for propagation (Figure 2 8) An increase in propagation wort gravity

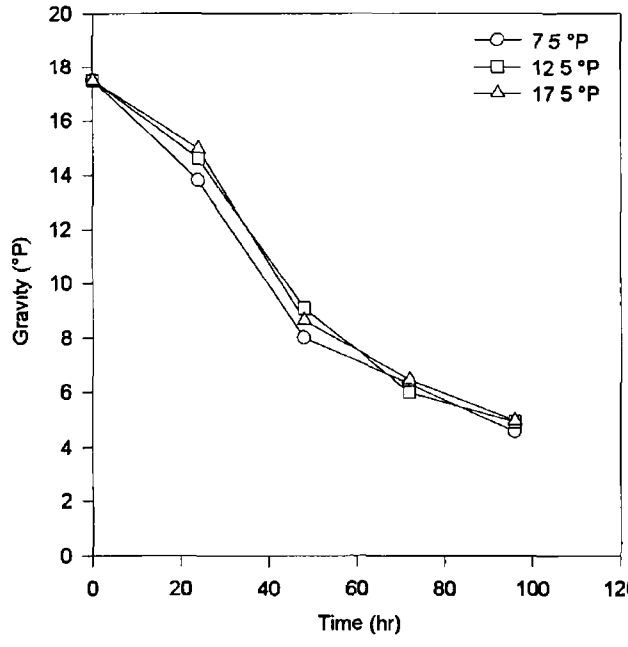

Figure 2 6 Fermentation profile (17 5°P) using ale yeast 1164 propagated over a range of wort gravities Pitching was on a cell number basis

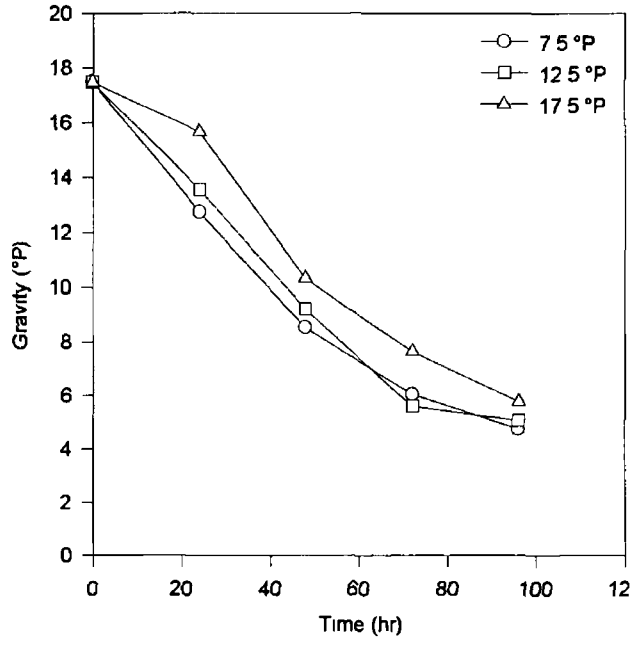

Figure 2.7. Fermentation profile (17 5°P) using ale yeast 1164 propagated over a range of wort gravities Pitching was on a weight per volume basis

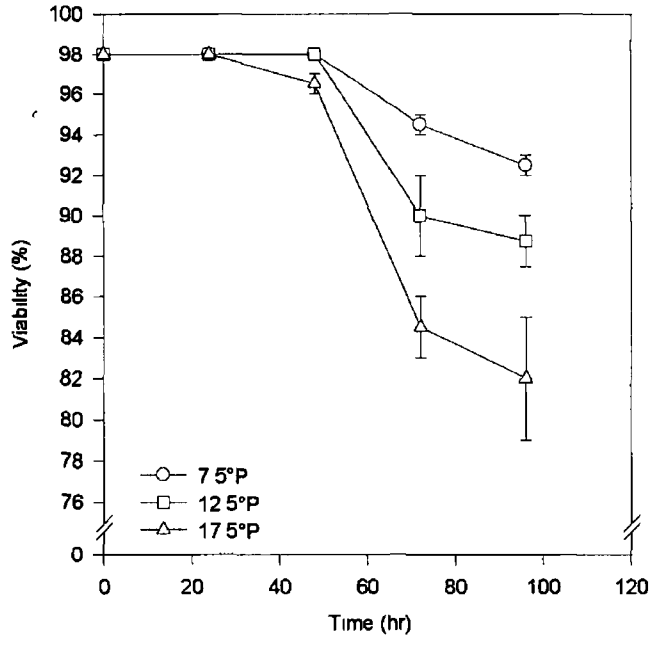

Figure 2.8 Ale yeast 1164 viability profile during fermentation (17 5°P) using yeast propagated over a range of wort gravities

has a deleterious effect on yeast quality during fermentation This effect observed in fermentation is not evident during propagation, where the viability of yeast cells at the end of propagation for all wort gravities did not decrease below 98% This negative impact on fermentation is most likely a result of increased yeast stress during propagation in high-gravity wort

2.3 3 Fermentation: bottom fermenting yeast

The effect of wort propagation gravity on fermentations was examined at 12 5°P and at 17 5°P Lager yeast (7012) was propagated at 7 5, 10 and 12 5°P wort and subsequently pitched (on both a number and weight basis) into 12 5°P wort for fermentation In contrast to ale fermentations, the specific gravity profiles of all lager fermentations were similar, whether pitched on a number or weight per

volume basis (data not shown) Furthermore, the viability of the yeast at the end of all fermentations (12 5°P) was greater than 95% There was no direct relationship between yeast viability at the end of fermentation and the wort OG used for propagation (data not shown) High-gravity propagation and fermentation trials were undertaken for the lager yeast (7012) propagated in 7 5, 12 5 and 17 5°P and subsequently pitched into lager wort at 17 5°P on a number and weight basis Yeast viability during propagation remained above 98% in all cases In contrast to the findings for high-gravity ale fermentations, the specific gravity profiles were similar for all lager fermentations (data not shown) This is in agreement with previously reported data indicating that lager yeast are generally more robust than ale yeast in high-gravity fermentations (Stewart *et al*, 1997) Yeast viability was monitored throughout each fermentation to examine the effect of wort propagation OG on yeast viability The results indicate that high gravity wort used for propagation has a negative effect on yeast viability during high-gravity fermentation Yeast propagated in wort gravities of 7 5, 12 5 and 17 5°P resulted in viabilities of 98, 95 and 89% respectively at the end of fermentation (230 hr) The observed decrease in yeast viability during high-gravity fermentations is similar for both ale and lager yeast and is related to increasing wort gravity during propagation The deleterious effect of high wort OG during propagation on yeast is manifested during high-gravity fermentation (17 5°P)

Propagation of ale and lager yeast in wort gravity in the range 7 5 to 17 5°P consistently produces yeast with a viability in excess of 98% However, the biomass yield (expressed as wet weight g/L) and yeast mean cell volume increase

with increasing wort gravity The viability of ale and lager yeast propagated in 17 5°P wort decreases during high-gravity fermentation The fermentation rate is also reduced when pitching on a weight per volume basis, as this results in underpitching of viable yeast cell numbers at the start of fermentation Conversely, the reduction of the mean cell volume of ale yeast during storage results in the over-pitching of fermentations pitched on a weight per volume basis (Cahill *et al*, 1999) High-gravity wort is not optimal for propagation of yeast used in high-gravity fermentations The data presented suggest that the optimal propagation gravity is in the range 7 5 to 12 5°P for both top and bottom fermenting yeast strains

Altered fermentation characteristics have been reported for yeast subjected to stress prior to pitching (stored aerobically at 25°C for 18 hr) (O'Connor-Cox *et al*, 1996) This observation has been attributed to the decreased production of sterols for cell membrane synthesis compared to fresh yeast and also reduced regeneration of glycogen reserves towards the end of fermentation (O'Connor-Cox *et al*, 1996) The observations reported in this work for both ale and lager yeast support these findings, insofar as the viability of yeast during fermentation decreases as the stress before pitching increases

In practical terms, the findings presented in this chapter are of significance to breweries engaged in high-gravity brewing It is imperative that the yeast crop from the first generation fermentation is of the highest quality In general, all of the yeast collected from the first generation fermentation must be used for re-pitching

in order to eliminate the old yeast stock from the brewery The viability of the first generation yeast in high-gravity fermentations is adversely affected by increasing propagation wort OG Therefore, the recommended best practice is to propagate yeast in wort with an OG in the range 7 5 to 12 5°P For breweries engaged in high-gravity brewing, this requires dilution of casting worts for propagation

2 4 CONCLUSIONS

Propagation of yeast in various wort gravities has a significant effect on cell volume For all yeast examined, the mean cell volume increases with increasing wort gravity Pitching of fermentations based on cell numbers can produce similar fermentation profiles for yeast propagated in worts of 7 5 to 17 5°P Optimal wort gravity for yeast propagation is in the range 7 5 to 12 5°P Pitching of fermentations on a weight per volume basis results in underpitching of fermentations when high-gravity wort is used for yeast propagation Significant decreases in yeast viability have been observed during high-gravity ale and lager fermentations (17 5°P) using yeast propagated in high-gravity wort Therefore, high-gravity wort used for yeast propagation alters cell volume and has a deleterious effect on the quality of first generation cropped yeast

Having quantified changes in cell volume during propagation and its impact on fermentation performance, the application of the image analysis techniques to measure yeast morphology in other brewing processes could prove beneficial, for example, yeast storage and fermentation Bearing in mind that most fermentations are pitched with yeast stored from a previous fermentation (as opposed to

propagated yeast) it would be useful to establish if changes in cell volume occur during the storage of pitching yeast and what impact (if any) these changes have on fermentation performance

CHAPTER 3

IMPROVED CONTROL OF BREWERY YEAST PITCHING USING IMAGE ANALYSIS

3.1 INTRODUCTION

Yeast management in breweries is continuously being studied and improved (Noble, 1997, O'Connor-Cox, 1997, O'Connor-Cox *et al*, 1996, Quain, 1988, Smart and Whisker, 1996), and recent research on yeast handling has led to a re-assessment of yeast cropping regimes (Deans *et al*, 1997, Quilliam, 1997) Recommendations for storage of pitching yeast include simple handling mechanisms and short holding times (O'Connor-Cox, 1997)

Incremental improvements in beer production technology are ongoing and the brewing industry endeavours to increase output with minimal capital expenditure High-gravity brewing is widespread in the industry and this undoubtedly has some deleterious effects on the brewing yeast (Casey and Ingledew, 1983) Central to maintaining consistent fermentations in any brewery is the quality of its yeast and the way in which it is handled Brewery yeast management requires both process monitoring and control similar to that typically achieved in other parts of the brewery, which now benefit from on-line analysers for dissolved oxygen, carbon dioxide and nitrogen The introduction of an on-line biomass probe (Pateman, 1997) into many breweries world-wide has improved monitoring and control of yeast pitching rates

Image analysis technology is being used extensively for (non-brewing) fermentation monitoring and control (Vecht-Lifshitz and Ison, 1992) Systems are rapidly becoming faster and more sophisticated due to improvements in computer processor speeds Image analysis techniques have been applied to yeast (Huls *et al*, 1992, O'Shea and Walsh, 1996, Pons *et al*, 1993, Vicente *et al*, 1996, Zalewski and Buchholz, 1996), fungal (Adams and Thomas, 1988, Cox and Thomas, 1992, Guterman and Shabtai, 1996, Reichl *et al*, 1992, Thomas, 1992, Vanhoutte *et al*, 1995) and bacterial (James *et al*, 1995, Mueller *et al*, 1992) systems in order to provide information regarding the physiological condition of these microorganisms Systems have further developed as on-line monitoring systems for free-cell (Suhr *et al*, 1995) and immobilised (Muller *et al*, 1988) fermentation systems Image analysis has the potential to improve brewery yeast management by providing near real-time information regarding yeast quality On-line imaging systems are in common use in industry, for example, in the Guinness Brewery at St James's Gate, for the inspection of beer kegs prior to filling

Yeast storage begins in the cone of a fermentation vessel (FV) when the yeast first starts to sediment from primary fermentation The yeast can remain in the cone for several days as the fermentation proceeds and during the final cooling of the fermenter 'Storage' in an FV cone is far from ideal and cannot be compared with 'normal' chilled storage in a yeast storage vessel (O'Connor-Cox, 1997) Temperature control cannot be maintained in an FV plug and the problem is further exacerbated by heat generation by the yeast Yeast plug temperatures of up to 18°C have been reported during lagering at 0°C, even with cooling of the cone

(Crabb and Maule, 1978) Information regarding the quality of the yeast being cropped and stored for subsequent fermentations is central to an effective yeast management program

In this study, changes in yeast cell morphology are reported during storage using image analysis These cellular changes affect fermentation performance but can be accounted for in a novel pitching regime based on image analysis data

3 2 EXPERIMENTAL

3 2.1 Yeast strains

Ale and lager fermentations were conducted using two brewery strains of *Saccharomyces cerevisiae* The yeast strains are identified as ale yeast 1164 and lager yeast 7012

3.2.2 Yeast storage

The method of storage was based on standard practices in Guinness brewery, St James's Gate, Dublin Both yeast strains were stored as a yeast slurry (30% w/w wet solids) at 4°C in an enclosed stainless steel vessel (D = 21 cm) The batch of yeast slurry was continuously mixed using a propeller mixer (D = 5 cm) at 200 rpm The purpose of mixing was to keep the yeast from sedimenting and to ensure an even temperature distribution within the slurry The mixing regime was gentle and no vortexing or entrainment of air occurred during storage The yeast was

stored with an air headspace at atmospheric pressure with no active air changes in the headspace

3 2.3 Image analysis

Samples of yeast were observed using a Nikon Optiphot microscope (Nikon, Japan) at 400X magnification The system was calibrated using a stage reticle The resolution obtained was 0 26 μm x 0 26 μm per image pixel Microscopic images of the yeast samples were recorded using a Panasonic F15 colour video camera and processed using a Leica Q500MC image analyser (Leica, Cambridge, England) The image analyser produces a digitised image (720 x 512 square pixels) with grey scale values assigned to each pixel (0 to 255) The system was programmed to enhance the grey images to enable detection of all yeast in samples Once detected, the resulting binary images were enhanced using erosion and dilation steps, hole filling and finally segmentation to separate touching and budding cells The single yeast cells in processed binary images were measured for individual cell length, breadth and cross sectional area

3.2.4 Pitching regime

Prior to pitching, a sample of stored yeast slurry was pressed to produce a yeast cake The pitching weight of yeast cake was added to wort to resuspend the yeast and the cream was then added to the fermentation vessel The pitching rate (expressed as pressed weight) was 2 5 g/L for ale and 3 g/L for lager The percentage viability of the stored yeast was taken into account when used for

pitching, therefore, 2 5 g/L and 3 g/L respectively of viable yeast were added to each fermentation

The pitching rate of ale fermentations was modified where indicated, based on the reduction in mean cell volume of the stored yeast The mean cell volume on day 0 of storage was measured using image analysis and this value was used as a reference for subsequent pitchings The modified pitching rate incorporating image analysis data was equal to the normal pitching rate multiplied by the initial mean cell volume divided by the mean cell volume after storage Pitching by weight rather than volume can be used as yeast cell density is not a strong function of cell size or condition (Burkhardt and Annemuller, 1998, McCarthy *et al*, 1998) The above procedure ensured that the pitching rate was based on constant viable cell numbers This modified pitching regime was then compared to the conventional pitching regime described above

The Aber^{TM} biomass probe (Aber Instruments Ltd Aberystwyth, UK) was used to pitch some ale fermentations as indicated This instrument operates on the principle that live yeast have an inherent capacitance when exposed to a radio frequency electrical field, while dead yeast have no significant capacitance (Pateman, 1997) The instrument was calibrated for ale yeast slurry and the units of measurement were % viable solids (w/w) The instrument does not count individual cells but estimates cell numbers by measurement of the capacitance of a sample Separate calibrations are required for different yeast strains due to differences in cell size, cell membrane capacitance and internal conductivity The extent of the effect of

changes in cell size on measurement accuracy using the Aber™ instrument is not known, although capacitance generally increases with cell size Changes in cell size were used to modify the pitching rate for Aber™ pitched fermentations in a manner as detailed above

3.2 5 Yeast enumeration and measurement of viability

Cell counts were performed in duplicate on all samples using a Thoma counting chamber In all cases, the viability of yeast was measured using the methylene blue staining technique (Pierce, 1970)

3.2.6 Glycogen measurement

Glycogen was determined by measurement of the optical density of iodine/potassium iodide stained yeast cells using a newly developed image analysis technique as described in Chapter 4

3.2.7 Wort

A single batch of either standard production ale or lager wort at 10°P was used for each pitching trial using ale yeast and lager yeast, respectively Each batch of wort was dispensed into 5 L aliquots and sterilised by steaming for two hours Further fermentations were undertaken using 10, 12 5 and 15°P ale wort prepared in the above manner The present gravity of the wort was measured using a Paar Density meter (Model DMA46, Anton Paar KG, Austria)

3.2.8 Fermentation conditions

Fermentations were conducted in 5 L Braun Biostat bench scale fermenters (B Braun, Melsungen, Germany) The fermentation conditions are outlined in Table 3 1 The dissolved oxygen concentration was measured prior to pitching using an Orbisphere DO meter (Model 26131, Orbisphere Laboratories, Geneva Switzerland) The agitation rate was set at 50 rpm to maintain all yeast in suspension Additional ale fermentations as indicated in the text were conducted in 2 L EBC tall tube glass fermenters using 10°P, 12 5°P and 15°P ale wort at 24°C

3 2 9 CO_2 evolution rate (CER) measurement

The fermentation performance of the stored yeast was assessed by monitoring the CER of the yeast in 10°P wort for 40 hr for ale fermentations and for 70 hr for

Table 3.1. Fermentation operating parameters

	Ale Trial	**Lager Trial**
Wort	Ale	Lager
OG (°P)	10	10
Temperature (°C)	24	13
Volume (L)	4	4
Pitching Rate (g/L)	2 5	3
Initial D O (ppm)	5 - 6	7 - 8

lager fermentations The CER was measured using an in-house constructed instrument incorporating a Brooks 5860i mass flow meter (Brooks Instrument b v Veenendaal, The Netherlands) The instrument measured CER and cumulative volume The meter was calibrated in the range 0 to 250 ml/min for CO_2 The accuracy was within 0 5% throughout the range of the meter

3.3 RESULTS AND DISCUSSION

3.3 1 Effect of sample size on mean cell volume measurement

Image analysis techniques were used to calculate the mean cell volume of ale and lager yeast strains during storage at 4°C It was assumed that the yeast cell generally conforms to the shape of a prolate ellipsoid (Lord and Wheals, 1981) To confirm this, the measured cross-sectional area of yeast cells was compared to the cross-sectional area of an ellipse calculated from the cell length and width In all samples tested, the results agreed to within 3% or better It was necessary to determine the minimum sample size required to yield a statistically significant measurement of mean cell volume Figure 3 1 presents data from a sample of 4500 cells where the sample data was randomised before sub-sampling populations of 50 cells, 100 cells, 200 cells etc In order to achieve an accuracy of $\pm$ 3%, a minimum sample size of 600 cells is required The typical analysis time for a sample of greater than 600 cells was less than 10 minutes using a PC powered by an Intel 486 DX2 (66MHz) processor This compares favourably to other systems studying filamentous organisms with processing times of 1 5 hr (O'Shea and Walsh, 1996) and 24 hr (Adams and Thomas, 1988) per sample Near real-time processing is possible using the latest processors on the market

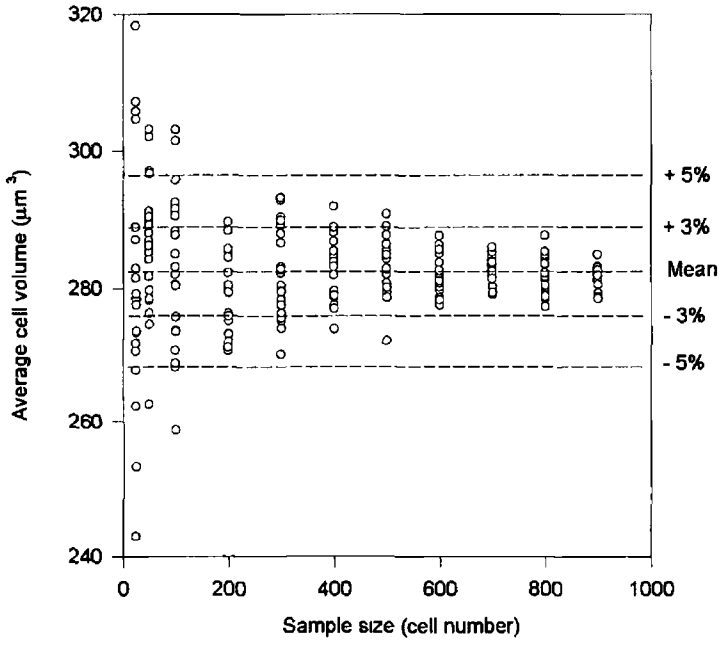

Figure 3.1. Effect of sample size on the accuracy of mean cell volume measurements

3.3 2 Effect of prolonged storage on fermentation performance

A 30% (w/v wet solids) slurry of ale yeast 1164 was stored as described and its fermentation activity was measured at regular intervals The pitching rates, yeast viability and mean cell volume are presented in Table 3 2 During prolonged storage the quality of the yeast deteriorates and the percentage viability decreases accordingly The total pitching rates are correspondingly increased as viability decreases (the viable pitching rates remain constant, i e 2 5 g/L) Interestingly, the mean cell volume also decreases The decrease in cell volume was observed across the entire range of cell sizes The results, presented in Figure 3 2, indicate the changes in the cell volume distribution during storage The modal value for the yeast cell population decreases with storage time The overall shape of the curves

Table 3 2 Storage data for ale yeast 1164

Days Stored	% Viability	Total Pitching Rate (g/L)	Mean Cell Volume (μm^3)	Normalised Volume
0	97	2 58	302	1 00
4	90	2 78	278	0 92
7	85	2 94	265	0 88
14	75	3 33	244	0 81

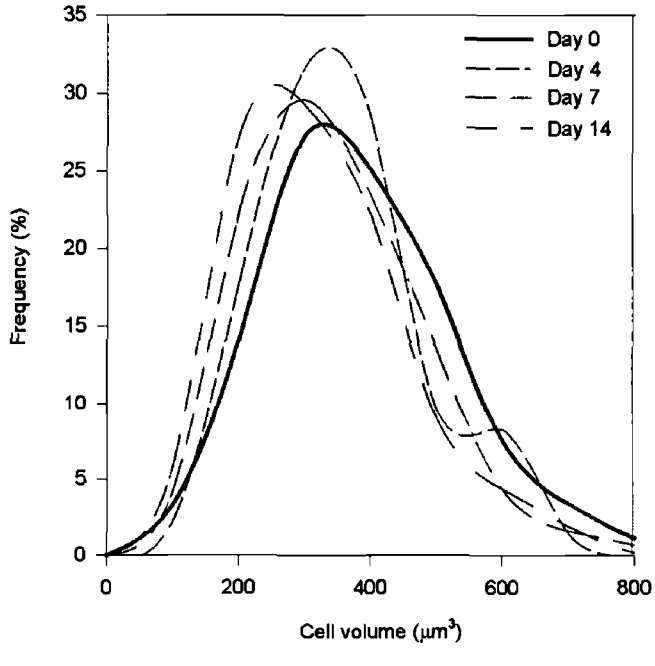

Figure 3.2. Changes in cell volume distribution of ale yeast during storage

remains unchanged indicating that all cells, whether large or small, decrease in size during storage The stored yeast was sampled, pressed and used to pitch fermentations (taking methylene blue viability into account) The cumulative CO_2 evolution profiles of the yeast during storage are illustrated in Figure 3 3

Viability data for lager yeast are presented in Table 3 3 The mean cell volume decreases with prolonged storage in a manner similar to ale yeast The fermentation activity of stored ale and lager yeast was unexpected, as reduced fermentation activity was expected with deteriorating yeast quality However, the rates of fermentation increased significantly for ale yeast compared to the normal day 0 profile The fermentation profiles for lager yeast presented in Figure 3 4 were not significantly altered during the entire storage period Both the ale and lager

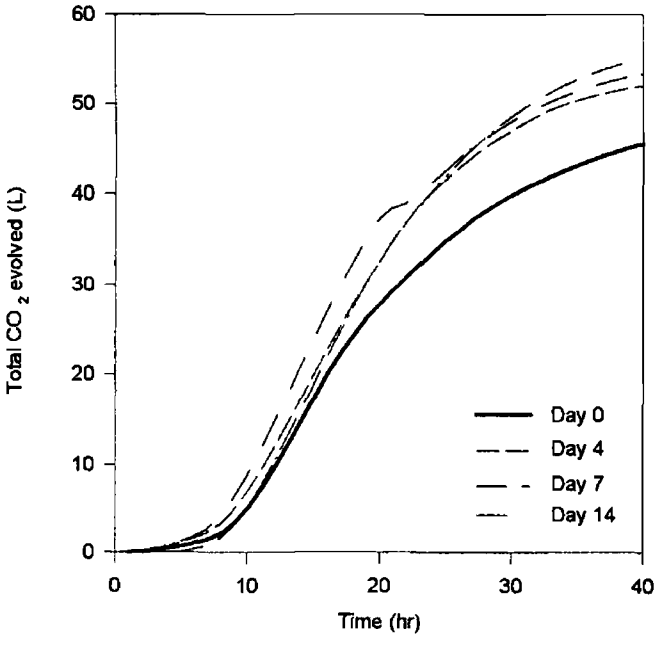

Figure 3 3 Fermentation activity of stored ale yeast re-pitched into 10°P wort

Table 3.3. Storage data for lager yeast 7012

Days Stored	% Viability	Mean Cell Volume (μm^3)	Normalised Volume
0	95	208	1 00
4	94	200	0 96
8	93	181	0 87
12	91	198	0 95
17	90	194	0 93

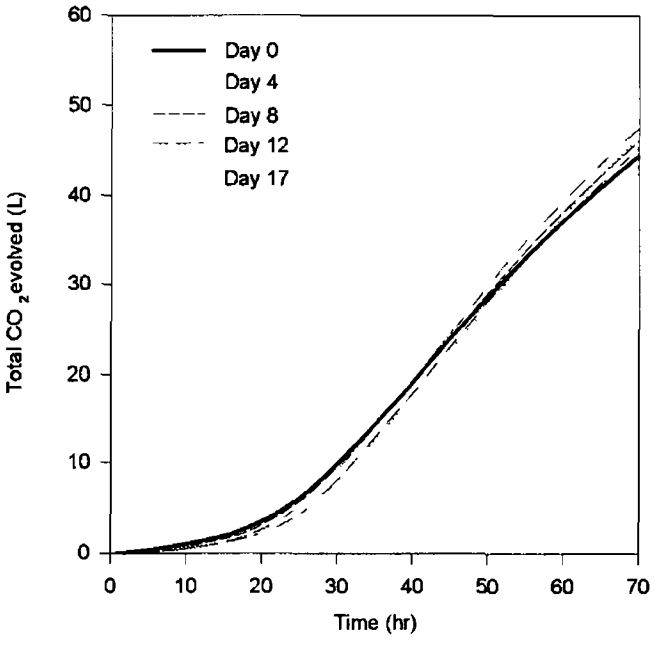

Figure 3.4. Fermentation activity of stored lager yeast re-pitched into 10°P wort

yeast strains used in this study showed reductions in mean cell volume during storage, although the decrease in mean cell volume for the top fermenting yeast strain was more pronounced than that of the bottom fermenting strain (19% versus 7%). However, it was only in the case of the ale strain that a significant effect on fermentation performance was observed. This is surprising as a decrease in fermentation performance would be expected for all brewing strains, as storage time increases. The different fermentation performance results may reflect the significant genetic differences between ale and lager strains (Coakley *et al.*, 1996). It is also possible that the differences are associated with differing physiological responses of these particular strains to the general differences in fermentation conditions between ale and lager fermentations such as temperature and wort composition. Further storage and pitching trials were not undertaken for lager yeast.

Increased rates of fermentation, caused by overpitching, are not always beneficial as this can lead to lower viability yeast crops, loss of bitterness, off flavours, filtration problems, oxygen limitations, reduced hop aroma, increased risk of autolysis, excessive fobbing and poor vessel utilisation in FV (Aries and Kirsop, 1977; Edelen *et al.*, 1996; Pagh-Rasmussen, 1978). Pitching of an FV entails the addition of a known weight, volume or number of yeast cells into a known volume of wort. Standard pitching regimes in Guinness breweries are based on either Aber™ biomass probe measurements or on laboratory analysis of stored yeast for wet solids content and percentage viability. However, if the yeast mean cell volume decreases during storage (as detailed in Tables 3.2 and 3.3) then pitching on a w/v

or v/v basis will lead to overpitching of FV's in terms of number of yeast per ml The density of yeast cells does not alter significantly even with changes in its physiological state or condition (Burkhardt and Annemuller, 1998, McCarthy *et al*, 1998) This implies that pitching on a w/v or v/v basis can result in the addition of excess numbers of yeast cells into FV as the mean cell volume decreases Improvement of pitching control was therefore studied using current pitching regimes in conjunction with image analysis measurement data The degree of overpitching based on total and viable cell numbers is compared to the maximum CER in Table 3 4 Overpitching results in the excessive fermentation rates noted early in ale fermentations This is clearly evident in Table 3 4 where there is a direct relationship between maximum CO_2 evolution rate and the number of viable yeast added to the fermentation at pitching

3.3.3 Modification of pitching regime using image analysis

Further storage trials using ale yeast were conducted in which image analysis data were used to determine pitching rates As cell size decreased during storage, the pitching rate was correspondingly reduced to ensure that the pitching rate based on cell number was constant The CO_2 evolution profile of each fermentation was recorded and compared to the 'ideal' profile of the day 0 fermentation The results presented in Figure 3 5 outline the 'ideal' day 0 profile based on a number of fermentations with fresh yeast All of the day 0 data falls within the upper and lower limits presented Over a period of two weeks, four pairs of fermentations

Table 3.4. Comparison of pitch numbers and CER for stored ale yeast 1164

Days Stored	Total Cell Numbers[a] at pitching (Normalised)	Viable Cell Numbers[b] at pitching (Normalised)	Maximum CER (Normalised)
0	1 00	1 00	1 00
4	1 17	1 08	1 03
7	1 30	1 14	1 12
14	1 60	1 24	1 17

a The normalised total cell numbers are calculated by dividing the total pitching rate by the mean cell volume (See Table 3 2)

b The normalised viable cell numbers are calculated by dividing the viable pitching rate (2 5 g/L in all cases) by the mean cell volume (See Table 3 2)

were conducted using the batch of stored yeast At regular intervals, one fermentation was pitched on a weight basis using methylene blue viability data (MB) and the second fermentation was pitched using mean cell volume data (using image analysis) in conjunction with viability data (MB&IA) All of the data for each pitching regime during the two week trial falls within the maximum and minimum limits outlined in Figure 3 5 The fermentation profile of the MB pitched fermentations differed from the day 0 profile to a greater extent than the MB&IA pitched fermentations The MB fermentations tended to ferment at a faster rate than the day 0 profile while the MB&IA fermentations adhered more closely to the day 0 profile

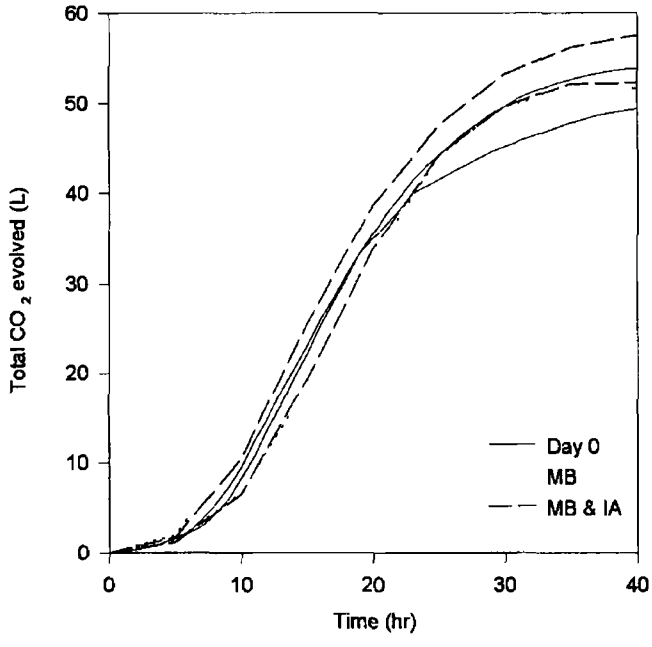

Figure 3.5 Minimum and maximum deviation from the fresh ale yeast (day 0) fermentation profile Pitching of fermentations was on a weight basis only (MB) and on a weight basis which was modified according to changes in mean cell volume (MB & IA) All fermentation data for each pitching regime falls within the maximum and minimum limits outlined

Further trials were conducted using the Aber™ biomass probe measurements (Pateman, 1997) to control the pitching rate used for ale fermentations A slurry of ale yeast was stored for two weeks as described previously and used to pitch four pairs of 10°P fermentations at regular intervals Fermentations were pitched using Aber™ biomass probe data only (Aber) and in parallel fermentations, the pitching rate was determined using the biomass probe data with corrections for yeast size from image analysis data (Aber & IA) The percentage viable solids output from the Aber™ meter was assumed to remain unaffected by changes in mean cell volume during storage The trial results are presented in Figure 3 6 The results are

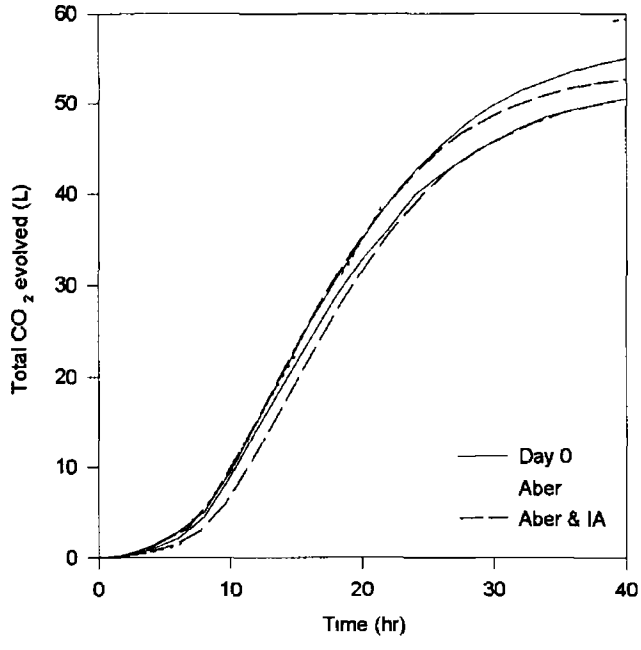

Figure 3.6. Minimum and maximum deviation from the fresh ale yeast (day 0) fermentation profile Pitching of fermentations was based on Aber™ biomass probe measurements (Aber) and Aber™ measurements which were modified according to changes in mean cell volume (Aber & IA) All fermentation data for each pitching regime falls within the maximum and minimum limits outlined

similar to pitching based on pressed solids insofar as correcting for mean cell volume ensures that the fermentation profile adheres more closely to the day 0 profile

These results show that pitching of fermentations based on viability data and using a pitching regime based on yeast mass leads to significant overpitching based on cell numbers It is worth noting that the reliability of methylene blue viability measurements below 95% is questionable (O'Connor-Cox, 1997) However, irrespective of which method of viability measurement is used, overpitching occurs if yeast size is not taken into account Using this method of pitching, it has been

demonstrated that fermentations will adhere more closely to the desired fermentation profile

Changes in yeast mean cell volume were recorded for a series of ale fermentations in 2L EBC tall tube fermenters Similar results were obtained for all wort gravities examined (10, 12 5 and 15°P) A typical time course of mean cell volume during fermentation is presented in Figure 3 7 The pattern of volume change is similar to reported changes in yeast glycogen levels during fermentation (Murray *et al*, 1984) Initially the yeast mean cell volume is large and decreases significantly until t = 24 hr As fermentation proceeds, the mean cell volume increases up to a maximum at 70 hr before decreasing in volume during the latter stages of

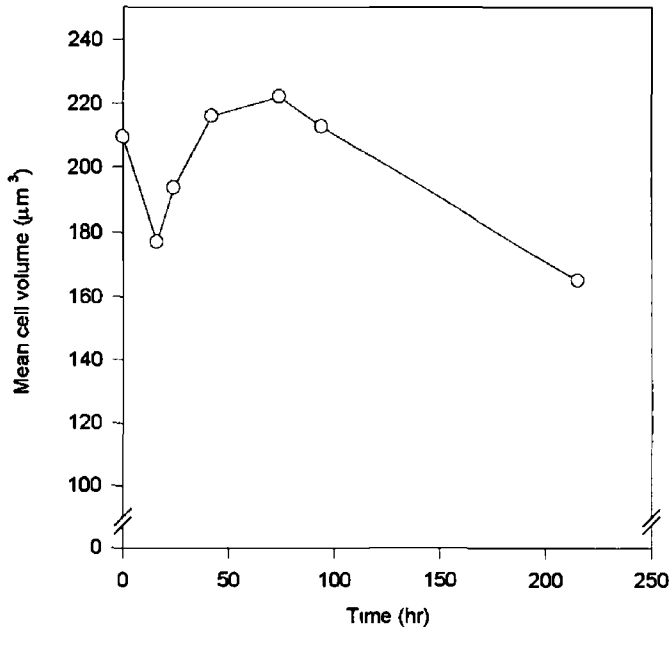

Figure 3.7 Typical changes in mean cell volume of ale yeast during fermentation

fermentation Normal fermentation times for this yeast are in the range 70 - 100 hr However, these fermentations were allowed to proceed for 200 hours in order to examine the effect of holding yeast in fully fermented beer The similarity in trends between mean cell volume and reported yeast glycogen content during fermentation (Murray *et al*, 1984) prompted an examination of the relationship between yeast mean cell volume and glycogen content (expressed as optical density of iodine stained yeast) The data presented in Figure 3 8 clearly indicates a relationship between mean cell volume and glycogen content for ale yeast, where larger yeast mean cell volume corresponds to greater concentrations of cellular glycogen Other factors may have an effect on yeast mean cell volume

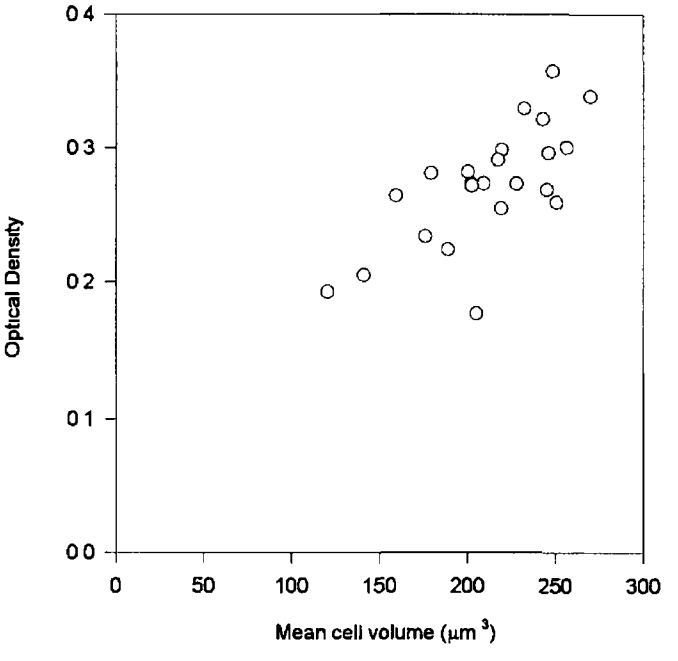

Figure 3.8. Correlation between ale yeast glycogen content (expressed as optical density) and mean cell volume during fermentation Data from several fermentations

during fermentation including cell bud formation, and changes in osmolarity due to increasing ethanol concentration or decreasing sugar concentration (See Table 2 4)

It is widely reported that the viability and glycogen content of yeast decreases during storage (McCaig and Bendiak, 1985a, McCaig and Bendiak, 1985b, Murray *et al*, 1984, O'Connor-Cox *et al*, 1996, Quain and Tubb, 1982) The mean cell volume of both yeast strains used in this study decreased during storage and it is likely that this is linked to utilisation of internal glycogen reserves by the yeast Furthermore, a correlation between mean cell volume and viability of stored ale yeast is illustrated in Figure 3 9 Mean cell volume decreases as yeast viability decreases A linear relationship has been reported elsewhere (McCaig and Bendiak,

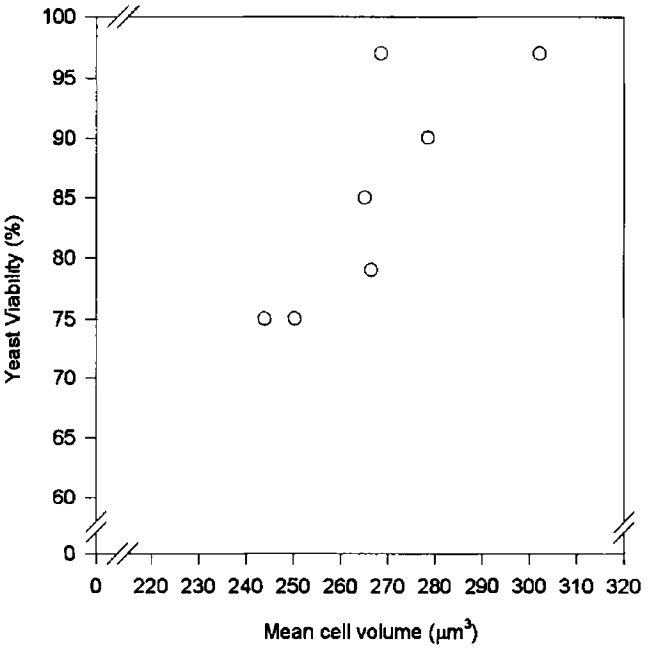

Figure 3 9. Correlation between mean cell volume and methylene blue viability of stored ale yeast

1985b) for glycogen content versus percentage viability of stored ale yeast slurry The reported findings indicate that the glycogen content of stored yeast decreased linearly with percentage viability (McCaig and Bendiak, 1985b) While it is not suggested that individual cell size relates to the viable status of the cell, it is evident that there exists a relationship between the mean cell volume of a population and both its viability and glycogen content

Cell size has previously been related to cell age whereby young cells are smaller and get progressively larger as they produce new generations of buds (Barker and Smart, 1996) It is not inferred that large cells are viable and vital and that small cells are not However, it is suggested that for a given population of stored yeast, a decrease in mean cell volume is indicative of glycogen depletion and this in turn increases the likelihood of cell death - hence the relationship observed between mean cell volume and viability Loss in cell dry weight during storage has been directly associated with glycogen depletion (Martens *et al*, 1986)

Cell size reduction is not restricted to storage of yeast under cold conditions The quality of the cropped yeast can be affected by fermentation conditions and factors associated with yeast flocculation and sedimentation Pitching yeast quality is therefore not a function of storage regime alone and information regarding yeast quality at cropping is valuable to the brewer Storage of yeast in a brewery actually commences in the base of a fermentation vessel as soon as yeast starts to form a yeast plug The profile of yeast age and quality throughout the layers of an FV plug have been characterised (Deans *et al*, 1997) It has been reported that the lower

layers on an FV plug consists of older yeast cells and the age profile of the yeast population tends towards younger cells towards the top of the plug It has also been reported that yeast plug or slurry temperatures cannot be controlled to any great extent due to poor thermal conductivity of the yeast plug and metabolic heat generation (Crabb and Maule, 1978, Lenoel *et al*, 1987, Noble, 1997, O'Connor-Cox, 1997, O'Connor-Cox, 1998a, O'Connor-Cox, 1998b) Stored yeast (either in plug or slurry) is in a temperature-dependent catabolic state Elevated temperatures can lead to autofermentation which depletes glycogen reserves and generates heat, CO_2 and ethanol Autofermentation is considered more harmful than autolysis (McCaig and Bendiak, 1985a) It is therefore suggested that hours of 'storage' in FV plug can correspond to days of chilled storage in yeast storage vessel Accurate mean cell volume measurement using image analysis can serve as an indicator of yeast quality and can ensure consistent pitching numbers in subsequent fermentations

3.4 CONCLUSIONS

Yeast mean cell volume has been demonstrated to decrease significantly for ale yeast during storage at 4°C This can lead to overpitching in a brewery system if pitching is solely based on percentage viable solids (w/v or v/v) A novel development in the control of pitching of wort involves the use of an image analysis system to accurately measure the mean volume of the stored yeast cells This information coupled with viability data ensures that consistent pitching rates are achieved based on cell number This protocol leads to more consistent pitching and better fermentation control

CHAPTER 4

DETERMINATION OF YEAST GLYCOGEN CONTENT BY INDIVIDUAL CELL SPECTROSCOPY USING IMAGE ANALYSIS

4.1 INTRODUCTION

Glycogen is a polymer of glucose and serves as an intracellular store of carbohydrate in yeast cells for energy and metabolic intermediates (Quain and Tubb, 1982). It has been directly associated with yeast vitality and in the optimisation of yeast addition rates to commercial fermentations (O'Connor-Cox *et al.*, 1996; Quain and Tubb, 1982). Reduced reserves of cellular glycogen in yeast added to commercial alcoholic fermentations result in reduced fermentation performance (Quain and Tubb, 1982). Glycogen is dissimilated early in alcoholic fermentations in the presence of dissolved oxygen and serves as the sole carbon source for sterol and fatty acid synthesis - important cell membrane components in yeast enabling cell division. (Quain *et al.*, 1981). A decrease in cellular glycogen reserves is indicative of yeast deterioration, and therefore monitoring of cellular glycogen during all stages of brewer's yeast management has been recommended in order to optimise yeast handling regimes (O'Connor Cox, 1998b).

Brewer's yeast harvested from a fermentation is stored cold (up to several days) and added as required at the beginning of subsequent fermentations. Both the viability and vitality of the yeast harvested at the end of fermentation are central to the quality of brewery fermentations (Walker, 1998). Significant improvements in

fermentation consistency and quality have been achieved using yeast addition rates based on the glycogen content of yeast cells (Quain and Tubb, 1982)

Many methods have been reported for the analysis of cellular glycogen ranging from NIR spectroscopy to enzymatic hydrolysis techniques (Mochaba *et al*, 1994, Parrou and Francois, 1997, Quain, 1981) Glycogen is an iodophilic polysaccharide and the absorbance maximum for brewer's yeast stained with I_2 KI solution is in the range 430 – 480 nm (Archibald *et al*, 1961, Quain and Tubb, 1983) There is considerable difference in the staining intensity of yeast cells with high and low reserves of glycogen, corresponding to dark brown and pale yellow staining respectively (Murray *et al*, 1984) A rapid spectrophotometric assay for cellular glycogen has been developed involving measurement of the optical density (at 660 nm) of yeast cell suspensions stained with I_2 KI solution (Quain and Tubb, 1983) However, all of these assays determine the mean glycogen content of the yeast cells

Image analysis techniques have been developed for the morphological examination of filamentous organisms (Cox and Thomas, 1992, McNeil *et al*, 1998, Reichl *et al*, 1992, Treskatis *et al*, 1997), dimorphic organisms (McCarthy *et al*, 1998, O'Shea and Walsh, 1996) and yeast (Cahill *et al*, 1999, Pons *et al*, 1993, Vicente *et al*, 1996, Zalewski and Buchholz, 1996) Microbial morphology during fermentation can have a direct impact on fermentation productivity (Priede *et al*, 1995) Automated systems capable of quantifying the morphological characteristics of commercially important fermentation microorganisms can be used to optimise

fermentation systems Furthermore, image analysis systems have been coupled to automatic sampling systems providing near-real-time data throughout the entire course of fermentations (Zalewski and Buchholz, 1996) Characterisation of yeast during immobilised (Muller *et al*, 1988) and free-cell (Suhr *et al*, 1995) fermentations has been reported using fluorescent probes and cameras installed in the wall of the fermentation vessel Image analysis techniques have also been used to study the effect of cell morphology on the filtration characteristics of fermentation broths (McCarthy *et al*, 1998) Further development of image analysis includes the colour processing of images which increases the sensitivity of detection compared to monochrome analysis (Vanhoutte *et al*, 1995) More comprehensive information regarding microbial physiology during fermentation can be obtained from a combination of image analysis and conventional staining techniques (Drouin *et al*, 1997, Pons *et al*, 1993) and the use of combinations of stains which requires sophisticated colour image processing (Pons *et al*, 1998) These techniques identify sub-cellular regions of interest based on stain uptake Enhanced image processing coupled with staining techniques allows the quantification of staining intensity as opposed to the identification of stained and unstained cellular regions Fluorescent staining has been used successfully to determine the intracellular pH of yeast in batch fermentations by examination of the fluorescent intensity ratio of cells (Imai and Ohno, 1995)

This chapter reports on the development of a rapid technique to determine the glycogen content of intact yeast cells by measuring the optical density of I_2 KI stained yeast cells Such a system can determine the distribution of glycogen within

a yeast population as well as the mean glycogen content of the yeast cells Near real-time measurements of both of these parameters provide valuable information regarding the quality of the yeast at all stages of the fermentation process

4 2 EXPERIMENTAL

4 2 1 Strain

Ale fermentations were carried out using a Guinness brewery strain of *Saccharomyces cerevisiae* identified as ale yeast 1164

4.2.2 Culture conditions

A batch of ale wort was diluted to a specific gravity of 12 5 °P for yeast propagations One litre aliquots of diluted wort were dispensed into 2 L Erlenmeyer flasks and sterilised by steaming for 2 hours Yeast propagations (aerobic) were conducted out in these flasks and incubated in an orbital shaker at 24°C and 120 rpm for 48 hr For all propagations, the inoculation rate was 5 x 10^6 viable cells per ml Cell counts were performed in duplicate on all samples using a Thoma counting chamber In all cases, the viability of yeast was measured using the methylene blue staining technique (Pierce, 1970)

For each fermentation trial, a batch of ale wort (17 5°P) was used Each batch of wort was dispensed into 2 L aliquots and sterilised by steaming for 2 hours All fermentations (anaerobic) were conducted in 2 L European Brewery Convention tall-tube glass fermenters at 24°C (Institute of Brewing Methods of Analysis,

1997) The fermentation medium was aerated to 8 ppm dissolved oxygen at the start of each fermentation The dissolved oxygen concentration was measured prior to pitching using an Orbisphere DO meter (Model 26131, Orbisphere Laboratories, Geneva Switzerland) For all fermentions the inoculation rate was 1 x 10^7 viable cells per ml The present gravity of the wort was measured using a Paar Density meter (Model DMA46, Anton Paar KG, Austria)

4 2.3 Glycogen measurement

The glycogen content of yeast was determined using the method of Parrou and Francois (1997) Fermentation samples, once collected, were stored at -20°C until analysed Once thawed, the samples were maintained at 0 - 4°C to minimise the metabolism of glycogen reserves within the yeast cells Yeast cells (4 – 20 mg dry weight) were collected by centrifugation at 5000 g for 3 minutes The pellet was resuspended in 0 25 ml of 0 25M Na_2CO_3 using screw-capped eppendorf tubes The tubes were incubated at 95°C for 4 hours to disrupt the cells After cell disruption, the pH was adjusted to 5 2 by the addition of 0 15 ml of 1M acetic acid and 0 6 ml of 0 2M sodium acetate Half of the suspension was incubated overnight with 1 2 U/ml amyloglucosidase (Boehringer Mannheim, Germany) at 57°C in an agitated waterbath The suspension was centrifuged for 3 min at 5000 g and 20 µl of the resulting supernatant was analysed for glucose using a glucose oxidase diagnostic kit (Sigma Diagnostics Inc , St Louis, MO, USA) Samples were analysed using an ELISA plate reader (Biorad Model 450, Japan) The glycogen content of yeast was expressed as % glycogen (on a wet weight basis)

4.2.4 Staining of cellular glycogen

Dilution of yeast cells in saline (9 g/L NaCl) has been shown to cause significant cell shrinkage (See Chapter 2) Therefore, fermentation samples were diluted as required with a clarified portion of the fermentation medium to achieve a concentration of 100 – 300 cells per field at 400X magnification One ml of Lugol's solution (I_2 KI solution, Sigma-Aldrich Ireland Ltd) was added to 1 ml of yeast suspension and mixed thoroughly Samples were examined under 400X magnification using a Nikon brightfield microscope and a series of 20 images were recorded as image files for subsequent processing (typically 1000 cells) The staining intensity of yeast cells varies depending on their glycogen content (Murray *et al*, 1984) Glycogen-replete yeast cells stain dark brown, while yeast with low glycogen reserves stain a pale yellow colour

During this study, the occurrence of intensely stained yeast cells in stored yeast was associated with the viable status of the cell This phenomenon was investigated by monitoring the changes in distribution of Lugol's staining intensity of admixtures of viable and non-viable yeast cells An actively fermenting yeast culture was re-suspended in 9 g/L NaCl (95% viable) and was divided into 3 x 10 ml aliquots One aliquot was stored at 4°C (control), the second aliquot was steamed for 5 minutes at 100°C and subsequently chilled to 4°C and to the third aliquot $CuSO_4$ $5H_2O$ was added to give a final concentration of 10mM Cu^{++} The yeast strain is resistant to 1 5mM Cu^{++}, but is inactivated at 10mM Cu^{++} after 1 hour Aliquots of 17 5°P brewer's ale wort (10 ml in 25 ml Erlenmeyer flasks) were inoculated with 1 x 10^7 cells per ml of the control suspension, a 50 50

mixture of control cells and heat-inactivated yeast cells and a 50 50 mixture of control cells and $_{CuSO4}$-inactivated yeast cells (these cells were centrifuged and washed twice in 9 g/L NaCl to remove $CuSO_4$ from the yeast suspension) Each flask was incubated at 20°C at 120 rpm on an orbital shaker for 4 hours Samples were then withdrawn and stained using Lugol's solution

4.2.5 Image analysis – microscopy

Microscopic images of the yeast samples were recorded using a JVC KY-F55B colour video camera (Victor Company of Japan Ltd , Japan) attached to a Nikon Optiphot microscope (Nikon Corp , Tokyo) at 400X magnification The images were stored as RGB files (red, green and blue bands with intensity ranges of 0 – 256) and processed with Optimas 6 1 image analysis software (Media Cybernetics, Washington, USA) using a Dell Optiplex GX1 PC (300 Mhz) The system was calibrated using a stage reticle and the resolution obtained was 0 37 x 0 37 μm per image pixel The image analyser produced a digitised image of yeast samples (768 x 572 square pixels) with intensity values assigned to each pixel (0 to 255) The background illumination was maintained at an intensity of 200 (±5) for all images

4.2.6 Image analysis – sample illumination

Yeast samples stained with Lugol's solution were analysed for glycogen content using image analysis This technique is described as Individual Cell Spectroscopy (ICS) as it measures the optical density (OD-L) of individual yeast cells stained with Lugol's solution Conventional spectroscopy utilises a light source of a single

wavelength In order to fully explore the potential of ICS, a range of light sources were examined to improve the resolution and accuracy of the system

Filtered light sources were evaluated in an attempt to increase the sensitivity of the assay The absorbance maximum of glycogen occurs at approximately 430 nm when stained with I_2 KI solution The concentration of glycogen found in yeast during fermentation can be up to 50% of the cell dry weight (Chester, 1963, Murray *et al*, 1984, O'Connor-Cox *et al*, 1996, Quain *et al*, 1981) Therefore, use of light with a wavelength of maximum absorbance is likely to render the assay insensitive to high concentrations of cellular glycogen due to an oversaturation of colour intensity A wavelength of 660 nm in a conventional spectrophotometer was recommended to measure the glycogen content of yeast suspensions (Quain and Tubb, 1983) This wavelength was a compromise between reduced sensitivity to the stained glycogen while allowing increased concentrations of yeast suspensions to be analysed It was also suggested that lower wavelengths could be used to increase the sensitivity of the assay All images for size analysis and ICS measurements were recorded using illumination from a 50 W halogen bulb (Philips Type 7027, Germany) with a neutral density filter (Nikon ND2) (termed white light) In order to assess the effect of illumination source on OD-L measurement, a series of identical images of yeast stained with Lugol's solution for ICS were also recorded in red light using a narrow band filter (620 nm ± 10) (Omiga Optical Inc Brattleboro, USA), and in green light using a broad band filter (490 – 630 nm) (Olympus Optical, Tokyo, Japan) Images recorded using red and green light were processed in a similar manner to white light, except that instead of converting the

images to monochrome, single-band colour processing was conducted on the images as appropriate

The distribution of cell OD-L was similar for white light and broad band green light (data not shown) However, illumination using a narrow band red light source (620 nm) resulted in reduced sensitivity to staining intensity with a 50% decrease in the mean OD-L for the sample, emphasising the insensitivity of light at 620 nm to glycogen iodine complexes Furthermore, the use of a red light source resulted in the detection of a reduced proportion of cells with high OD-L values A red light source is therefore insensitive to high concentrations of glycogen in cells Consequently, all further microscopic observations were undertaken using white light

4 2.7 Image analysis – algorithm

Monochrome digital images have a grey intensity scale ranging from 0 (black) to 255 (white) The illumination intensity of pixels within yeast cells can be expressed as optical density units by calculating the log inverse grey value for each pixel The optical density (OD) of a pixel is defined as (Russ, 1995)

$$OD = \text{Log}_{10} (I / I_o)$$

where I = Maximum luminance (255)

I_0 = Grey value of pixel

An algorithm was developed to process the images recorded in white light and measure geometric parameters and OD-L values of individual yeast cells (Figure 4 1) An image was initially converted from colour to monochrome and a copy of this image stored in the PC memory Using operator intervention, for the first image only, the image contrast was adjusted and the optimum grey level threshold selected to detect yeast cells Using these settings, each of the 20 sample images in turn was automatically converted to a binary image where the outline of the yeast was detected The binary outlines of the yeast cells were filled and touching or budded cells were separated Budded or touching cells were treated as separate single yeast cells All objects were assessed using classifiers to identify each as yeast or as non-yeast (e g trub or debris) The classification criteria for yeast were

Projected area (A)	$16\ \mu m^2 < A < 100 \mu m^2$
Circularity (C)	< 18

where C = (perimeter length)^2 / projected area (the circularity of a circle is $4\ \pi$)

The centroid data of each individual yeast cell in the processed binary images were superimposed on the original monochrome image stored in memory With all yeast in the image identified, it is necessary to establish measurement zones in the image in order to measure the OD-L value of each cell

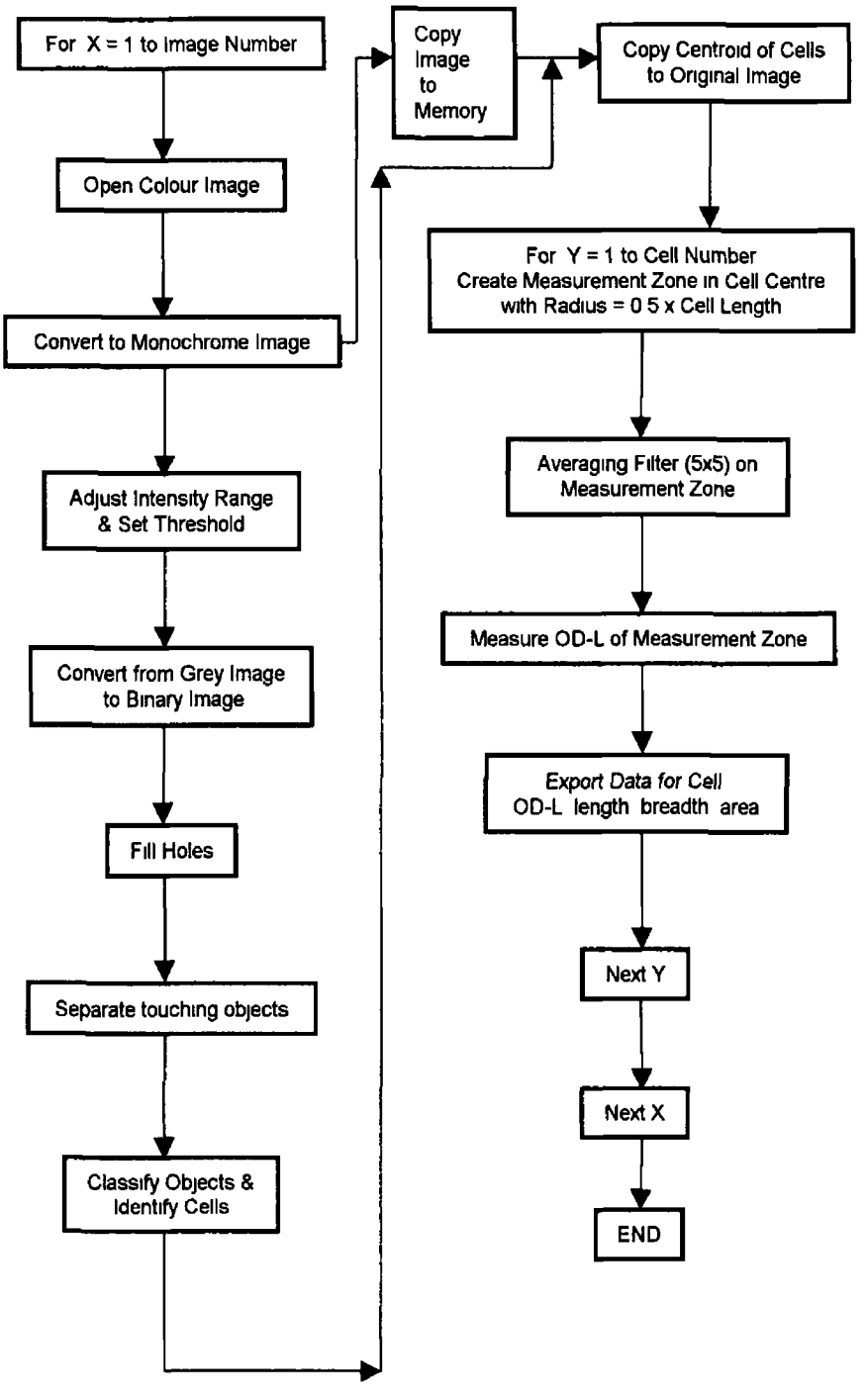

Figure 4.1 Process algorithm indicating the key steps involved in Individual Cell Spectroscopy using image analysis

Examination of stained yeast cells at pixel level clearly demonstrates that the outer edge of the yeast cell appears dark whilst the interior of the cell may have various degrees of staining intensity The OD-L profile of a cross section of two yeast cells of different staining intensities is presented in Figure 4 2 It is clear that the outer edges of both yeasts appear dark but the difference is more pronounced with cells of lower OD-L (i e those having less glycogen (A)) compared to cells with high OD-L (B) (glycogen replete cells) Calculation of the OD-L value of the entire area of the yeast cell results in an over-estimation of the mean value

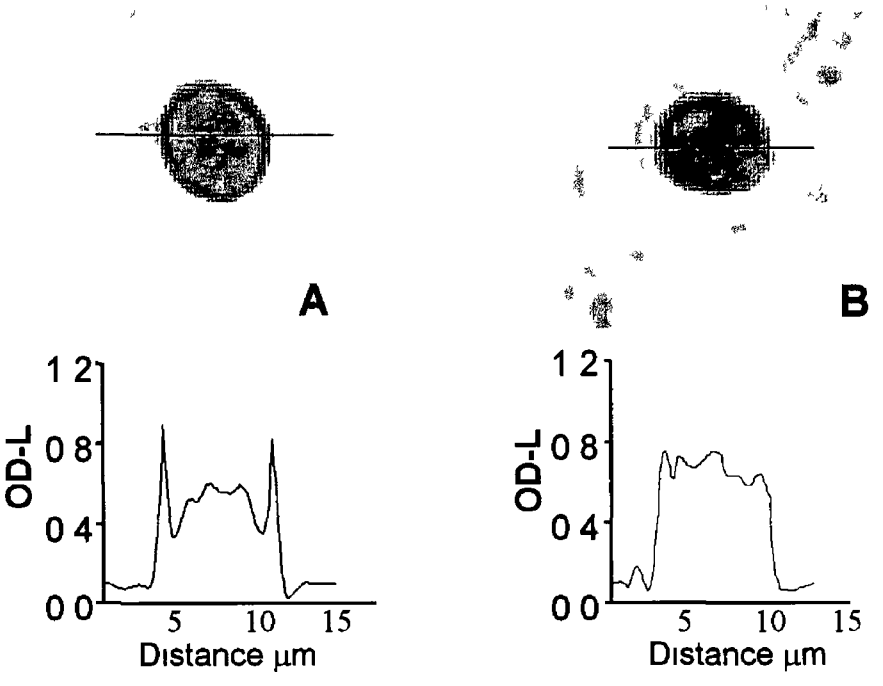

Figure 4.2 Optical density profile of a cross section of yeast cells stained with Lugol's solution Cell A has low glycogen reserves and cell B is glycogen-replete

In order to overcome this phenomenon in images of yeast cells, the OD-L of the central region of each cell was measured, instead of measuring the OD-L of the entire cell area The yeast cells in an image (Figure 4 3a) were initially identified as outlined in the process algorithm (Figure 4 1) Having superimposed the centroid locations for each cell back onto the original image, a circular measurement zone (with a diameter equal to half the cell length) was superimposed on each cell (Figure 4 3b) The centroid of this measurement zone was the same as that of the corresponding cell The interior of the yeast cells stained with Lugol's solution appears grainy and non-uniform In order to smooth out this variation, a 5 x 5 averaging filter was applied to the measurement zone in each cell The OD-L of each pixel in the measurement zone of the cell was measured and the arithmetic mean value recorded for each cell in the image

The OD-L values and the geometric parameters of each cell (length, breadth and projected area) were exported to a spreadsheet The processing time for each sample of approximately 1000 cells was typically 8 minutes The volume of each yeast cell was calculated assuming that yeast cells generally conform to the shape of a prolate ellipsoid (Lord and Wheals, 1981) Cell volume (V) is defined as

$$V = L B^2 \pi/6$$

where L and B are cell length and breadth respectively The volume of unstained yeast cells was measured using a simpler algorithm without measurement of OD-L values

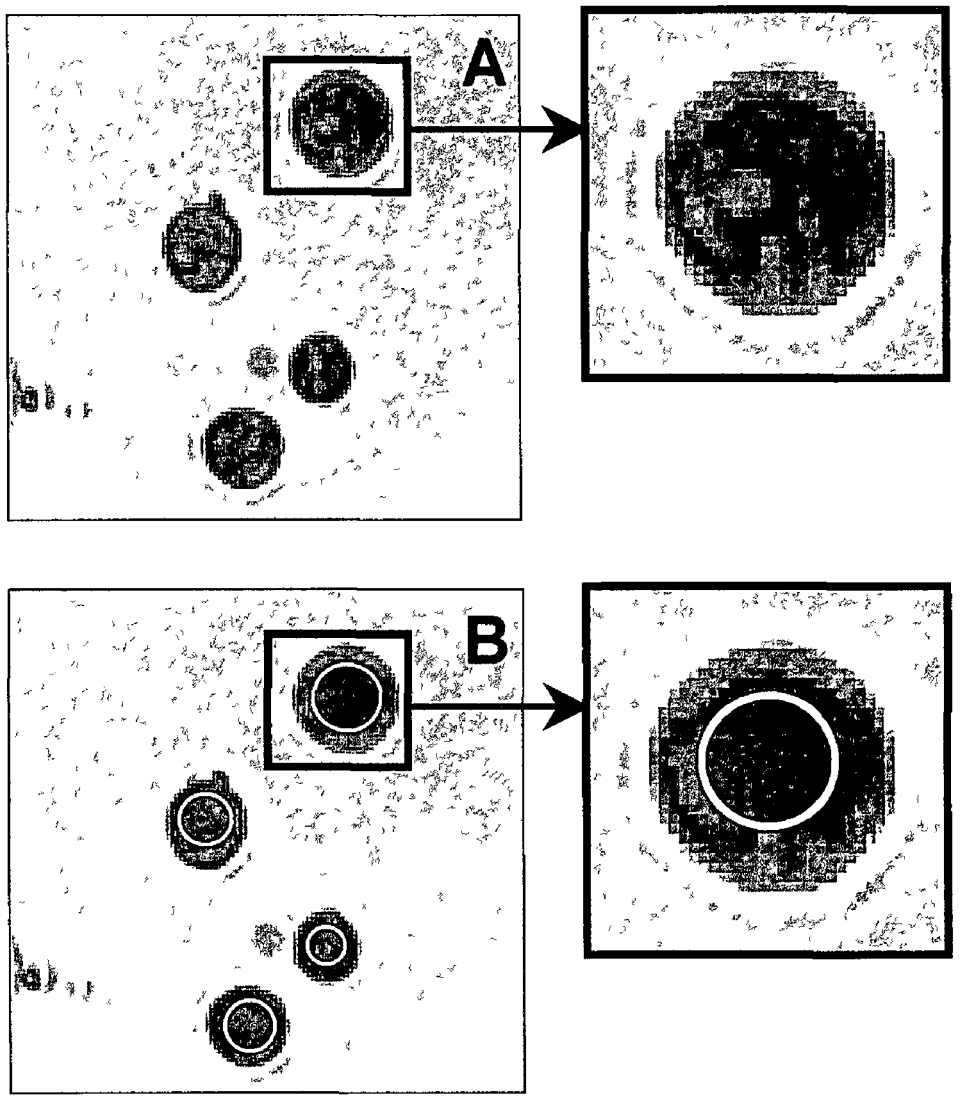

Figure 4.3. Identification of yeast cells and creation of measurement zone in the centre of each cell Original image of yeast with enlargement of a cell highlighting intracellular texture variation (A) Original image with outline of measurement zones and enlargement of a cell highlighting the effect of an 5x5 averaging filter on the cell interior prior to measurement of OD-L (B)

4.3 RESULTS AND DISCUSSION

4.3.1 Determination of minimum sample size

In order to ensure that the mean cell volume and OD-L measurements of samples were accurate, it was necessary to determine the minimum sample size required to yield a statistically significant result Figure 4 4 presents data from a sample of 3300 yeast cells where the data were randomised prior to sub-sampling populations of 50 cells, 100 cells, 200 cells etc The data indicate that an accuracy of better than 3% is attainable using a minimum sample size of 600 yeast cells In addition, data from a sample of 4300 yeast cells stained with Lugol's solution indicates that an accuracy of 3% or better for mean cell OD-L is attainable with a sample size of 600 cells Typically, 1000 cells were analysed for each measurement

4.3.2 Calibration of ICS

The ICS system was calibrated by analysing a series of fermentation samples for glycogen content using the method of Parrou and Francois (1997) and comparing these values to the mean OD-L of a sample of yeast cells stained with Lugol's solution A linear correlation (r^2 = 0 77) was observed as outlined in Figure 4 5 The microscope focus was adjusted prior to image acquisition to ensure that the outer edge of the yeast was in focus However, the degree of focus was dependent on the size of individual cells and therefore not all cells in an image were in the focus plane There is a trade off between magnification (image resolution) and degree of focus The best compromise was found using a magnification of 400 X

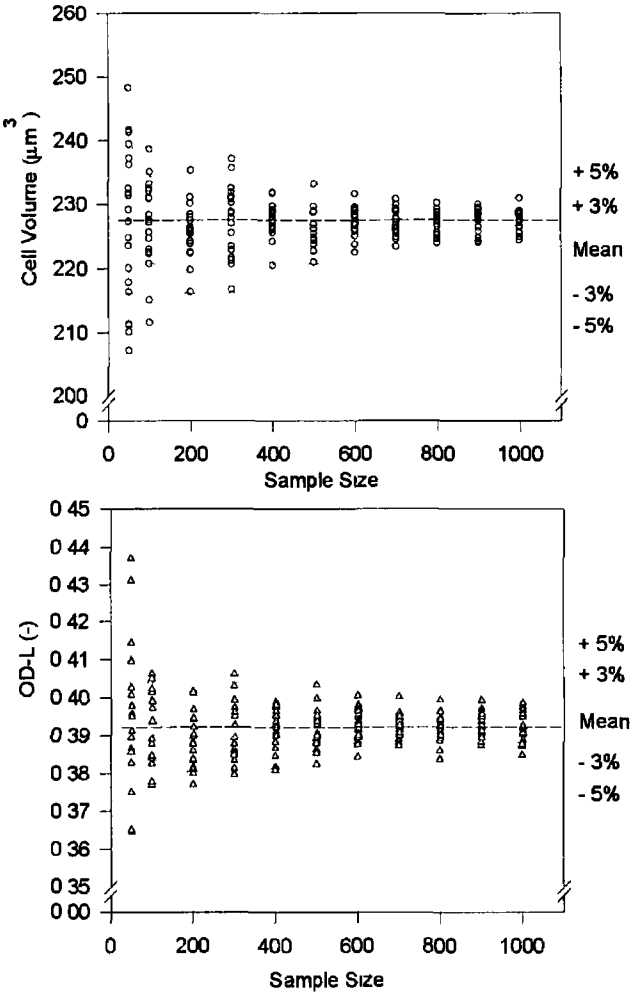

Figure 4.4. The effect of sample size on the accuracy of mean cell volume measurement and mean cell OD-L measurement of a yeast cell population

4 3 3 Mean cell OD-L during fermentation

The technique was applied to the measurement of mean cell glycogen content during ale fermentations The mean cell OD-L values together with the specific gravity during fermentation are presented in Figure 4 6 and follow a typical

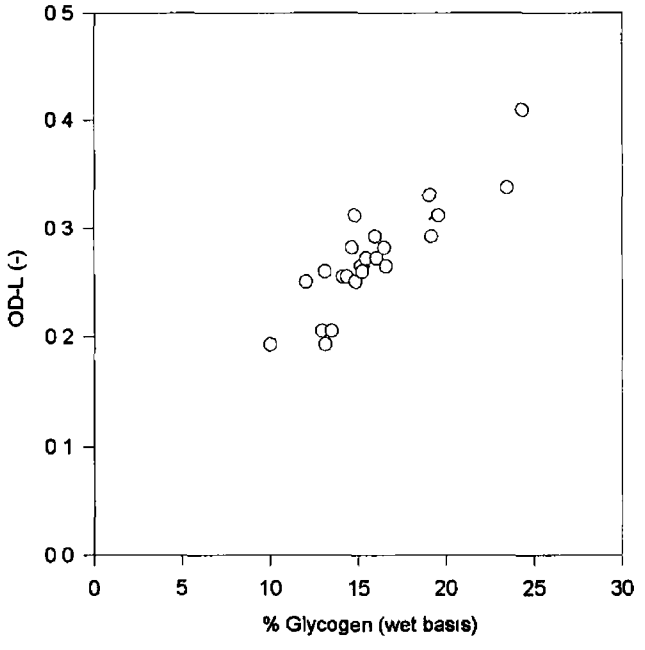

Figure 4 5 Calibration curve for mean cell OD-L of yeast stained with Lugol's solution versus glycogen content ($r^2 = 0\ 77$)

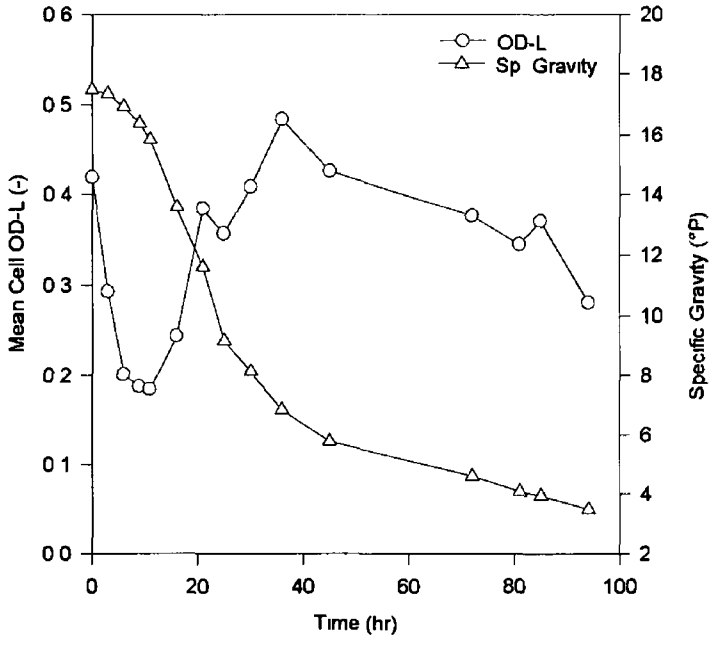

Figure 4.6 Typical time course of mean cell OD-L during ale fermentation

glycogen profile as reported elsewhere (Murray *et al*, 1984, Quain *et al*, 1981, Quain and Tubb, 1982,) Initially the yeast cells, in the presence of 8 ppm dissolved oxygen (added at the start of fermentation), utilise their internal reserves of glycogen to produce sterols and fatty acids for cell membrane synthesis As fermentation proceeds and the sugar concentration in the wort (measured in °P) diminishes, the yeast cells start to accumulate internal reserves of glycogen This continues until maximal glycogen reserves are achieved mid-way through fermentation (40 hr) As the nutritional content of the wort decreases towards the end of fermentation, the yeast cells start to utilise internal glycogen reserves

4 3.4 Distribution of glycogen within yeast populations

There are considerable advantages to using an automated ICS system to measure cellular glycogen compared to conventional methods The analysis is simple and rapid (20 min) compared to 2 days for the method of Parrou and Francois (1997) which required great care in order to achieve sufficient accuracy and consistency Furthermore, this image analysis technique is capable of indicating the distribution of glycogen content within the yeast population

The variation in staining intensity observed for stored yeast can be quite significant (Figure 4 7) Cell A appears glycogen-replete in contrast to cell B In the author's experience, there is batch to batch variation in the distribution of OD-L of stored yeast Some batches of stored yeast have a normal OD-L distribution, while others have a non-normal distribution with a greater proportion of yeast cells with a

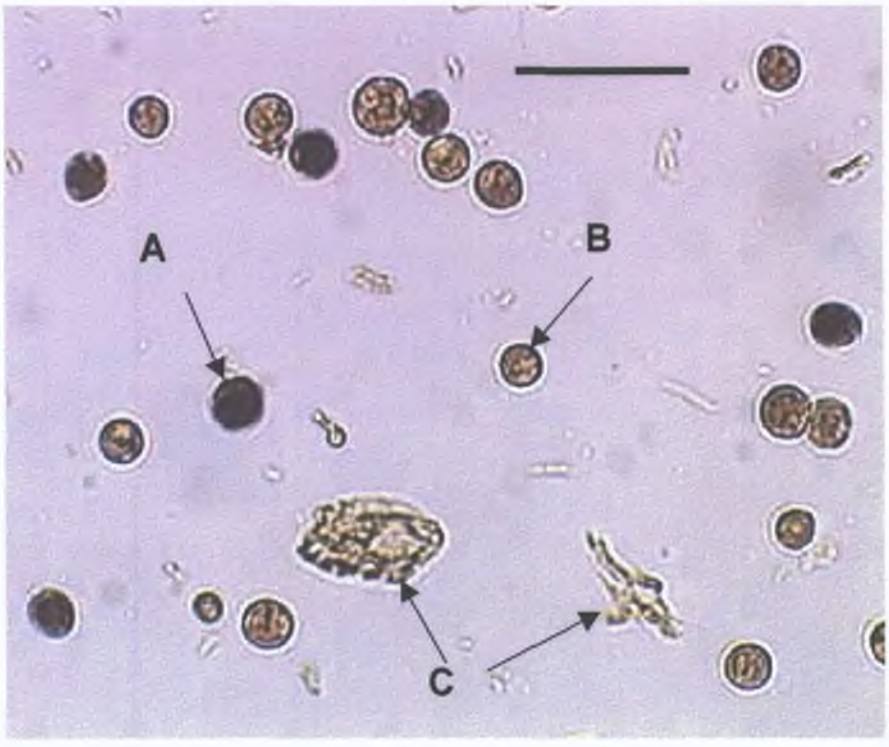

Figure 4.7. Stored yeast stained with Lugol's solution indicating the degree of variation in glycogen content within a population. Glycogen-replete cell (A), low glycogen cell (B), debris (C). (Bar indicates 20 μm).

high OD-L value. Conventional analysis yields only a mean glycogen concentration for yeast samples. This information is of use if the glycogen content of all yeast cells is similar or is normally distributed throughout the yeast cell population. It is clear from Figure 4.7 that there is considerable variation in the staining intensity of individual yeast cells. Using ICS, it is now possible to examine the glycogen content of a yeast population at an individual cell level and therefore indicate the distribution of glycogen content within a yeast population.

The viability of propagated yeast typically exceeds 98% while the viability of stored yeast is usually in excess of 80%. Staining of non-viable cells was examined

using yeast cells inactivated by heat (100°C for 5 min) and chemically (10 mM $CuSO_4$ for 1 hr) The mean OD-L of cells inactivated by steam increased by 25% on average while the OD-L value of $CuSO_4$-inactivated yeast cells remained unchanged There was a corresponding 20% shrinkage in steamed yeast cells and a 20% increase in the mean volume of $CuSO_4$-treated cells Incubation of chemically and heat-inactivated yeast cells for a period of 4 hours resulted in close to a 2-fold increase in OD-L values The increase in OD-L values for prevailing non-viable yeast cells is possibly due to the deterioration of the cell membrane and cell wall of the yeast The viability of the yeast samples used for calibration of the system was in excess of 95% Furthermore, it was generally observed that high viability yeast samples did not contain individual yeast cells with an OD-L value > 0 6

There are obvious differences in the distribution of cellular glycogen of yeast propagated for 48 hours (>98% viable) compared to yeast harvested at the end of plant-scale fermentations (80% viable cropped yeast) The OD-L data outlined in Figure 4 8 indicate a normal distribution for propagated yeast with a reduced mean OD-L value compared to cropped yeast Propagated yeast may be considered homogenous due to the mixing characteristics during propagation However, the OD-L measurements of cropped yeast cells are not normally distributed and there is a greater fraction of cells with elevated OD-L values Yeast sedimenting to the base of a fermentation vessel has been shown to consist of layers of yeast of different cell ages and physiological condition (Deans *et al*, 1997) Furthermore, some yeast cells remain in suspension for longer during fermentation, others ascend

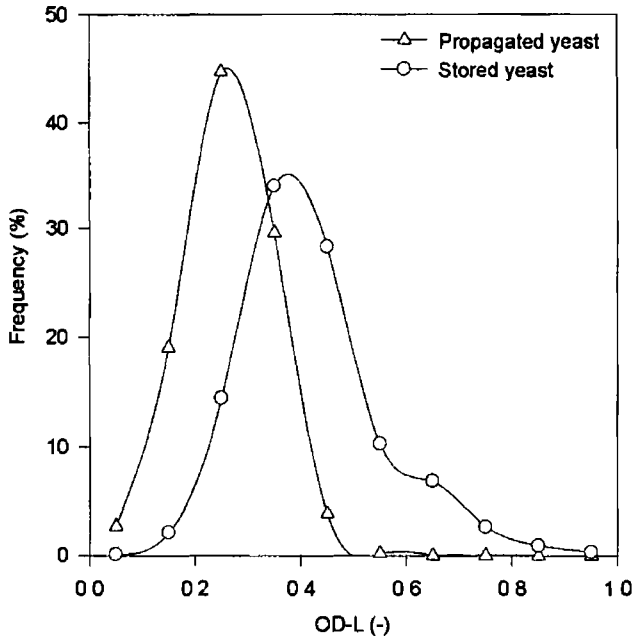

Figure 4 8 Glycogen distribution of propagated yeast compared to storage yeast

into the foam on top of the fermentation liquor (yeast head) and subsequently return to the fermentation liquor towards the end of fermentation

The mean OD-L values used in the calibration curve (Figure 4 5) extend to approximately 0 4 and it was generally observed that mean OD-L values did not exceed 0 5 during fermentation In contrast to propagated yeast, the distribution data for cropped yeast (Figure 4 8) highlight a fraction of the cell population with OD-L values in excess of 0 6 The difference in the percentage viability of propagated and cropped yeast is significant and it is probable that cells with high OD-L values (>0 6) are non-viable It is worth noting that the percentage of cells with OD-L values > 0 6 is similar to the percentage of non-viable cells in the cropped yeast (i e 20%) In order to investigate the effect of yeast viability on

OD-L distribution, the dissimilation pattern of admixtures of viable and non-viable yeast cells was studied under aerobic fermentation conditions The OD-L distribution of actively-fermenting yeast (with a viability of 95%) is normally distributed around the mean value (Figure 4 9) The OD-L distribution of the viable yeast and a 50 50 mixture of viable and non-viable yeast (heat inactivated) was measured after 4 hours incubation under aerobic conditions The OD-L distribution of the viable yeast decreases as expected due to glycogen dissimilation in the presence of wort nutrients and dissolved oxygen In contrast, the OD-L distribution of the admixture of viable and non-viable yeast cells is bimodal The first peak at an OD-L value of 0 25 refers to viable yeast cells whilst the second peak is due to the presence of a high proportion of non-viable yeast cells Similar findings were observed for chemically-inactivated yeast cells (data not shown) OD-L values in excess of 0 6 are due to prevailing dead cells in the yeast population where the Lugol's staining intensity has been altered

The occurrence of a non-normal (as in Figure 4 8) or bi-modal OD-L distribution (as in Figure 4 9) is therefore an indication of the presence of non-viable yeast cells which contain glycogen but which are incapable of utilising their cellular reserves Based on several fermentation trials, it has been observed that these cells remain intact during the course of the fermentation and therefore the glycogen is compartmentalised and is unavailable to other yeast cells

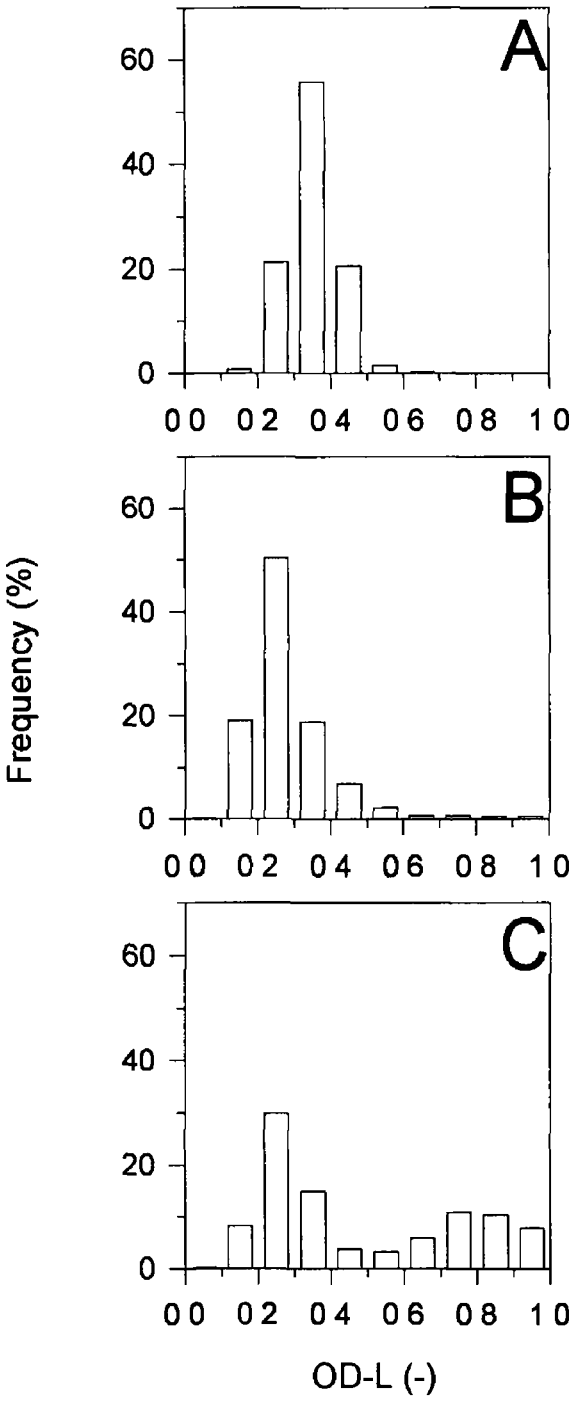

Figure 4.9. OD-L distribution of viable and non-viable yeast, actively fermenting viable yeast harvested from fermentation at t = 45 hr (A) Viable yeast after aerobic incubation in wort for 4 hr (B) Mixture (50 50) of viable and non-viable yeast cells (heat inactivated) after aerobic incubation in wort for 4 hr (C)

In order to assess the impact of a non-normal glycogen distribution (measured as OD-L) on the fermentation performance of yeast, the dissimilation of glycogen during early fermentation was studied using cropped yeast with a viability of 80% The findings outlined in Figure 4 10 indicate that glycogen dissimilation is not uniform throughout the population Within 3 hr, over 70% of the population has an OD-L of ≤ 0 3 The remainder of the cells have OD-L values which extend up to 1 0 This implies that a significant proportion of the yeast population has not utilised any of its glycogen reserves during the aerobic phase of fermentation This sub-population consists of intact non-viable yeast cells where the Lugol's staining intensity has been increased due to cell death It is interesting to note that the cropped yeast used to inoculate the fermentation comprised of 20% dead cells Furthermore, the addition of yeast into wort of high specific gravity can lead to the immediate loss of viability of a fraction of the cell population (Casey and Ingledew, 1983) This non-normal OD-L distribution profile persists until t = 9 hr At 16 hr there is an increase in the mean cell OD-L as cells commence building up internal reserves of glycogen This trend continues until t = 45 hr and further fermentation leads to a decrease in cellular glycogen reserves The glycogen distribution at t = 45 hr is similar to the distribution of the cropped yeast used for pitching The occurrence of glycogen-replete yeast cells which do not utilise their glycogen reserves early in fermentation casts significant doubts on the usefulness of measuring the mean glycogen content of yeast used to inoculate brewery fermentations

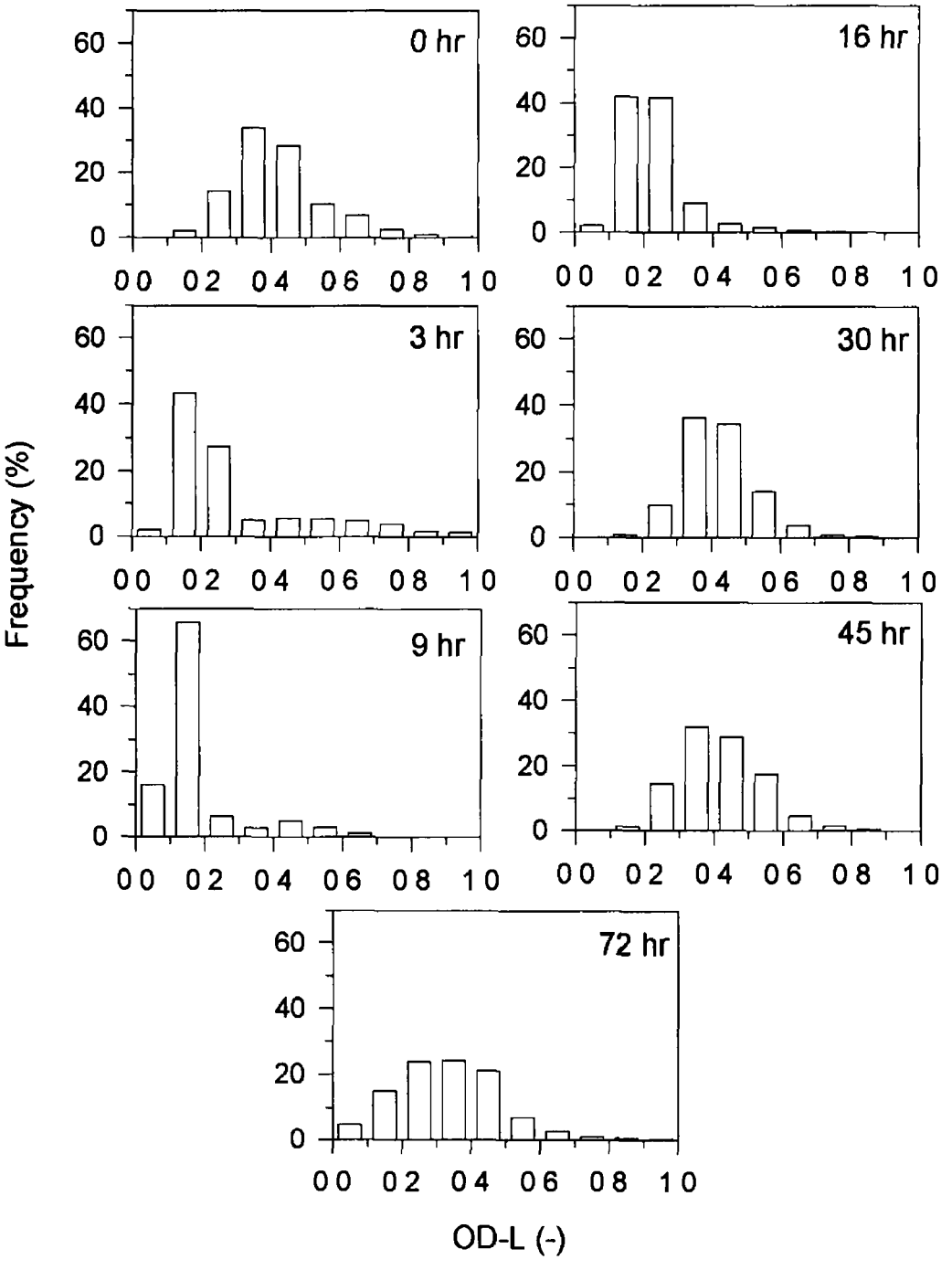

Figure 4.10. Distribution of cellular glycogen reserves during fermentation

The distribution of glycogen (measured as OD-L) in a yeast population serves as a useful indicator of yeast quality A normal OD-L distribution with uniform staining generally indicates high cell viability and homogeneous glycogen distribution In contrast, a non-normal OD-L distribution indicates reduced viability and heterogeneous yeast quality This information may be concealed using conventional glycogen analysis which determines the mean glycogen content

4 3.5 Cell size and glycogen content

The ICS system simultaneously measures individual cell OD-L values and cell dimensions Previously it was reported that the mean cell volume of a yeast population during fermentation and during storage of yeast correlated with expected glycogen concentrations (Cahill *et al*, 1999) Analysis of 15 fermentation samples demonstrated that Lugol's solution causes a 30% reduction in mean yeast cell volume However, the extent of cell shrinkage was consistent and similar changes m cell volume were observed during fermentation for both stained and unstained yeast cells In order to study the effect of glycogen content on individual cell volume, individual cell OD-L was compared to the corresponding (stained) cell volume for yeast growing aerobically in propagations and during (anaerobic) fermentation Typical data of OD-L versus cell size for 100 cells sampled at random is presented in Figure 4 11 for aerobically growing (r^2 = 0 20) (A) and anaerobically fermenting (r^2 = 0 56) (B) yeast cells This data refers to yeast samples of high viability (> 95%) and individual yeast cells with an OD-L value of in excess of 0 6 were not observed There is no direct relationship between

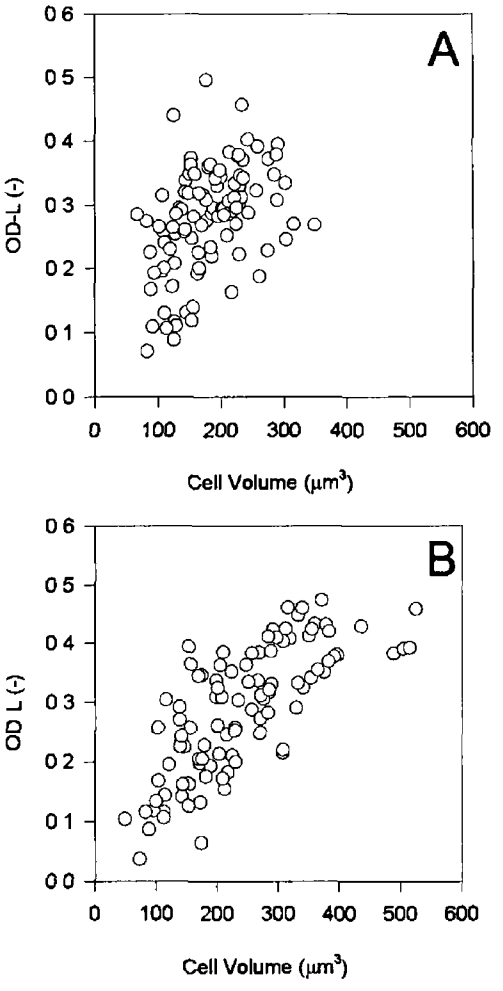

Figure 4.11. Comparison of cell OD-L versus size for aerobically cultivated yeast (t = 48 hr) (A) and anaerobically fermenting yeast (t = 120 hr) (B)

individual yeast cell volume and the corresponding glycogen content during propagation Similar results were observed in the early stages of fermentation Cell proliferation and glycogen utilisation occur during propagation and early fermentation due to the presence of dissolved oxygen and readily available nutrients Oxygen is known to act as a trigger for glycogen dissimilation in yeast cells (Pickerell *et al*, 1991) However, a more direct correlation between cell glycogen content and cell size was observed later than 48 hours in fermentation As the sugar content of the wort decreases, cells build up internal reserves of

glycogen Previous findings indicate that the mean cell volume of a yeast cell population is related to its mean glycogen content (Cahill *et al*, 1999) However, it is further suggested that the volume of individual yeast cells during anaerobic fermentation, correlates with the cellular glycogen content Glycogen can account for 40 – 50% of the cell dry matter during fermentation (Chester, 1963, Murray *et al*, 1984, O'Connor-Cox *et al*, 1996, Quain *et al*, 1981) and this appears to directly affect the volume of yeast cells

The glycogen content of yeast cells is indicative of yeast vitality and viability However, there has been a mixed response to using glycogen content as an indicator of yeast vitality and to control the yeast addition rates to brewery fermentations (Slaughter and Nomura, 1992) The findings in this study indicate that data on the mean glycogen content of a yeast sample is of limited use The dissimilation of glycogen reserves by yeast can be non-uniform, resulting in quantities of glycogen remaining un-metabolised by some yeast and this glycogen is unavailable to other metabolically active yeast cells However, ICS provides near-real-time detailed information regarding the distribution of glycogen within the yeast population and the dissimilation pattern of glycogen during fermentation

4.4 CONCLUSIONS

This rapid and simple method provides information on both the mean glycogen content and the distribution of glycogen within the yeast population The glycogen content of propagated yeast (measured as OD-L) is normally distributed, but the distribution in stored yeast can vary from batch to batch Analysis of the

distribution of glycogen during fermentation indicates that a portion of the population does not dissimilate any glycogen due to reduced vitality or cell death Furthermore, the pattern of glycogen distribution within a population serves as a useful indicator of yeast quality Determination of mean cell glycogen content using conventional assays is of limited use as an indicator of yeast vitality if the yeast viability is reduced or if the yeast is used to pitch high-gravity fermentations where cell death may occur upon inoculation

CHAPTER 5

A STUDY OF THERMAL GRADIENT DEVELOPMENT IN YEAST CROPS

5.1 INTRODUCTION

Breweries routinely crop yeast from the cone of fermentation vessels (FV) and store the yeast cold until it is used to pitch subsequent fermentations Yeast handling procedures have a direct impact on fermentation quality and consistency All aspects of brewery yeast management have been reviewed extensively (O'Connor-Cox, 1997, O'Connor-Cox, 1998a, O'Connor-Cox, 1998b) Yeast storage essentially commences as soon as yeast starts to sediment from fermentation into the cone of an FV However, significant thermal gradients occur in yeast slurry or yeast plugs due to poor thermal conductivity and metabolic heat generation (Noble, 1997) Yeast plug temperatures of 14°C have been reported during lagering at 0°C (Crabb and Maule, 1978), highlighting the magnitude of the problem Elevated temperatures in stored yeast can lead to significant deterioration in yeast quality, resulting in reduced viability and vitality Poor quality pitching yeast can adversely affect fermentations (O'Connor-Cox, 1997), resulting in extended fermentation times and off-flavours This chapter examines the thermal gradients which develop in un-mixed yeast slurry during chilled storage An examination of the effect of yeast metabolic activity on the development and magnitude of thermal gradients was undertaken using freshly cropped, acid-washed and $CuSO_4$-treated yeast slurries

5 2 EXPERIMENTAL

5.2.1 Cooling apparatus

In order to mimic the thermal gradients in yeast plugs, a stainless steel cooling tube (D =150 mm) was fabricated as outlined in Figure 5 1 The tube consisted of two separate sections 1) a small chamber with glycol recirculating to a refrigeration unit and 2) a large chamber containing 23 L of yeast slurry The temperature of the refrigerant was held at 1 5°C in all experiments The storage section was maintained at a pressure of 0 5 bar (g) for all experiments using CO_2 Three Pt100 temperature probes were fixed to the base of the yeast chamber at distances of 0 1, 0 6 and 1 2 m from the cooling surface These probes are nominally identified as 0 1 m, 0 6 m and 1 2 m respectively and the temperature reading from these probes was continuously logged The tube was insulated with 50 mm of insulation suitable for cold applications

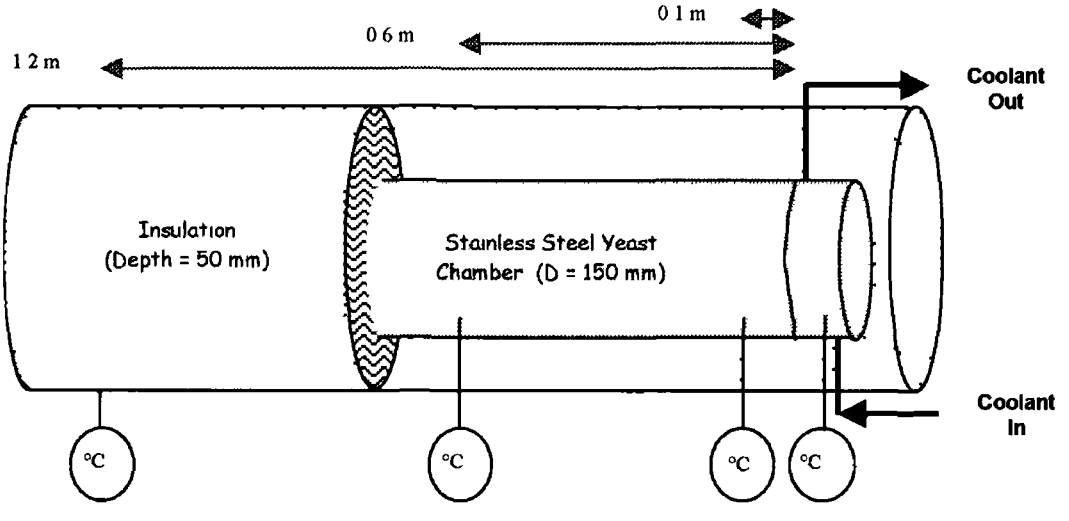

Figure 5 1. Schematic of yeast cooling apparatus

5.2.2 Yeast slurry

Trials were conducted using Guinness ale yeast (strain 1164) Yeast of three different qualities was used 1) freshly cropped yeast, 2) acid-washed yeast and 3) fresh yeast inactivated with 10mM $CuSO_4$ The yeast strain used in these studies is normally resistant to 1 5mM $CuSO_4$ The wet solids content of the yeast slurry was 30% (w/w) in all cases

5 2 3 Solids determination

Duplicate 100 g samples of yeast slurry were vacuum filtered using a Buchner flask and filter funnel through 3 sheets of filter paper (1 x Whatman No 113 followed by 2 x Whatman No 2, Whatman International Ltd UK) The solids content was expressed on a % w/w basis

5.2.4 Yeast viability measurement

Yeast viability was measured using methylene blue staining (Pierce, 1970)

5 3 RESULTS AND DISCUSSION

A series of storage trials was conducted using the cooling apparatus described in Figure 5 1 The temperature profile at each distance from the cooling surface is presented in Figures 5 2 to 5 6 The maximum thermal gradient measured during the cooling of water was 0 5°C (Figure 5 2) The convection currents, which occur in water during cooling, provided sufficient mixing to minimise thermal gradients

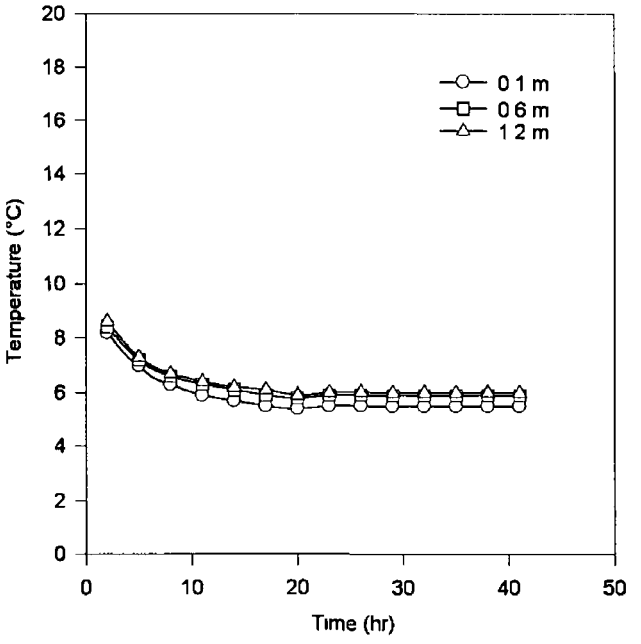

Figure 5 2 Temperature data for water

In contrast, the cooling profile of freshly cropped yeast was significantly different (Figure 5 3) The temperature of the yeast slurry at 0 1m dropped from 8°C to 7°C in the first 45 hr During this period, temperatures as high as 14°C were recorded at 0 6 m and 1 2 m Further temperature increases up to 15°C and 17°C were recorded at 0 6 m and 1 2 m respectively and the temperature at 0 1 m increased to a maximum of 9°C Yeast sampled after 110 hr storage from 0 1 m and 1 2 m had a viability of 80% and 70% respectively This illustrates the damaging effect of elevated local temperatures on yeast viability Yeast storage at 0 - 5°C has been reported as satisfactory, but as the storage temperature reaches 10°C and above, rapid yeast deterioration occurs (Lenoel *et al* , 1987)

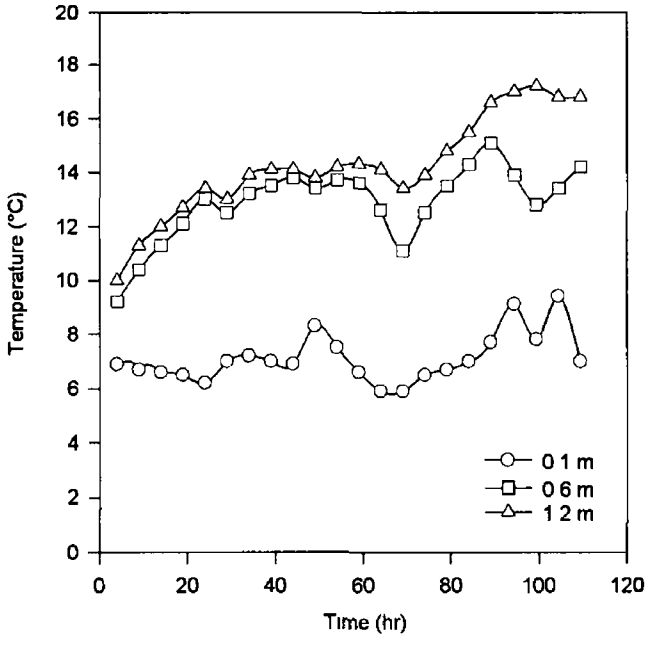

Figure 5 3 Temperature data for freshly cropped yeast

The magnitude of thermal gradients in acid-washed yeast slurry (Figure 5 4) was not as great as for freshly cropped yeast The temperature measured at 0 1 m remained below 6°C while the temperature increased to > 9°C within 10 hr at 0 6 m and 1 2 m A gradual rise in temperature was recorded at 0 6 m and 1 2 m for the duration of the trial up to a maximum of 10°C and 12°C respectively The thermal gradients observed during storage of $CuSO_4$-treated yeast (Figure 5 5) were almost as great as those for freshly cropped yeast up to 40 hr However, after 40 hr, the temperatures at 0 6 m and 1 2 m decreased from 12 - 14°C to approximately 10°C at 110 hr This is the only observed decrease in temperature at 0 6 m and 1 2 m It is likely that the yeast has been inactivated to a great extent by the $CuSO_4$ after 40 hr and that, in the absence of significant metabolic heat generation, the slurry started to cool

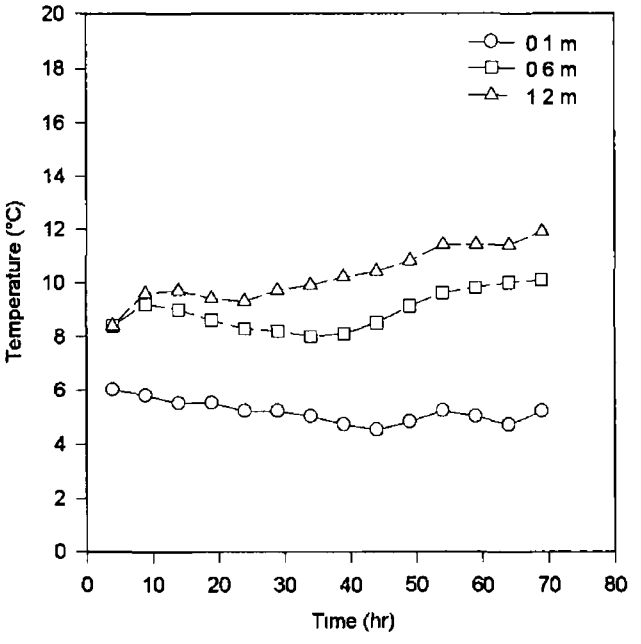

Figure 5.4. Temperature data for acid-washed yeast

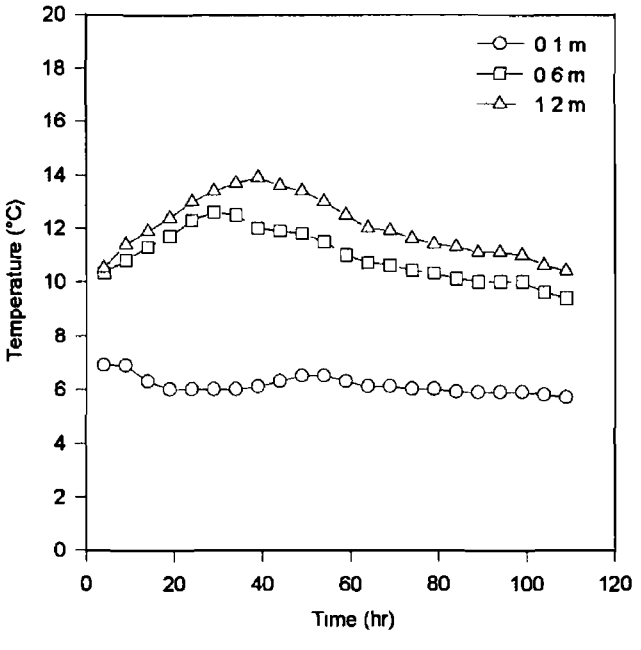

Figure 5.5. Temperature data for $CuSO_4$ treated yeast

Development of thermal gradients in freshly cropped yeast is more clearly observed by expressing the thermal data versus the distance from the cooling surface (Figure 5 6) Initially, the slurry close to the cooling surface starts to cool but within 5 hr there is a 3 5°C temperature difference between the yeast at 0 1 m and at 1 2 m This thermal gradient increases with time and is greatest between 0 1 m and 0 6 m, with the gradient levelling off between 0 6 m and 1 2 m The maximum thermal gradients recorded between 0 1 m and 1 2 m are listed in Table 5 1 The results indicate a correlation between the maximum measured thermal gradients and yeast activity These findings emphasise the significance of metabolic heat generation by yeast and how it can result in temperature increases in yeast plugs

Temperature readings from probes fitted close to the vessel wall suggest adequate temperature control of un-mixed yeast slurry However, jacket cooling has little

Table 5.1 Maximum thermal gradients recorded during each cooling trial

	Maximum Thermal Gradient (°C)
Water	0 5
Freshly cropped yeast	11
Acid-washed yeast	7
$CuSO_4$ treated yeast at 40 hr	8 (decreasing to 5°C at 110 hr)

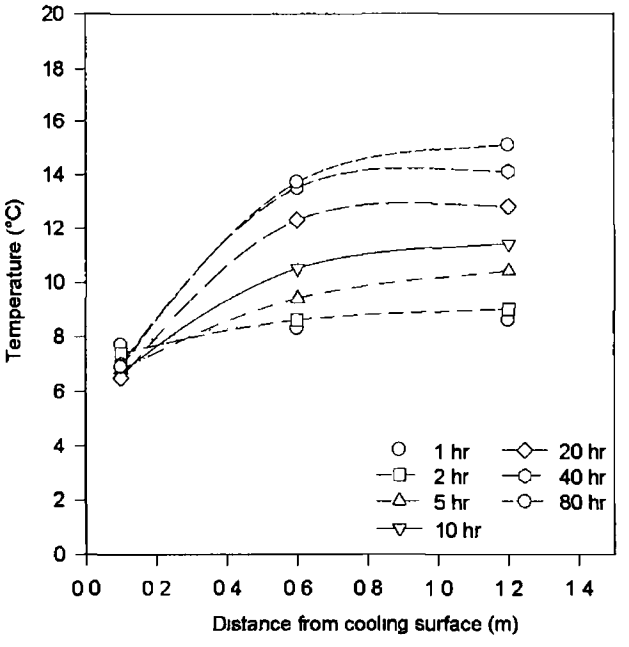

Figure 5 6 Thermal gradient development in freshly cropped yeast

effect on the internal temperature of un-mixed yeast slurry and significant thermal gradients rapidly develop Yeast in an un-mixed system rapidly becomes a heterogeneous mixture of sub-elements with different viability and vitality due to temperature differences It has been reported previously that there is an axial gradient throughout FV yeast plugs in terms of cell age and fermentation performance (Deans *et al*, 1997) The findings reported above outline a second gradient of heterogeneity A radial gradient of yeast quality exists in un-mixed yeast slurry, consisting of higher quality yeast in a cool zone close to the vessel jacket and poorer quality yeast in a warmer local environment towards the interior of the vessel When water is cooled, convection currents are generated thus ensuring uniform temperatures However, heat transfer by convection does not occur in yeast slurry to any great extent due to its high viscosity (Lenoel *et al*, 1987)

The rapid development of temperature gradients suggests that yeast cropping from FV should occur as soon as the yeast plug develops or that cropping should occur in stages if plug development is slow Yeast quality deteriorates during storage and therefore it is necessary to ensure adequate storage conditions to minimise losses in viability (Martens *et al*, 1986, McCaig and Bendiak, 1985a, McCaig and Bendiak, 1985b, O'Connor-Cox, 1998a, Quain, 1988) Lenoel *et al* (1987) reported that yeast storage at 0 - 5°C was satisfactory but, if the storage temperature reached 10°C or above, then rapid deterioration of yeast was evident It is important that brewers should distinguish between the reading from vessel temperature control probes and the temperature gradients which occur throughout the entire yeast crop

These trials were conducted using chilled yeast It is reasonable to suggest that temperature gradients in FV plugs are more damaging as temperatures increase above fermentation temperature Stored yeast is in a catabolic state and as the local temperature increases, the rate of metabolism of the yeast increases accordingly (McCaig and Bendiak, 1985a, McCaig and Bendiak, 1985b) The findings in these trials support the notion that a yeast crop is a collection of separate portions of yeast rather than a homogeneous unit It is the objective of the brewer to obtain the highest proportion of these elements in the best condition for pitching subsequent fermentations In major breweries, early cropping from FV has been identified as a major contributor to improved yeast quality, resulting in improved fermentation consistency and beer quality (Loveridge *et al*, 1997, O'Connor-Cox, 1997)

5.4 CONCLUSIONS

The temperature of un-mixed yeast slurry cannot be controlled at distances of 0 1 to 0 6 m (and greater) from a cooling surface The magnitude of thermal gradients which occur in un-mixed yeast slurry is proportional to the metabolic activity of the yeast Rapid development of thermal gradients is likely in yeast plugs in FV and in un-mixed stored yeast Temperature probes fitted close to the wall of yeast storage vessels or FV cones are of limited use Such probes indicate the local temperature of the un-mixed yeast plug or slurry close to the cooling surface but give no indication of yeast temperatures in the interior of the vessel

The magnitude of thermal gradients in un-mixed yeast slurry is substantial highlighting an aspect of yeast management which can result in significant yeast deterioration These findings warrant an examination of the thermal gradients which occur during mixed storage of pitching yeast

CHAPTER 6

A STUDY OF THE VARIATION IN TEMPERATURE, SOLIDS CONCENTRATION AND YEAST VIABILITY IN AGITATED STORED YEAST

6.1 INTRODUCTION

In order to ensure consistent quality of fermentation, a storage regime for pitching yeast must maintain yeast viability and vitality for a period of days. Low storage temperatures ensure that yeast metabolism is kept to a minimum and a uniform distribution of solids improves the accuracy of pitching of fermentations (O'Connor-Cox, 1998a). The exclusion of oxygen is also considered beneficial as its presence triggers glycogen dissimilation (O'Connor-Cox, 1998a), resulting in pitching yeast with reduced glycogen reserves and subsequent poor fermentation performance (Quain and Tubb, 1982).

There are a number of problems associated with trying to maintain the viability and vitality of pitching yeast during storage. These are mainly due to the fact that yeast slurry is a viscous, pseudoplastic (shear-thinning) fluid of high solids concentration (typically > 40% w/w). Due to the high viscosity, heat transfer by convection does not occur (Leonel *et al.*, 1987). Therefore, cooling in the absence of mixing can only occur by conduction, which is ineffective. Consequently, mixing of yeast slurry is required in some form to enable heat transfer by forced convection. Difficulties in temperature control are compounded by the fact that significant heat generation occurs in yeast slurries and plugs due to yeast

metabolism (Boughton, 1983, Crabb and Maule, 1978, Noble, 1997) Temperature control of yeast slurry is therefore hindered by its high viscosity and exothermic nature

Mechanical agitation of stored yeast has been reported using a diverse range of agitator systems, including large diameter, slow speed mixers (Kawamura *et al*, 1999), small diameter impellers (Cholerton, 1995, Murray *et al*, 1984) and off-centre mixers (Munday and Dymond, 1998) Interestingly, intermittent agitation of yeast slurry has been reported to be more effective for cooling yeast slurries than continuous agitation (Andersen, 1998) Yeast is also stored unmixed in insulated vessels in coldrooms prior to pitching (O'Connor-Cox, 1998a), which can result in gradients of solids concentration and temperature throughout the stored yeast Recirculation of yeast slurry using an external loop has also been used both as a mixing mechanism and to effectively de-carbonate the slurry during storage This is achieved by recirculating the slurry over a flat conical disc which spreads the slurry out into a thin film, allowing the release of dissolved carbon dioxide (O'Connor-Cox, 1998a) Increased levels of dissolved CO_2, either in fermenter or in yeast storage vessel, have a deleterious effect on yeast cells (Arcay-Ledezma and Slaughter, 1984, Knatchbull and Slaughter, 1987, O'Connor-Cox, 1998a)

The recommended temperature of storage for yeast slurry is in the range 2 – 5°C (Lenoel *et al*, 1987, Martens *et al*, 1986, McCaig and Bendiak, 1985a, O'Connor-Cox, 1998a) Determination of the precise storage temperature of the

entire storage vessel contents is difficult Based on a single temperature probe situated close to the vessel wall, the temperature of a yeast slurry may be within specification However, significant increases in slurry temperature have been measured at distances from the cooling surface for un-mixed yeast slurry (See Chapter 5 and Lenoel *et al* , 1987)

For this work, a customised 10 hL yeast storage vessel was commissioned to study the gradients of temperature, yeast viability and solids concentration which occur during yeast storage Measured differences in temperature, viability and solids concentration of yeast slurries are reported for pitching yeast stored with different modes of mixing including mechanical agitation and recirculation using an external pump

6.2 EXPERIMENTAL

6.2 1 Yeast strain

All storage trials were conducted using a brewery ale strain of *Saccharomyces cerevisiae* (strain 1164) Yeast was harvested by centrifugation from FV at the end of fermentation and 8 hL of yeast slurry (approximately 40 % w/w) was used for each trial unless otherwise stated The slurry was collected after in-line chilling using a plate heat exchanger

6.2.2 Solids determination

Duplicate 100 g samples of yeast slurry were vacuum filtered using a Buchner flask and filter funnel through 3 sheets of filter paper (1 x Whatman No. 113 followed by 2 x Whatman No. 2, Whatman International Ltd. UK). The solids content was expressed on a % w/w basis.

6.2.3 Viability determination

Yeast viability was determined using methylene blue staining (Pierce, 1970).

6.2.4 Yeast storage vessel (YSV)

6.2.4.1 Rationale of design

Yeast slurry with a wet solids content above 40% pressed solids exhibits non-Newtonian (shear-thinning) rheological behaviour (Lenoel *et al.*, 1987). Therefore, agitation using a small diameter impeller can result in a reduction in the apparent viscosity of the slurry close to the agitator, with little effect on the slurry viscosity close to the vessel wall. The effectiveness of mixing using impellers can be improved using baffles (installed on the base of the vessel or on the vessel wall), or by using an off-centre mixer (Coulson *et al.*, 1983). The installation of baffles in the vessel was considered inappropriate from a hygiene point of view, as the slurry would prove difficult to remove completely using a normal cleaning-in-place (CIP) regime. Similarly, large paddles were not considered due to the difficulty of cleaning and also the increased power consumption compared to smaller diameter impellers.

Traditionally, yeast was screened upon harvesting to remove lumps and reduce the amount of dissolved CO_2 (Andersen, 1998) Decarbonation can have beneficial effects on the yeast and therefore mixing by recirculation was evaluated However, the slurry was not decarbonated using a Chinese hat diffuser as described by O'Connor-Cox (1998a), in order to compare the recirculation regime to mechanical agitation, which does not decarbonate the slurry to the same extent

6.2.4.2 Vessel specifications

A customised 10 hl pilot-scale Yeast Storage Vessel (YSV) was fabricated as outlined in Figure 6 1, to study the temperature distribution of yeast slurry during storage The vessel details are summarised in Table 6 1 Temperature control was achieved using a proportional integral and derivative (PID) temperature controller (Datalogic ND Series, Datalogic SRL, Bologna, Italy) linked to a single, surface mounted Pt100 temperature sensor on the vessel wall (See Figure 6 1) The coolant was recirculated through the full length dimple jacket to a heat exchanger system using a recirculation pump with a capacity of 2000 L / hr (Grundflos Model CR2-20, Grundflos (Irl) Ltd Dublin) The heat exchanger system consisted of a plate heat exchanger supplied with glycol (at -3°C) followed by an in-line electrical heating element Heating or cooling was activated as required by the temperature controller The vessel was equipped with an off-centre, variable speed agitator with an agitation range of 50 to 250 rpm (Lightnin Model No LC37VH, Lightnin Mixers Ltd Cheshire, UK) Two triple blade impellers (D = 325 mm) were fitted to the agitator shaft as outlined in Figure 6 1 Mixing by recirculation was achieved using a variable speed, positive displacement lobe

Table 6 1 Technical specifications of YSV

	Specification
Working Volume	10 hl
Diameter	1000 mm
Height	1500 mm
Agitator	Variable speed, 2 x triple blade impellers, 50 – 250 rpm
Recirculation Pump	Variable speed, Lobe Pump, 200 – 1950 L/hr
Cooling Jacket	Full dimpled jacket
Minimum Coolant Temperature	- 3°C
Temperature Control	PID with surface mounted Pt100 fitted to vessel wall
Temperature Probes	4 x customised probes with 4 Pt100 sensors in each

pump (SSP Pump Model No SR/2/013/LS/3A, Alfa Laval Pumps Ltd UK),with a pumping range of 200 to 1950 L/hr The slurry was pumped from the base of the vessel and returned via a tangential inlet towards the top of the vessel (See Figure 6 1) The rate of recirculation is expressed as the time taken to pump the contents of the vessel through the loop (vessel turnover time) Sample probes were fitted at the top and bottom of the vessel allowing sampling of yeast 50 mm from the vessel wall and in the centre of the vessel (500 mm from the vessel wall)

The vessel was fitted with 4 custom-built probes accommodating 4 x Pt100 temperature sensors in each Each probe consisted of 4 separate Pt100 sensors at

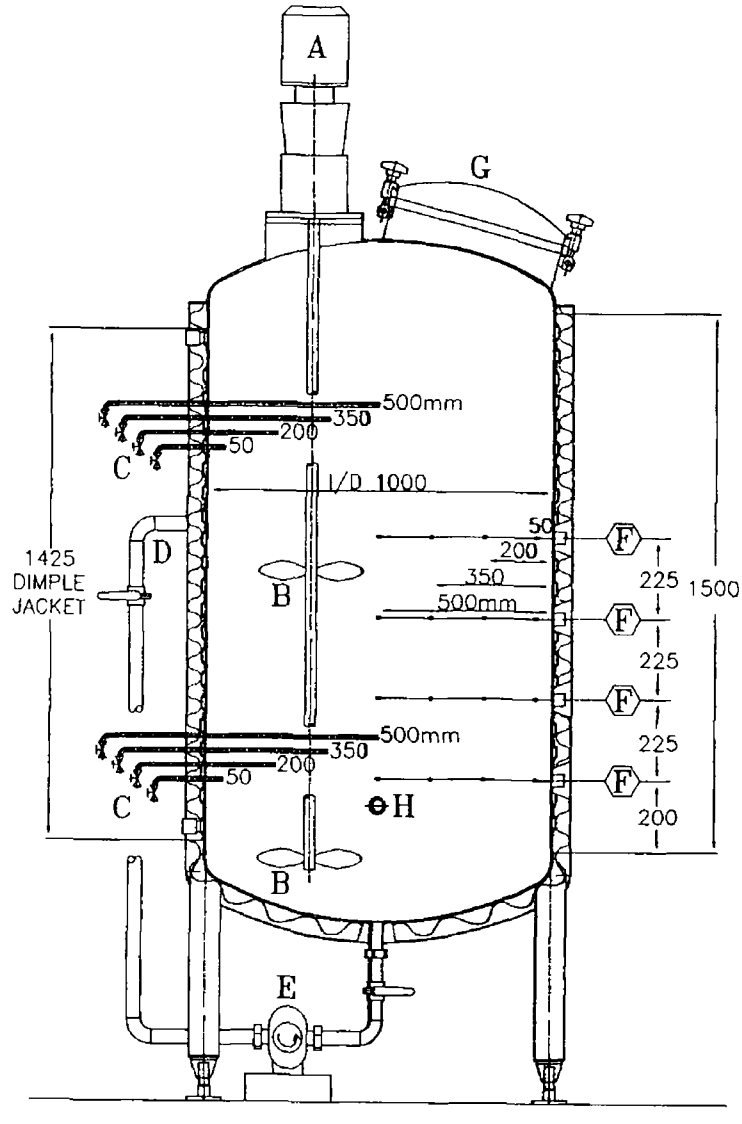

Figure 6 1. Schematic of the 10 hl customised YSV with the following features variable speed agitator (A), triple blade impellers (B), sample points (C), recirculation loop (D), variable speed positive displacement recirculation pump (E), customised temperature probes each with 4 measurement points (F), manway (G) and surface mounted Pt100 temperature control probe for vessel (H)

distances of 50, 200, 350 and 500 mm from the vessel wall The 4 x 4 array of temperature sensors covered half of the cross-section of the vessel The accuracy of each Pt100 sensor was ± 0 1°C The temperature setpoint for all storage trials was 4°C and the vessel was maintained at a top pressure of 0 5 bar using N_2

6.2 5 Calculation of isotherms

Using the data from the 16 measurement points within the vessel, a map of isothermal contours was prepared at various storage times A grid reference point was calculated for each temperature sensor location consisting of an x-value (distance (in mm) of the sensor from the vessel wall) and a y-value (distance (in mm) of the sensor from the base of the vessel) Using Sigmaplot™ Version 5 0 (SPSS ASC GmbH, Erkrath, Germany), XYZ triplet data, consisting of measured temperature values (Z data (°C)) and their corresponding horizontal (X data (mm)) and vertical (Y data (mm)) co-ordinates within the vessel, were used to calculate interpolated mesh data SigmaPlot™ uses an inverse distance method to generate Z values for an evenly spaced XY grid from XYZ triplet data The weighting used for these calculations sets the effect of distant points in the interpolation of values The effect of changing the weight value depends on the differences in the Z values, the ratio of the number of original data points to the number of interpolated data points, and the number of grid lines (X and Y values) Using a smaller weight places a degree of emphasis on distant data points and results in a mesh that passes further away from the original data A larger weight places greater emphasis on the nearest data points, resulting in a mesh passing closer to the data For the number of data points measured in this study, a weighting value of 3 produced the optimal contour profile An off-centre agitator

was used during the study, which results in different degrees of mixing across the horizontal plane of the vessel However, for illustration purposes, the temperature data was mirror-imaged to reflect the temperature distribution across the entire vessel

6 3 RESULTS

6 3 1 Cooling of water

A series of trials were conducted using the 10 hL pilot scale YSV as outlined in Table 6 2 Trials using water were conducted initially to establish a baseline for agitated and un-agitated systems Thermal gradients of approximately 1 5°C were measured during the cooling of water from 24°C to 4°C without agitation A

Table 6.2. Description of YSV trials

Trial	Fluid	Agitation Type	Agitation Rate	Volume (hL)*
1	Water	None	N/A	10
2	Water	Twin Impeller	50 rpm	10
3	Yeast Slurry	None	N/A	8
4	Yeast Slurry	Recirculation	2 hr vessel turnover	8
5	Yeast Slurry	Recirculation	0 5 hr vessel turnover	5
6	Yeast Slurry	Twin Impeller	50 rpm	7
7	Yeast Slurry	Twin Impeller	200 rpm	8

* The volume of yeast slurry collected varied due to fobbing during collection

thermal profile after 15 hours of cooling indicates the temperature distribution in the YSV (Figure 6 2) Layers of water at different temperatures occurred in the vessel The coldest areas were at the top of the vessel and water at 4°C (maximum density) remained in the bottom section In contrast, the maximum thermal difference measured in water under mild agitation conditions (50 rpm) was considerably reduced throughout the cooling period A maximum thermal gradient of 0 3°C was observed (data not shown)

6 3 2 Storage of yeast without agitation

Storage of yeast without agitation demonstrates the extent to which yeast slurry can become heterogeneous in terms of temperature and consistency The temperature distribution throughout the YSV at t = 24, 48 and 72 hr is illustrated

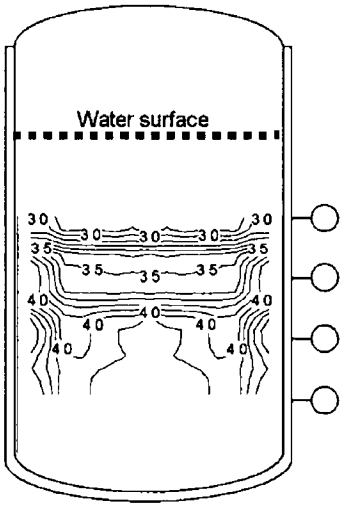

Figure 6.2. Temperature distribution profile for water without agitation after 15 hours cooling

in Figure 6 3 The initial mean temperature of the slurry was 5 9°C However, after 24 hours of cooling, the temperature of the slurry increased, ranging from 7 5 - 9 5°C The warmest regions were in the lower sections of the vessel with horizontal layers as indicated by the isotherms Surprisingly, the solids distribution in the vessel changed from a mean value of 40 6 % (w/w) initially to 58% in the upper levels of the vessel and 35% in the base of the vessel within 24 hr This change in solids distribution was accompanied by a 25% increase in the observed volume of slurry in the vessel due to CO_2 evolution from yeast metabolism The evolution of CO_2 caused a flotation of biomass to form a dense layer on the surface of the yeast slurry which persisted for the duration of the trial The temperature distribution measured at 48 hr indicates cool zones at 4°C close to the jacket However, temperatures in excess of 7 5°C were observed in the dense yeast layer

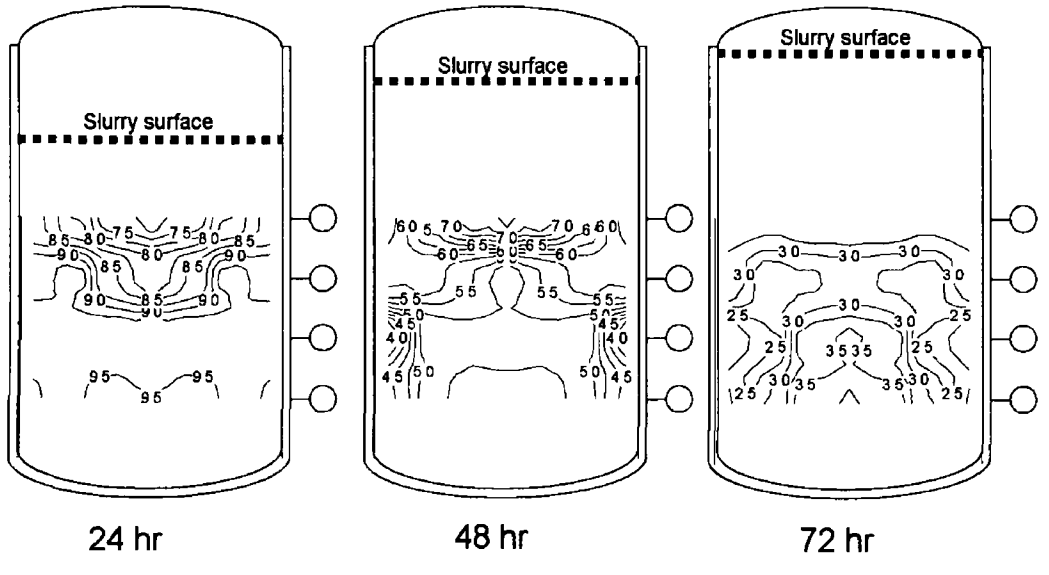

Figure 6.3. Temperature distribution profile for yeast slurry without agitation at t = 24, 48 and 72 hr

at the top of the vessel The initial solids content of the slurry was 40% However, after 72 hr of chilled storage, the sampled solids concentration at the base of the vessel was 34% and at the surface of the slurry was 73% solids The corresponding yeast slurry temperature ranged from 2 5 - 3 5°C

6 3.3 Storage of yeast with mixing by recirculation

Recirculation of the vessel contents using an external loop as a mixing mechanism was evaluated using a vessel turnover time of 2 hr The temperature distribution at t = 24, 48 and 72 hr is illustrated in Figure 6 4 The initial slurry temperature was 5 8°C and cooling was improved using a vessel turnover time of 2 hr compared to un-mixed yeast slurry While the arithmetic mean of all temperature measurements at 24 hr was 2 6°C, the slurry temperatures ranged from

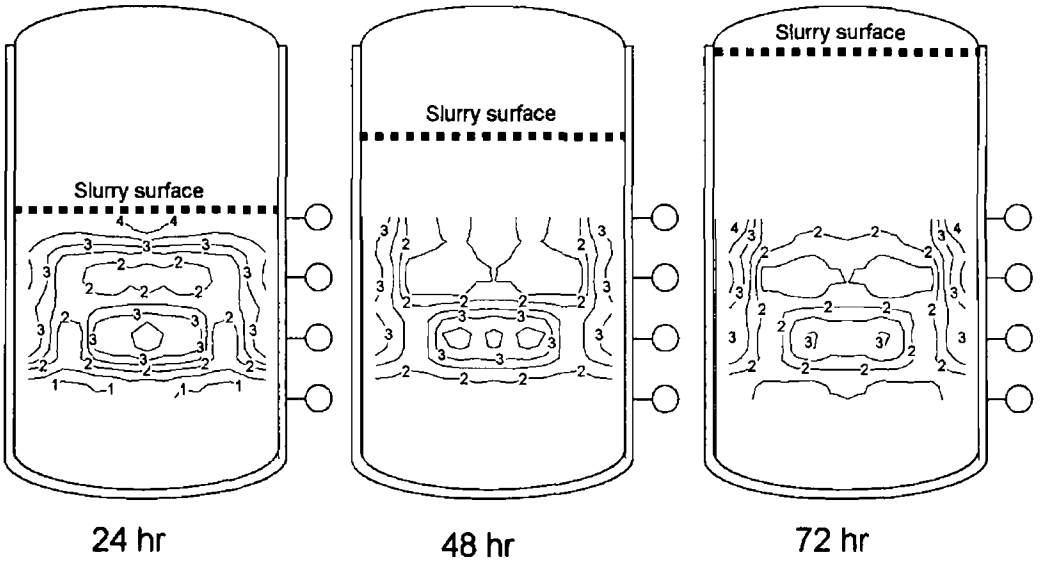

Figure 6.4. Temperature distribution profile for yeast slurry with a vessel turnover time of 2 hr at t = 24, 48 and 72 hr

1 - 4°C The coldest area was in the base of the YSV with increasing temperature towards the centre and also towards the top of the vessel As observed during un-agitated storage, a dense layer of yeast formed on the surface of the vessel contents during storage The solids content of this layer at 72 hr was 65% compared to 28% in the bottom of the vessel The temperature distribution data after 48 and 72 hr indicates a warm zone in the centre of the vessel with the coldest yeast slurry at the top of the vessel This is because the lower section of the vessel is being mixed due to recirculation while the upper region of the vessel (with increased solids content due to flotation of biomass) remains almost stagnant No significant heat generation was recorded in this poorly-mixed region In contrast to the un-mixed trial, the yeast slurry remained chilled from the start However, the range of temperatures and solids concentrations measured in the vessel are indicative of poor homogeneity of the slurry

Decreasing the vessel turnover time to 0 5 hr did not improve the temperature control or homogeneity of the slurry The temperature distribution data at t = 24, 48 and 72 hr is illustrated in Figure 6 5 At 24 hr the slurry temperature ranged from less than 1 5°C to 5°C This profile indicates that the lower region of the vessel was mixed by recirculation and was, in fact, being over chilled as stagnant regions in the upper regions of the vessel remained above 3°C Overcooling may have occurred as a result of stagnation of the yeast slurry in the vicinity of the temperature probe used to control the vessel temperature As observed with the previous trials, the solids content of the upper layer of the slurry increased to 65 % at 72 hr, while the corresponding solids concentration in the base of the YSV

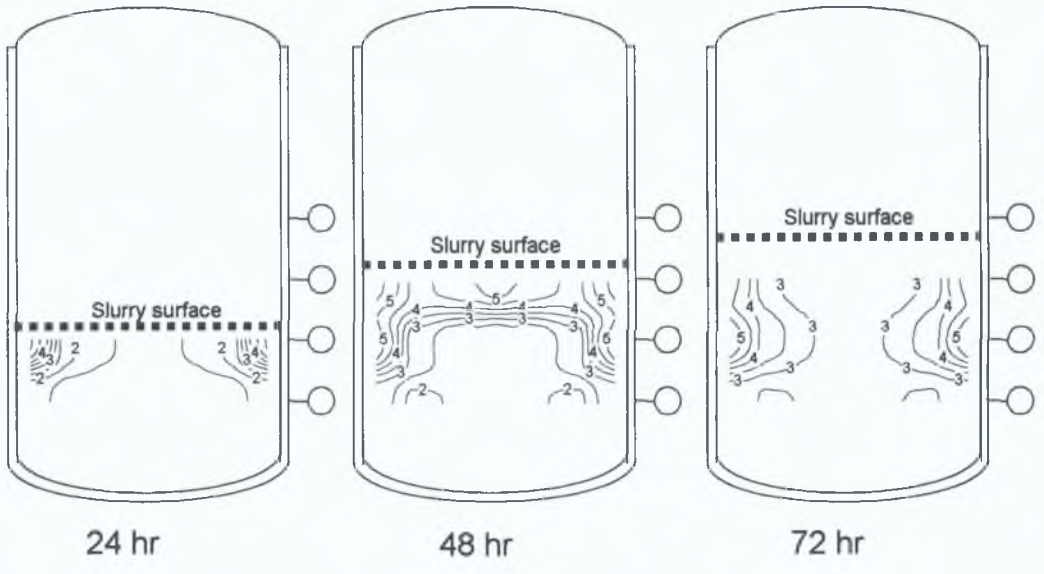

Figure 6.5. Temperature distribution profile for yeast slurry with a vessel turnover time of 0.5 hr at t = 24, 48 and 72 hr.

decreased to 23%. Recirculation as a mixing mechanism does not result in homogeneous slurry in terms of both temperature and solids content.

6.3.4 Storage of yeast with mechanical agitation

Mixing of yeast slurry using the described twin impeller at 50 rpm was studied. The initial mean slurry temperature was 5.8°C and the temperature distribution data at t = 24, 48 and 72 hr is illustrated in Figure 6.6. The slurry temperature was maintained at approximately 4°C with 50 rpm agitation after 24 hr, but the range of temperatures measured was 3.5 - 5.5°C. The warmest region was at the top of the vessel where a dense layer of yeast formed as observed in all previous trials. The maximum solids content of this layer was 75%. The overall temperature of the slurry at 48 hr decreased compared to 24 hr but the upper stagnant region of

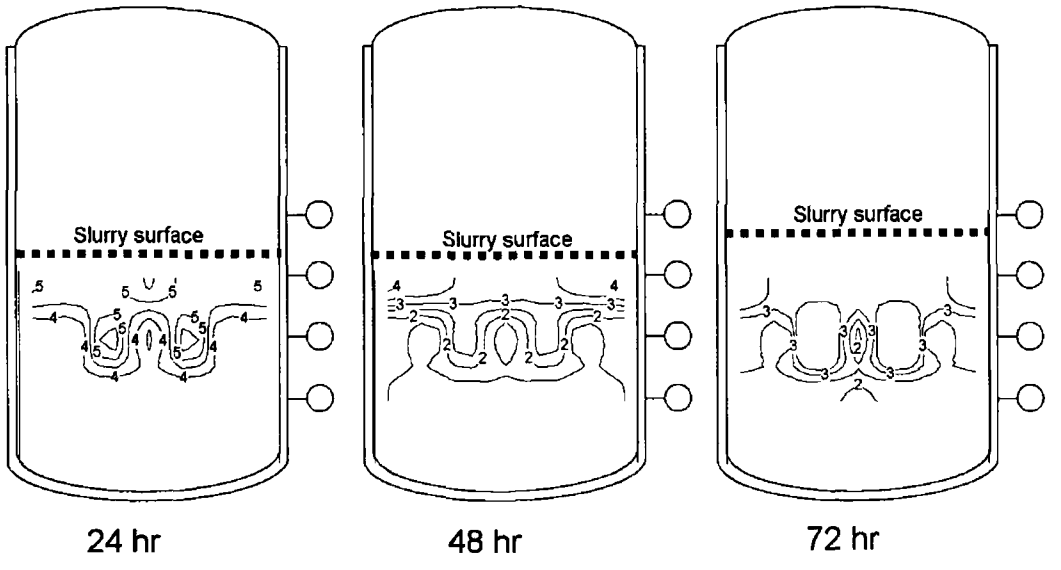

Figure 6.6. Temperature distribution profile for yeast slurry with an agitation rate of 50 rpm at t = 24, 48 and 72 hr

the vessel remained the warmest with the lower mixed region becoming over-chilled The temperature range at 48 hr storage was 1 5 - 4°C The temperature distribution at 72 hr indicates a warmer region at the top of the vessel but the inner core of the vessel had also developed a warm region due to metabolic heat generation and inadequate mixing at 50 rpm

A further trial using an agitation rate of 200 rpm indicated improved temperature control in the vessel The temperature range at 24 hr was 2 - 2 3°C and at 48 hr was 2 7 - 2 9°C (Figure 6 7) The thermal gradient remained constant for 72 hr of chilled storage i e 2 0 - 2 3°C Despite the improved agitation rate and a reduction in temperature gradients, the solids distribution in the vessel was not

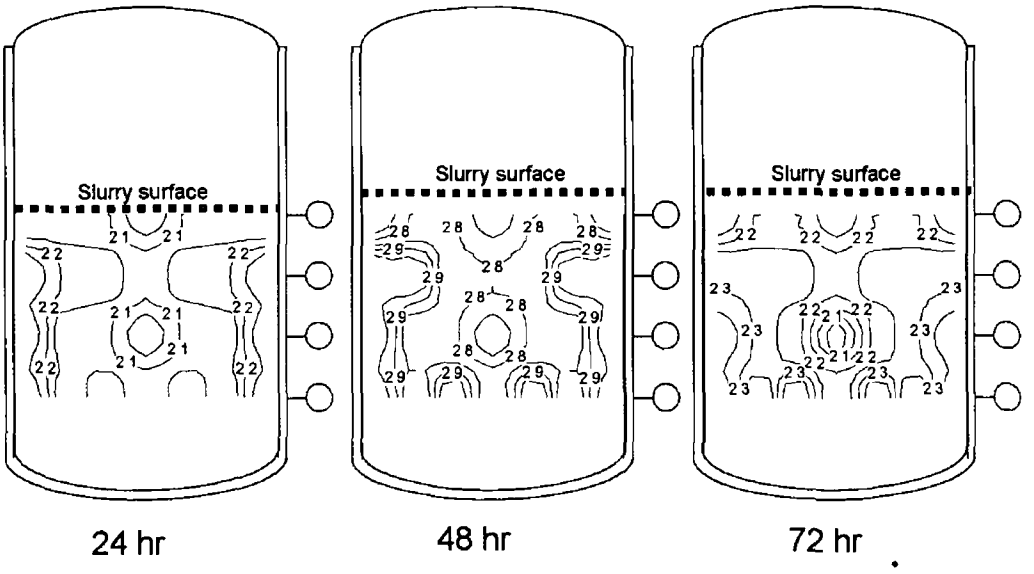

Figure 6.7. Temperature distribution profile for yeast slurry with an agitation rate of 200 rpm at t = 24, 48 and 72 hr

homogeneous The bulk contents of the vessel remained at approximately 40% solids, while the solids content of the upper surface of the slurry reached 59%

The maximum thermal gradients measured during the course of each storage trial are summarised in Table 6 3 The maximum thermal gradient measured for stored yeast without mixing (3 9°C) occurred after 48 hr of storage The gradient subsequently decreased to less than 2°C at 72 hr The effectiveness of recirculation as a mixing mechanism is poor, with thermal gradients in excess of 3°C measured at 24 and 72 hr An improvement in mixing, with a corresponding reduction in measured thermal gradients, was not observed with a decrease in vessel turnover time from 2 hr to 0 5 hr Thermal gradients of similar magnitude were measured for yeast stored with an agitation rate of 50 rpm The thermal

Table 6.3. Summary of temperature distribution in stored yeast

	Initial temperature (°C)	Maximum temperature difference at t = 24 hr	Maximum temperature difference at t = 48 hr	Maximum temperature difference at t = 72 hr
Water (unmixed)	24 5	1 9	ND	ND
Water (50 rpm)	26 5	0 3	ND	ND
Yeast (Unmixed)	5 9	2 5	3 9	1 8
Yeast (2 hr vessel turnover)	5 8	3 7	2 6	3 3
Yeast (0 5 hr vessel turnover)	7 0	4 1	3 6	3 1
Yeast (50 rpm)	5 8	2 3	3 1	4 4
Yeast (200 rpm)	5 5	0 3	0 3	0 4

ND Not determined

gradients increased during storage from 2 3°C at 24 hr to 4 4 at 72 hr The formation of a dense stagnant layer of yeast at the surface of the slurry was primarily responsible for such thermal gradients The greatest degree of temperature control was achieved with an agitation rate of 200 rpm The maximum thermal gradients observed throughout the trial did not exceed 0 4°C, which compares favourably with the thermal data for water at 50 rpm (0 3°C)

6.4 DISCUSSION

Yeast viability and the concentration of solids in the slurry were determined for samples taken from 4 separate locations in the vessel during each trial (Figure

6 1) The sample locations were at the top of the vessel 50 mm from the cooling jacket, at the top of the vessel in the centre, at the bottom of the vessel 50 mm from the cooling jacket and at the bottom of the vessel in the centre The maximum differences in solids distribution are summarised in Table 6 4 The minimum solids content measured in the base of the slurry can serve as an indication of the size of the dense upper layer of yeast As the dense upper layer increases in size, the solids concentration of the bulk contents of the vessel will correspondingly decrease The greatest decrease in solids concentration in the lower section of the vessel was observed for un-mixed yeast slurry and vessel turnover times of 2 hr and 0 5 hr Mechanical agitation resulted in improved uniformity of solids content with greater uniformity at the higher agitation rate The maximum difference in yeast viability for each trial follows similar trends The largest difference in yeast cell viability during storage corresponds to the largest differences in solids content, which re-emphasises the poor degree of mixing and homogeneity achieved with recirculation In general, yeast with the lowest measured viability was in the dense layer at the surface of the yeast slurry Poor temperature control due to inadequate mixing of this yeast layer is the most probable cause of the viability decrease An improvement in overall storage conditions can therefore be achieved by minimising or eliminating the development of such a layer by adequate agitation

In all storage trials, a dense layer of yeast biomass formed in the upper layer of the slurry irrespective of the agitation regime used The dense layer consisted of a

Table 6.4. Summary of solids and viability differences during yeast storage using different mixing regimes

	Initial solids (% w/w)	Maximum solids content (% w/w)	Minimum solids content (% w/w)	Maximum difference in solids (% w/w)	Maximum difference in viability (%)
Yeast (Unmixed)	40 6	73 4	28 6	44 8	13
Yeast (2 hr vessel turnover)	43 9	69 0	28 6	40 4	12
Yeast (0 5 hr vessel turnover)	29 4	65 6	23 1	42 5	12
Yeast (50 rpm)	39 6	74 6	35 4	39 2	4
Yeast (200 rpm)	40 7	59 5	37 8	21 7	3

mixture of solids and entrapped CO_2 (with a heavy, mousse-like consistency) The solids concentration was measured on a w/w basis, thus eliminating any entrapped CO_2 from the sample Although the solids concentration on a w/v basis would be less than on a w/w basis, the data demonstrates a transfer of solids from the bulk slurry to a layer at the surface

The volume of slurry in the vessel increased as a result of CO_2 evolution by the stored yeast, which resulted in foaming at the slurry surface The yeast used for all of these trials was a top fermenting ale yeast, and it is reasonable to assume that

the evolution of CO_2 during storage has a flotation effect on the yeast solids, thus forming a dense yeast layer on the surface of the slurry It is unlikely that such a phenomenon would occur with bottom fermenting yeast However, due to their flocculation characteristics, it is possible that bottom fermenting yeast would develop gradients of solids concentration, with the greatest concentration in the base of the YSV The increase in volume of the yeast slurry during storage was recorded and expressed in terms of relative volume increase compared to the initial volume of yeast slurry This data is presented in Table 6 5 The magnitude of volume increase is proportional to the metabolic activity of the stored yeast which results in the production of CO_2 The observed increase in slurry volume is greatest for un-mixed yeast slurry and yeast mixed by recirculation (vessel turnover time of 2 hr) These storage regimes allow the greatest degree of yeast metabolism during storage, which will decrease the yeast glycogen reserves and result in yeast with reduced viability and vitality A small improvement is observed with a decrease in vessel turnover time to 0 5 hr Mechanical agitation, both at 50 and 200 rpm, resulted in little change in the volume of stored yeast Evolution of CO_2 and foaming by the yeast slurry was kept to a minimum which serves to indicate reduced metabolism by the yeast as a result of improved temperature control throughout the bulk of the slurry Mechanical agitation has been demonstrated to be more effective than recirculation alone in maintaining a greater degree of uniformity throughout the slurry of temperature, yeast viability, and solids concentration This level of control results in a reduction in the rate of yeast metabolism and subsequent CO_2 evolution during storage

Table 6.5. Percentage increase in volume of slurry during storage due to CO_2 evolution and foaming.

	Relative volume (% of initial volume)			
	t = 0 hr	**t = 24 hr**	**t = 48 hr**	**t = 72 hr**
Yeast (Unmixed)	100	125	150	162
Yeast (2 hr vessel turnover)	100	100	125	162
Yeast (0.5 hr vessel turnover)	100	100	130	150
Yeast (50 rpm)	100	100	100	107
Yeast (200 rpm)	100	102	106	106

Yeast storage plays a central role in any brewery yeast management programme The storage of yeast can extend from hours to several days and therefore the quality of yeast must be maintained as high as possible It is clear from this work that stored yeast exists as a collection of portions of yeast in a YSV and that different portions are, in effect, stored under different local conditions if the mixing regime is inadequate It is important that the environmental conditions of these localised intra-vessel portions should be within the acceptable storage conditions for pitching yeast (as defined by the brewer) Temperature readings from probes fitted close to the vessel wall can mislead the brewer into believing that the yeast crop is at a uniform temperature This phenomenon has been

reported for un-mixed systems previously (See Chapter 5 and Leonel *et al*, 1987) Variations in storage temperature can indirectly have an adverse affect on the quality of the stored yeast For example, ethanol tolerance is known to decrease with increasing temperature (O'Connor-Cox, 1997) Therefore, with high gravity fermentation systems, small elevations in storage temperature can result in increased ethanol stress on the yeast Representative sampling of a YSV to enable determination of the concentration of stored pitching yeast can also prove difficult It is impossible to determine the solids concentration of a YSV if the degree of mixing during storage is inadequate An estimation of a yeast concentration based on a single sampling location in a batch of stored yeast can result in inaccurate pitching rates (O'Connor-Cox, 1998a) The estimated weight (or volume) of slurry based on a 'mean value' for the YSV can consist of a range of values of yeast solids and viability Accurate pitching control can only be achieved in such circumstances based on measurement of wort biomass concentration during filling of the FV It is essential that brewers challenge the validity of temperature probe readings from yeast storage vessels and determine the actual effectiveness of their yeast storage regimes in maintaining uniform yeast quality throughout the entire stored yeast crop

6.5 CONCLUSIONS

The mode and degree of mixing employed during the storage of pitching yeast has a direct impact on the distribution of temperature, viability and solids concentration within the slurry Recirculation of the slurry from the base to the top of the YSV using an external pumping loop is least effective in maintaining uniform temperature within the vessel In addition, the solids distribution using

this regime is more heterogeneous than compared to the use of a mechanical agitator A high agitation rate of 200 rpm was required to ensure uniformity of temperature and solids content throughout the slurry Gradients of yeast slurry temperature can exist in YSV's even though the vessel temperature probe reading is within specification

CHAPTER 7

SUMMARY

7 1 CONCLUSIONS

The mean cell volume of brewing yeast strains increases when propagated in worts of increasing original gravity This phenomenon can result in inaccurate pitching rates when using indirect biomass measurements such as wet weight analysis Furthermore, propagation of yeast in wort gravities in excess of 12 5°P results in a deterioration of yeast quality in subsequent high-gravity fermentations Therefore, propagation of brewer's yeast is recommended in wort gravities of 12 5°P or below

During storage of pitching yeast, the mean cell volume of top and bottom fermenting yeast decreases with time The decrease in cell volume may be attributable to utilisation of cellular reserves of glycogen for maintenance metabolism during prolonged storage A decrease in mean cell volume during storage can result in over-pitching of fermentations when indirect biomass measurements are used, for example, wet weight analysis and an Aber^{TM} biomass probe The pitching rates can be corrected using image analysis data to determine the decrease in cell volume during storage This novel pitching regime results in fermentation profiles which match the ideal fermentation profile more closely than conventional pitching

Image analysis technology, combined with staining of yeast cells for intracellular glycogen (using Lugol's solution), has enabled the development of a rapid assay for the determination of yeast glycogen content (20 minutes versus 2 days) Unlike conventional glycogen assays, the newly developed technique can measure both the mean glycogen content and the frequency distribution of cellular glycogen throughout the yeast population The overall shape of the frequency distribution profile serves as a useful indicator of yeast quality Furthermore, the dissimilation of glycogen during the early stages of fermentation is not uniform, which can limit the usefulness of mean glycogen analysis as an indicator of yeast vitality

Significant thermal gradients occur in un-mixed yeast crops due to metabolic heat generation by yeast The magnitude of these gradients is directly linked to the metabolic activity of the yeast The potential for yeast deterioration in yeast plugs is significant and short residence times in FV are recommended Cone cooling has little effect on yeast crop temperatures at a distance of 0 6 m from the cooling surface

Inadequate mixing during storage of pitching yeast can result in significant gradients of temperature, viability and solids concentration throughout the batch Mechanical agitation resulted in improved uniformity throughout the vessel compared to mixing using an external recirculation loop Single temperature probes and sampling points do not give a good indication of the storage conditions

and slurry consistency, unless the mixing regime is effective in achieving a homogeneous suspension throughout the yeast batch

7.2 RECOMMENDATIONS FOR FUTURE WORK

(i) The combination of image analysis and Lugol staining has proved most useful in determining yeast quality Other vital staining techniques could be combined with imaging techniques to develop rapid viability and vitality assays The advantage of such a system would include speed of analysis and the ability to look at the frequency distribution of results throughout the yeast population

(ii) Using the developed rapid technique for yeast glycogen content, it would be useful to survey the entire yeast handling process within breweries to identify areas where significant losses in vitality occur, for example, plate heat exchangers, centrifuges and pumps

(iii) Image analysis techniques have been developed and applied to brewer's yeast Exploration of other applications for image analysis in other processes within the brewery could prove useful, for example, determination of raw material quality (grain size and shape, contamination), control of malt milling with feedback systems to adjust mill settings to achieve a uniform grist consistency

(iv) Examination of the distribution of solids concentration during storage of a slurry of a top fermenting yeast strain indicated significant transfer of solids to the surface of the slurry due to CO_2 flotation It would be interesting to observe what mass transfer occurs (if any) during the storage of a bottom fermenting yeast strain It is likely that the transfer of solids with inadequate mixing would occur in the opposite direction i e , sedimentation as opposed to flotation

(v) The mixing regime used for storage of pitching yeast has a dramatic effect on the uniformity of the slurry Further studies of the heterogeneity of yeast slurry during acid washing would be useful in terms of pH, temperature, yeast viability and levels of contamination

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