STRESS MARKERS AS INDICATORS OF FERMENTATIVE ABILITY OF A *Saccharomyces cerevisiae* BREWERY STRAIN

By

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DECLARATION

I, the undersigned hereby declare that the work contained in this thesis is my original work and has not previously in it's entirely or in part been submitted to any university for a degree

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SUMMARY

In the brewing industry yeast cells are re-used in successive fermentations. Consequently, the state of the cells at the end of each successive fermentation could impact on the quality of the subsequent fermentations. The use of markers to evaluate the fermentative ability of yeast to resist stress enables brewers to select populations of yeast for brewing. Yeasts are typically exposed to osmotic-, ethanol- and cold-stress during the high-gravity brewing process. In this study the vitality of the yeast cells was monitored during and after each successive high-gravity brewing fermentation. This was done by measuring the cell metabolites, which included glycerol, trehalose and glycogen. Others markers that were evaluated for yeast viability were the number of budding scars, the levels of activity of the enzymes neutral trehalase and esterase and the expression level of the heat shock protein Hsp12p. Coupled to these evaluations, the growth of the yeast and the utilisation of the sugars glucose, fructose, maltose and maltotriose were monitored during the fermentations. The experiments were conducted in 2-litre E.B.C. tubes at either 14 ^{o}C or at 18^{o}C using standard techniques.

Comparable growth patterns were obtained for different re-pitching fermentations, with fermentation 1 at 18ºC and 5 and 6 at 14°C being the most active fermentations. The higher temperature encouraged more rapid growth and a greater numbers of cells. The wort attenuation was more rapid at 18°C than at 14°C. Glucose and fructose in wort were utilised prior to maltose and maltotriose. At 18°C the yeast consumed the sugars faster, with mean utilisation values of 97.3% glucose, 100% fructose, 59.9% maltose and 65.6% maltotriose. At the lower temperature of 14°C high concentrations of residual sugars remained at the end of the fermentation. All re-pitching fermentations revealed lower viabilities at 18°C in comparison to the 14°C fermentations.

Simultaneously, a number of other markers were evaluated. The intracellular trehalose concentration per cell varied considerably with each fermentation. Trehalose levels at 18°C gradually increased in concentration from 48h until the end of the stationary phase.

Much lower trehalose concentrations were observed in fermentations conducted at 14°C. Higher and more consistent glycerol concentrations were found in fermentations at 14°C with mean concentrations of 12 mg/g dry weight at pitching. The expression of the heat shock protein Hsp12p level increased during the fermentation but no sharp increase was detected in any particular fermentation. No increase in yeast budding scar number was observed during re-pitching fermentations. Neutral trehalase and esterase activities in fermentations at 18°C were especially high at pitching. Neutral trehalase activities at 14°C were all generally lower than in the case of fermentations at 18°C.

The fermentation ability of flocculated yeast in slurry and yeast suspended in beer was investigated after exposure to various stresses. The aged yeast present in the slurry was generally found to be more resistant to stress, in particularly to osmotic stress, throughout the serial re-pitching process. The fermentation rates of both yeast types were especially sensitive to prior exposure to ethanol stress.

OPSOMMING

In die broubedryf word gisselle herhaaldelik gebruik vir agtereenvolgende fermentasies. Derhalwe kan die toestand van die gisselle teen die einde van elke agtereenvolgende fermentasie ‘n invloed hê op die kwaliteit van die daaropvolgende fermentasies. Deur gebruik te maak van merkers om die fermentasievermoë van gis om stres te weerstaan te evalueer, stel dit bierbrouers in staat om gispopulasies te selekteer. Gedurende die hoë-digtheid brouproses word giste tipies aan osmotiese-, etanol- en koue-stres blootgestel. In hierdie studie, gedurende hoë-digtheid fermentasies, is die lewensvatbaarheid van die gisselle gedurende en na elke agtereenvolgende fermentasie gemonitor deur die volgende selmetaboliete te bepaal: gliserol, trehalose en glikogeen. Bykomende merkers vir gis lewensvatbaarheidsbepalings was: die aantal botselletsels, die vlakke van aktiwiteit van die neutrale trehalose en esterase ensieme, en die uitdrukkingsvlak van die hitteskokprotein Hsp12p. As aanvullende evaluasies is die groei van die gis en die gebruik van die suikers glukose, fruktose, maltose en maltotriose gedurende fermentasies gemonitor:. Die proewe is in 2-liter E.B.C. buise uitgevoer, by ‘n temperatuur van 14^{o}C of 18^{o}C, deur van standaard tegnieke gebruik te maak.

Die groeipatrone van die verskillende herhaaldelike-inokulasie gistings was ongeveer dieselfde. Fermentasie 1 by 18ºC en fermentasies 5 en 6 by 14°C was die mees aktiewe fermentasies. Die hoër temperatuur het vinniger groei en ‘n groter aantal selle begunstig. Die wortattenuasie was vinniger by 18°C as by 14°C. Glukose en fruktose in mout is voor die maltose and maltotriose opgebruik. By 18°C het die gis die suikers vinniger opgebruik. Gemiddelde gebruikswaardes vir die sewe reeksgewyse fermentasies was die volgende: 97.3% glukose, 100% fruktose, 59.9% maltose en 65.6% maltotriose. Teen die einde van fermentasie by 14°C was daar hoë konsentrasies van die oorblywende suikers, hoofsaaklik na fermentasie 1. Alle herhaaldelike inokulasie fermentasies het lae lewensvatbaarheid by 18°C in vergelyking met 14°C fermentasies getoon.

Ander merkers is ook gelyktydig gebruik. In die verskillende fermentasies was daar ‘n groot verskil in die intrasellulêre trehalose konsentrasie per sel. Trehalose konsentrasies by 18°C het geleidelik toegeneem, vanaf 48 uur tot aan die einde van die stationêre fase.

Baie laer trehalose konsentrasies is gemeet vir fermentasies by 14°C. In fermentasies by 14°C was die gliserolkonsentrasies hoër en meer konstant. Gemiddelde konsentrasies was 12mg/g 14°droë gewig by inokulasie. Die uitdrukking van die hitteskokproteien Hsp12p vlak het gedurende fermentasie toegeneem, maar daar was geen skerp toename vir die afsonderlike fermentasies nie. Die bepaling van die aantal botselletsels per sel het daarop gewys dat die gemiddelde aantal nie toegeneem het met die veroudering van die gis gedurende reeksgewyse herhaaldelike inokulasie nie. Neutrale trehalase aktiwiteite in fermentasies by 18°C was besonders hoog, veral by inokulasie. Die neutrale trehalase aktiwiteite in die fermentasies by 14°C was in die algemeen laer as die by 18°C.

Die fermentasievermoë van die geflokkuleerde gis in die sediment en gesuspendeerde gis in die bier is ondersoek na blootstelling aan verskeie tipes stres. Die verouderde gis teenwoordig in die sediment was in die algemeen meer bestand teen stres, veral aan osmotiese stres, dwarsdeur die reeksgewyse herhaaldelike inokulasie proses. Etanolstres het die gistingstempo van beide giste dieselfde geaffekteer.

BIOGRAPHICAL SKETCH

Sabrina Boudler was born in Abidjan, Ivory Coast, on June the 8th, 1979. She grew up in the south of Belgium, and immigrated with her family to Port Elizabeth, South Africa in July 1995. She completed her secondary education in 1997 at Pearson Secondary School in Port Elizabeth. She then completed her biochemistry honours in 2002 at the University of Port Elizabeth.

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BREWING TERMINOLOGY

Adjunct	- Fermentable material (e.g: rice, maize) used as a substitute for traditional grains, to make beer lighter-bodied or cheaper.
Aeration	-Bubbling oxygen/air supply in the wort prior to yeast pitching.
Anaerobiosis	-Process lacking or depleted in oxygen
Attenuation	- Extent to which yeast consumes fermentable sugars (converting them into alcohol and carbon dioxide).
Autolysis	-The breakdown of yeast cells by "own-digestion" by its own hydrolytic enzymes, in suspension within the beer after the second fermentation is completed.
Barley	-A cereal grain that is malted for use in the grist that becomes the mash in the brewing of beer.
Cropping	-The collection of yeast from fermented wort for subsequent re-pitching.
Dextrin	- The unfermentable carbohydrate produced by the enzymes in barley. Lower temperatures produce more dextrin and less sugar, while higher temperatures produce more sugars and less dextrin.
Esters	- Esters are organic compounds that result from the interaction of acids and alcohol. The presence of esters can cause the fruity flavours and aromas, such as banana, blueberry, and pear that intentionally or unintentionally occur in some beers.
Extract	-The total amount of dissolved materials in the sweet wort after mashing and lautering malted barley and adjuncts.
Fermentation	- This is the process of producing alcohol and carbon dioxide through the actions of yeast on grain-based sugars. Conversion of sugars into ethyl alcohol (ethanol) and carbon dioxide, through the action of yeast.
Fermentation performance	-Evaluation of the yeast fermentative activity in terms of its vitality.
Final specific gravity	- Specific gravity of a beer when fermentation is complete (that is, all fermentable sugars have been fermented).

Flocculation -	-The process of yeast coming together, dropping to the bottom of a fermenter, is called flocculation. This term describes how fast or how well yeast clumps together and settles to the bottom of the fermenter after fermentation is complete.
FV	-Fermentation vessel shaped as a cylindroconical vessel (V-shaped).
Generation number	-The number of time the yeast has been pitched into the wort in consecutive fermentations.
Gravity (Specific)	- The weight of a liquid relative to the weight of an equal volume of water. Specific gravity must be checked before and after fermentation. Used as an indication of the amount of alcohol present in the finished beer.
Grist	- Brewers' term for milled grains, or the combination of milled grains to be used in a particular brew. Dry mixture of barley malts and adjuncts used in mashing.
Heat Exchanger	- A mechanical device used to rapidly reduce the temperature of the wort.
Hops	- Brewers use the aromatic flowers of the hops vine to provide bitterness, aroma & flavour to beer. The hops flowers can be used whole or in a pelletized/processed form. Different varieties of hops contribute varying levels of bitterness and different aromatic qualities. Depending on when hops are added to a brew, the contribution of aroma, bitterness or flavour can be controlled by a brewer.
Malt	- This key ingredient provides colour, body and fermentable sugar. The color and fermentability of malt is determined during kilning at the end of the malting process. The quantity of fermentable sugar contributed by malt is key factor in determining the alcoholic strength of the beer.
Malt Extract	-Syrups manufactured by evaporating excess water out of wort. The condensed wort from a mash, consisting of maltose, dextrins and, other dissolved solids. Either as a syrup or powdered sugar, it is used by brewers, in solutions of water and extract, to reconstitute wort for fermentation.
Malt (ing)	- The process by which barley is steeped in water, germinated, kilned (dried) to convert insoluble starch to soluble substances and sugar as the foundation ingredient of beer.

Mashing	-The preparation of the wort, the liquid base of beer. Mashing converts starches to sugars by mixing malted barley with hot water.
Mash tun	-The double-jacketed, stainless-steel vessel in which mashing occurs. A tank where grist is soaked in water and heated in order to convert the starch to sugar and extract the sugars and other solubles from the grist.
Necrosis	-The death of cells or tissues as a result of external damage.
Pitching	-The inoculation of yeast into wort, the mass of yeast slurry used per units of volume or the number of yeast cells inoculated per milliliter.
Plato, degrees	- Expresses the specific gravity as the weight of extract in a 100 gram solution at 64°F (17.5°C). Refinement of the Balling scale.
Propagation	-The step-wise generation of biomass from laboratory to plant scale to obtain sufficient biomass for production purpose.
Racking	- The process of separating the fermented beer from the yeast cells at the bottom of the fermenting vessel. Also the transfer of finished beer to kegs. Broadly, moving beer from one vessel to another.
Senescence	-Advancing age-the complex of ageing processes that eventually lead to death.
Sparging	- To spray grist with hot water in order to remove soluble sugars (maltose). This takes place at the end of the mash. Rinsing the mashed grains to ensure complete extraction of the sugars from the mash.
Specific gravity	- A measure of the density of a liquid or solid compared to that of water ((1.000 at 39°F (4°C)).
Slurry	-The sedimented/flocculated yeast from a previous fermentation. Yeast being stored in storage vessel as slurry.
Trub	-Proteins in barley filtered during the wort boil.
Viability	- Measure of the number of dead or living cells. The ability to grow, reproduce and interact with their immediate environment.
Vitality	- Measure of the activity or physiological state of the living cell in regards to the entire yeast population (mean vitality).The capacity to overcome or recover from physiological stress. This factor can

	influence the fermentation performance and the quality of the finished beer.
Water activity (a_w)	-Water activity is a measure of the amount of free unassociated water molecules in a system. It runs on a scale of 0 to 1.0, where pure water equals 1.0. This parameter can also be expressed as a percentage referred to as the relative humidity. It is influenced by dissolved solutes and insoluble food components which act to bind water, thus reducing the available free water and hence a_w value.
Wort	- The solution of grain sugars strained from the mash tun. At this stage, regarded as "sweet wort", later as brewed wort, fermenting wort and finally beer. The sweet liquid derived from mashing, or mixing malted barley with water. Wort is the beginning of all beers.

COMMONLY USED ABBREVIATIONS

AAT	Alcohol acetyl transferase
ADP	Adenosine diphosphate
AK	Adenylate kinase enzyme
AMP	Adenosine monophosphate
APT	Acidification power test
ATP	Adenosine triphosphate
CCT	Catalase T
CIP	Cleaning in place
DAK	Dihydroxyacetone kinase
DDR	DNA damage
E.B.C.	European brewery convention
ER	Endoplasmic reticulum
FAN	Free amino nitrogen
FG	Final gravity
FP	Facilitator protein
FV	Fermentation vessel
GAP	Glucose-induced acidification power
GIPE	Glucose-induced proton efflux
GPD	Glycerol-3-phosphate dehydrogenase
GPP	DL-glycerol phosphate phosphatase
GRE3	Aldo-keto reductase

GSY	Glycogen synthase
GPH	Glycogen phosphorylase
HOG	High osmolarity glycerol
HPLC	High performance liquid chromatography
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
LHS	Chaperone of the endoplasmic reticulum lumen
MAPK	Mitogen activated protein kinase
MIP	Major intrinsic protein
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phopshate
NTH	Neutral trehalase
NIR	Near infrared reflectance spectroscopy
OG	Original gravity
PBS	Phosphate buffer saline
PHB	Subunit of the prohibitin complex
PGM	Phosphoglucomutase
SG	Specific gravity
SSA / SSE	Heat shock protein of Hsp70p family
STRE	Stress response element
TPS / TSL	Trehalose phosphate synthase
RF	Re-pitching fermentation

UBI	Polyubiquitin enzyme
UDP	Uridine diphosphate
UDPG	Uridine diphosphate glucose dehydrogenase
UGP	UTP-glucose-1-phosphate uridylyltransferase
UTP	Uridine triphosphate
YCV	Yeast collection vessel
YPV	Yeast pitching vessel
WAP	Spontaneous acidification power

Chapter 1: Introduction and Objectives

1.1 Introduction

Yeast is the key source of the brewing process. A selected strain of the brewing yeast in pure culture, belonging to the genus *Saccharomyces cerevisiae,* is used for fermentation during the brewing process. Two different strains of brewer's yeast are found in the brewing industry depending on the type of beer brewed: the top fermenting yeast *Saccharomyces uvarum* is used for ale-type beer at fermentation temperatures between 18 and 22°C, whereas the bottom fermenting yeast *Saccharomyces cerevisiae* is used to produce lager-type beer (Stewart *et al,* 1984).

Yeast management in modern breweries has become increasingly critical since the introduction of a new technology called "high gravity brewing". In this process, fermentation of high substrate concentration wort supplied with extra sugar addition in the form of "adjunct" occurs, producing a final beer with an ethanol yield in proportion to the plato of the fermentable extract (Boulton and Quain, 2001). Fermentation times, energy, labour and capital costs are all proportionally simultaneously decreased.

The consistent production of a high quality beer is mostly dependent on the physiological status of the pitching yeast. Higher pitching rates and longer fermentation time are thus required to achieve the desired attenuation, resulting in a relative increase in yeast biomass (Fernández *et al*, 1985). The continuous fresh supply of yeast, grown weekly in a propagation process, has been established to ensure a regular renewal of pure culture yeast to prevent the emergence of mutations and infections (Boulton and Quain, 2001). Yeast after a first fermentation is cropped and stored at low temperatures to be re-used in successive fermentations – up to nine generations in cycles of serial re-pitching. The yeast is continuously exposed to various stresses during fermentation in high gravity worts, storage at low temperatures and from movement from one vessel to another through a high-pressure pipe system (Heggart *et al*, 1999). Fermentation stresses encountered include the following: high osmotic pressure present in wort, low water activity, and toxic effects from elevated

ethanol concentrations and nutrient limitation at the end of fermentation. All of these stresses inhibit yeast growth and, to a certain extent, the fermentation rate (Boulton, 1991). The ability of the yeast to endure stress and still perform under such conditions is defined as the vitality, which is critical to evaluate the fermentation performance.

During the cycle of re-pitching, one must be able to determine the fermentative activity of the yeast and to what extent the previous stresses negatively affect the physiological activity. Therefore, the yeast slurry during storage should be maintained under constant conditions otherwise inconsistent fermentation performance might occur. Standard yeast handling procedures, including the storage method used prior to re-pitching (storage time and temperatures or agitation) also need to be closely examined to ensure the best optimum fermentation performance (O'Connor Cox, 1998).

1.2 Objectives

The main aim of this research was to evaluate various techniques as indicators of the fermentation potential of a brewer's yeast during fermentation of high gravity wort. The fermentation performance and desired fermentation characteristics were to be determined by general methods such as yeast count, viability, decrease in plato gravity, ethanol production and yeast's vitality, by the measurement of different intracellular metabolites such as trehalose, glycerol, glycogen and the heat shock protein 12 (Hsp12p). The fermentative ability of the brewer's yeast after being exposed to selected levels of different types of stresses encountered during the course of the brewing process was also investigated.

1.3 General characteristics of brewer's yeast

The brewer's yeast belongs to the family of Saccharomycetaceae, and is classified under the subfamily *Saccharomycetoideae* (Phaff *et al*, 1978). The genus *Saccharomyces* is a member of this subfamily and is characterised as ellipsoidal or elongated vegetative cells occurring in pairs or in small clusters. They are classified by reproducing through multilateral budding as diploid or haploid depending on environmental conditions (Fig 1.1) (Phaff *et al*, 1978).

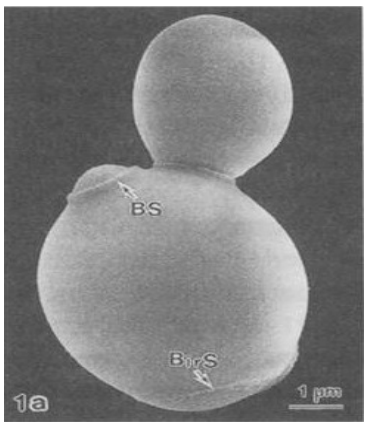


Fig 1.1: Illustration of a budding yeast cell, showing multiple budding scars represented by the budding scar arrows.

The brewer's yeast strains differ from the laboratory yeast strains in a number of ways. Distinct biochemical differences between lager and ale yeast have produced two types of brewing yeast: the bottom fermenting strain named *Saccharomyces carlsbergensis (uvarum)* that produce lager beers and the top fermenting strain is included in *Saccharomyces cerevisiae* to produce ale beers. Both are related to the laboratory strain *Saccharomyces cerevisiae* in their genome structure, with the top fermenting strain being closer related to the original laboratory strain (Zimmermann and Entian, 1997). These three strains differ mainly in properties like ethanol tolerance, osmotic sensitivity and flocculation patterns (Heggart *et al,* 1999). Various genetic and physiological differences between the laboratory and the brewing strains as can be viewed in Table 1.

Table 1.1: Genetic and physiological differences between laboratory- and brewing-yeast strains (Heggart *et al*, 1999).

	CRITERIA	ALE STRAIN	LAGER STRAIN	LAB STRAIN
GROWTH	**Rate**	Slow growth	Slow growth	Fast growth
	Flocculation	Weakly flocculent	Strongly flocculent	Generally non-flocculent
	Killer character	Non-killer	Non-killer	Various killer phenotypes
GENETICS	**Selected for**	Alcohol production	Alcohol production	Genetic studies
	Ploidy	Polyploid or aneuploid	Allotetraploid	Stable haploids or diploids
	Karyotype	Variable	Variable (2 types of same chromosome present)	16 chromosomes
	Sporulation	Poor	Poor	Good
METABOLISM	**Fermentation rate**	High	High	Low
	Sugar utilization	Melibiose not used	Melibiose used	Melibiose not used
		Glucose inhibition of maltose uptake	Glucose does not inhibit maltose uptake	Single maltose transporter
		Poor maltotriose transport	Good maltotriose transport	Variable maltotriose transport
	Valine transport	Poor uptake	Poor uptake	Good uptake
	Flavour compounds	Lack POF1-no ferulic acid decarboxylation	Lack POF1-no ferulic acid decarboxylation	POF1-allele ferulic acid decarboxylated→phenolic off-flavour
	Ferment at 37°C	Yes	No	Maybe

1.4 Yeast cell cycle:

Saccharomyces cerevisiae displays either a heterothallic or a homothallic life cycle with the same (homothallic) or different (heterothallic) mating types (a/a, α/α or a/α). Both heterothallic and homothallic diploid strains sporulate under conditions of nutrient starvation when the diploid cell undergoes meiosis producing four haploid

cells enclosed within an ascospore (Fig 1.2). Under more favourable nutrient conditions, two haploid ascospores of opposite mating types will conjugate together to form a zygote as a diploid (Fig 1.2) (Phaff *et al,* 1978).

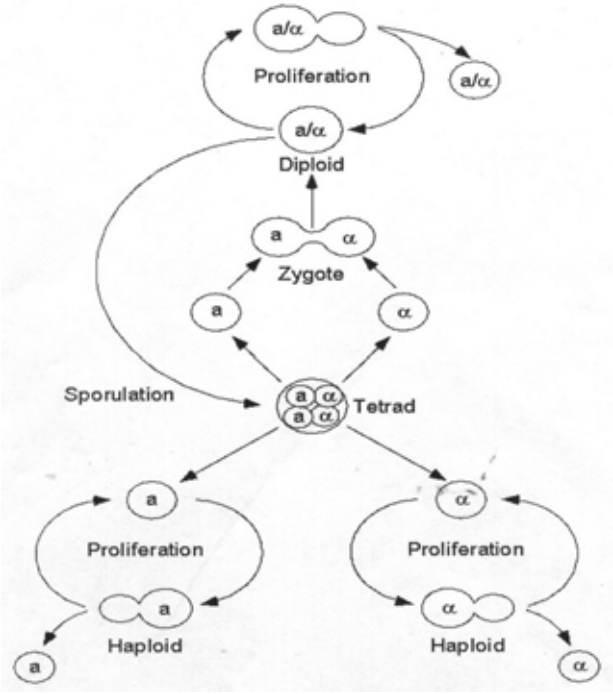


Fig 1.2: The life cycle of heterothallic *S. cerevisiae* strain (http://www.dbb.urmc.rochester.edu/labs/sherman_f/yeast13.html).

Brewer's yeast strains are polyploid and homothallic. They are directly related to laboratory strains of *S. cerevisiae,* in the sense that their homozygous diploid chromosomes conjugate together to form tetraploid of the same mating type (aa/αα and αα/aa) (Rose *et al*, 1995). Because of a lack of genetic mutations in reproduction genes and because of being homothallic, the brewer's yeast strains have poor sporulation ability.

The genome of most *S. cerevisiae* laboratory strains contains 16 haploid chromosomes with an approximate nuclear size of 13.5-Mb (Rose *et al*, 1995). The *S.carlsbergensis* genome has a large majority of the chromosomes found in *S.cerevisiae* reference strain but contains some different nucleotide sequences, different genes structures and some chromosomal regions being specific to *S.carlsbergensis* (Rose *et al*, 1995). *S. carlsbergensis* is an alloploid hybrid containing parts of a least two diverged genomes (*S. cerevisiae* and *S. monacensis*), which

various mutations have disturbed sexual reproduction resulting in poor mating ability and sporulation (Rose *et al*, 1995).

1.4.1 Life span of yeast

The budding yeast *S. cerevisiae* has a limited lifespan, in which each individual cell is capable of a limited number of divisions before it reaches senescence and leads to cell death (Barker and Smart, 1996). Termination of the brewing yeast lifespan due to exposure to physiological stress may lead to necrosis (the autolysis of the cell wall that causes the release of the cell's components and that leads to subsequent cell death) causing deterioration of cellular components. This deterioration may be reversible but prolonged stress exposure may cause permanent cellular damage to cellular components, promoting an irreversible loss of replicative activity (Smart, 1999).

The maximum number of divisions that a yeast cell can achieve is determined by both genetic and environmental factors, and by the maximum number of time the cell divides (Rodgers *et al*, 1999). Irreversible morphological and physiological changes have been observed with each generation time:

- An increase in bud scar numbers with each new daughter cell
- An increase in chitin deposition on the cell's surface
- An increase in cell size (20% by volume) and granular appearance
- Accumulation of surface wrinkles
- Eventual cell lysis

Chapter 2: Literature review

2.1 The brewing process

A process known as brewing produces European style beer. The two most important parts are wort preparation and fermentation.

2.1.1 Wort preparation:

Mashing is the process of converting starch from the milled malt into fermentable sugars released into the liquid extract, called wort. The malting floor is covered with about 25 cm of barley grains and sparged with water. During germination at a controlled temperature (15ºC) and oxygen concentration, the barley seed (embryo) is allowed to grow (Fig 2.1). The embryo secretes enzymes to degrade the protein and starch in the cell walls of the endosperm into amino acids (Hough, 1985). This initiates breakdown of the endosperm wall to release the principal enzymes converting starch and proteins into respective fermentable sugars and smaller peptides (Hough, 1985).

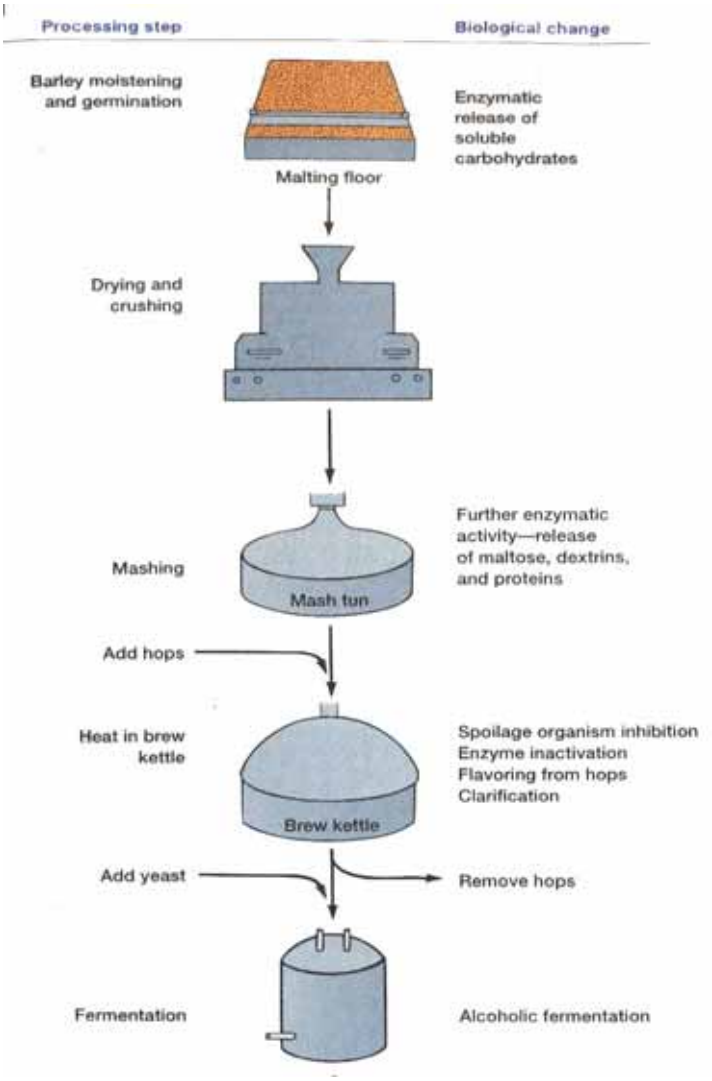


Fig 2.1: Summary of the brewing process (Prescott *et al*, 1996).

There are numerous enzymes present in germinating barley for the reduction of both proteins and carbohydrates. The two most important groups of enzymes are the proteolytic and starch degrading enzymes.

Proteolytic enzymes:

- Endopeptidases, which randomly cleave the chain of amino acids at any peptide linkage. Their activity increases up to 20-fold during germination (Hough, 1985).
- Exopeptidases including carboxypeptidases, cleave amino acids or simple proteins, and release amino acids at the free carboxyl end.

Starch-degrading enzymes:

There are two forms of starch present in barley grains, namely amylose and amylopectin, which are both glucose polymers (Fig 2.2). The glucose units of amylose are linked by α 1-4 linkages with only one reducing group at the end of the molecule (Hough, 1985). The amylopectin molecule is also linked by α 1-4 linkages, as well as by α 1-6 linkages at the branching points (Fig 2.3).

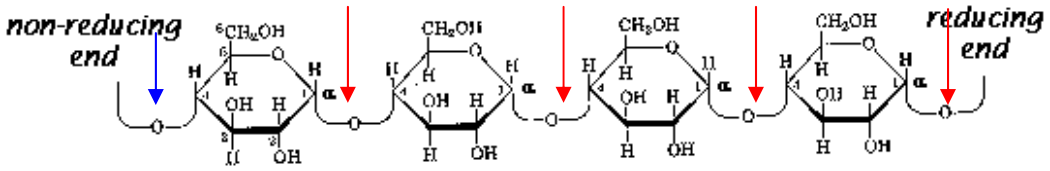


Fig 2.2: Structure of amylose, a polysaccharide of starch connected by α 1-4 linkages (Lenhinger *et al*, 1993). The arrow indicates where the amylase splits the bond.
→ ß amylase cleavage point, → α amylase cleavage point

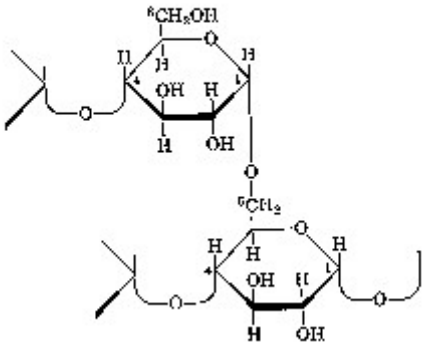


Fig 2.3: Structure of amylopectin, a polysaccharide of starch connected by α 1-4 linkages as well by α 1-6 linkages (Lenhinger *et al*, 1993).

The most important enzymes acting on starch are β- and α-amylase and debranching enzymes. The enzymatic action of β- and α-amylase is summarized in Table 2.1. Debranching enzymes attack α 1-4 links of amylopectin to give mixture of dextrins plus few sugars (Hough, 1985).

Table 2.1: Comparison of β- and α-amylase enzymes activities (Hough, 1985).

	α-AMYLASE	**β-AMYLASE**
1. Attack on starch chain	Randomly (except near chain ends and branch points); an endoenzyme	Cuts off maltose from non-reducing ends of molecules; an exoenzyme
2. Glycosidic link attached	α 1,4	α 1,4
3. Product of attack	Mainly dextrins, few sugars	β-maltose
4. Production of reducing groups	One per attack	One per attack
5. Production of non-reducing ends	One per attack	One per attack
6. General requirements	Calcium ions	Reducing conditions to maintain thiol groups
7. Inhibitors	Calcium sequestrants	Heavy metals and sodium iodoacetate
8. Optimum pH	5.5	5.2
9. Optimum temperature for most rapid actions	70	60
10. Presence before germination	Not present in mature barley grains, begins to form during germination	Present in mature barley grain but active enzyme increases during germination

The α-amylase is a metalloenzyme with an endo-action that randomly attacks starch molecules by hydrolysing α 1-4 links yielding shorter polysaccharide chains (Fig 2.2). When hydrolysing amylopectin, α-amylase produces a mixture of branched and unbranched starch molecules (dextrins). The β-amylase is a thiol-enzyme with an exo-action that attacks starch molecules at their non-reducing ends, and which is more selective towards dextrins and maltotriose by cleaving these substrates to produce the disaccharide maltose.

Once all the starch has been broken down, the liquid extract called wort is separated from the solids (spent grains and adjuncts) by filtration through a lauter tun and boiled in a wort kettle to deactivate the enzymes and kill any contaminants. At this stage, hops are added to improve the flavour of the beer. The liquid wort is clarify by filtration or passed through a whirlpool tank, cooled through a heat exchanger and aerated (Fig 2.1) (Young, 1996).

2.1.2 Fermentation of the wort

The composition of wort can exerts a significant effect on the yeast's fermentation performance and on the flavour of the beer. The basic wort constituents are:

(a) Fermentable carbohydrates: The disaccharides sucrose and maltose, the trisaccharide maltotriose and the monosaccharide glucose and fructose, together with unfermentable dextrin material, account for 90-92% of the wort solids (Amore *et* al, 1989). In the early stages of fermentation, the α 1-4 glycosidic bond of sucrose is split into the monosaccharide D-glucose and D-fructose by the action of the yeast periplasmic enzyme invertase and transported across the cell membrane (Amore *et al,* 1989). Maltose and maltotriose are taken up and enzymatically hydrolyzed within the cell to glucose as main fermentable sugars (Hough, 1985). The principle of high-gravity brewing is to increase the concentration of fermentable sugars in the wort, by the addition of adjuncts such as maize, honey, molasses or granulated sugar (Thomas *et al*, 1996). There are few advantages to high-gravity brewing: (1) increased plant efficiency and reduced energy, (2) reduced labour and capital costs, (3) increase in ethanol yields per unit of fermentable extract (Reilly *et al*, 2004). The original sugar concentration present in the wort is referred to as the original specific gravity (OG) and increases proportionally to the wort extract (the total amount of dissolved materials in wort after mashing and lautering malted barley and adjuncts). The wort gravity is often measured by the specific gravity in Degrees Plato (°P), which is a measurement of the wort extract as a percentage of sucrose in 100 grams solution at a temperature of 20°C and measured by a saccharometer or density meter. A normal gravity wort contains 11-12°P of dissolved solids (fermented to approximately 4% ethanol) compared to high-gravity wort containing 13-16°P (fermented to 7-8% ethanol) (Thomas *et al*, 1996). The final gravity (FG) indicates the end of fermentation where most sugars have been converted, and which corresponds to an average of 1/5 of the OG ((http://www.howtobrew.com/appendices/appendixA.html).

(b) Total nitrogen: Nitrogenous material in wort represent up to 4-5% of the total solids and includes compounds like ammonia, simple amines, amino acids, complex proteins and vitamins (MacWilliams, 1968). The nitrogen content (free amino acids or FAN) coming from the malt are identified mostly as alpha-amino acids and are used by yeast to synthesize new cellular and enzymatic proteins, stimulate growth and to

achieve maximum fermentation rates (Pierce, 1987). The yeast requires approximately 150 mg/l of soluble nitrogen for effective fermentation. If high amounts of cereal or sugar adjuncts are used, additional nitrogen can be added to the wort in the form of ammonium salts http://www.users.zetnet.co.uk/dpichett/docs/cba/cba3/hi3p3.htm

(c) Mineral salts including calcium, magnesium, sodium, potassium, iron, zinc, copper and manganese, chloride, sulphate, carbonate and inorganic phosphate and vitamins such as biotin, pantothenic acid, ionisotol, thiamin, pyridoxine and nicotinic acid are found in wort (Hough, 1985). Copper and zinc are mostly required to support the growth of brewer's yeast, used as co-factors in many enzymes reactions.

(d) Sterols and unsaturated fatty acids including esters, hydrocarbons and glycerides are required for membrane synthesis during yeast growth.

(e) Hops: The female flower of the hop plant (*Humulus lupulus*) has long been used in brewing of beer to which is added to the wort in the kettle just before boiling (http://www.stpats.com/hopsinfo.htm). The four main functions of hops are: (1) to contribute bitterness that is directly related to the alpha acids content through isomerization into more stable and soluble iso-alpha acids (2) to supply tannins that combine with unwanted proteins and (3) to lower the surface tension of the wort to allow longer boiling times (http://www.realbeer.com/hops/aroma.html). The bitterness consists of three main classes: (1) the iso-compounds derived from the resin components of the hops, (2) the hops oils and (3) esters and acids, which contribute to the total acids of wort (MacWilliams, 1968). The iso-compounds mainly consist of isohumulones, hulupones and unchanged humulones. The concentration of hulupones is low (less than 2 µg/l), while the humulones concentration varies depending on the hop boiling process (MacWilliams, 1968). There are 2 classes of hops oils differentiated by their volatility: the fixed oils are nonvolatile and do not contribute much to the aroma, and the volatile oils, making up 0.5-3% of the plant, that contribute to a large extent to the "nose" of the beer (http://www.realbeer.com/hops/aroma.html).

2.1.2.1 Wort conditioning and aeration

The resulting clear wort must be conditioned by boiling in a kettle at 100°C for minimum of 45 min to maximize hops extraction, protein precipitation, and antimicrobial activity. Following wort clarification through filtering, the wort is cooled down prior to the addition of the yeast. The wort is cooled through plate heat exchangers down to the fermentation temperature of approximately 14°C. Aeration of the wort is required to supply adequate amount of oxygen to the growth of the yeast during the early stages of fermentation. The typical oxygen requirement in high-gravity brewing ranges from 12 to 16 mg/ml, compared to 8 mg/ml in normal gravity wort.

2.1.2.2 Fermentation

The fermentation process consists of the conversion of the wort sugars into carbon dioxide and ethanol by a specific yeast strain. Two types of fermenting yeasts are used: Top fermenting yeast (*S. uvarum*), rise to the top of the vessel during fermentation at temperatures of 15-22ºC and produce ales beers. Bottom fermenting yeasts (*S. cerevisiae)*; settle to the bottom of the vessel during fermentation at temperatures of 8-15ºC and produce lager beers. The amount of yeast pitched into the cooled wort depends on the starting sugar concentration (gravity) of the wort. During the initial phase of fermentation, the yeast uses the oxygen present in the wort to convert their internal carbohydrate reserves into sterols and fatty acids required for cell growth. Once the oxygen had been depleted, the yeast in turn grow anaerobically by converting the wort sugars into ethanol, carbon dioxide and various alcohol by-products that result into flavour beer compounds (Hough, 1985). When attenuation is reached (all fermentable carbohydrates are utilised) as indicated by a drop in specific gravity, the fermentation vessel is cooled to stop yeast metabolism to allow the yeast to sediment to the bottom of vessel before being removed for storage and subsequent inoculation. A typical yeast fermentation cycle is illustrated in Fig 2.4. Yeast in the fermenter is removed from the bottom of the cone by a cropping pump to be temporarily stored in the cropping vessel. At the time of pitching, the yeast is transferred to the cooling vessel, from which the yeast is progressively pumped to a new fermenter, and simultaneously added together to the wort.

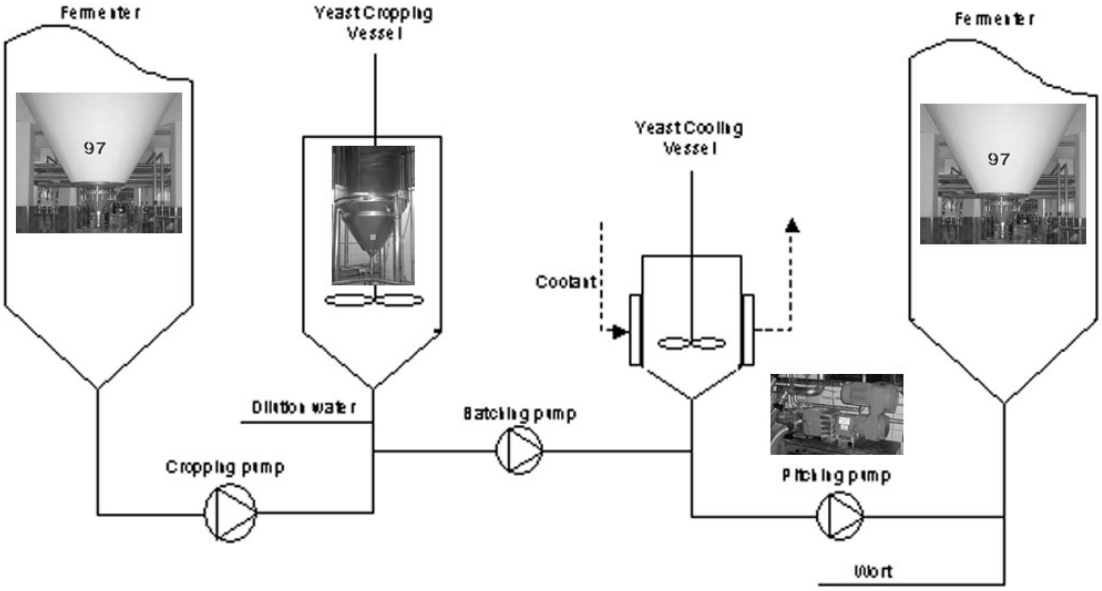


Fig 2.4: The fermentation process from the fermentation tank, removal of cells and particulate matter and re-pitching of a fermentation vessel (personal communication, STL Harrison).

2.2 Fermentation metabolism

Following inoculation of the yeast into the aerobic wort, the yeast grow and reproduce very rapidly by absorbing most of the wort sugars and other components like nitrogenous material and mineral salts (Hough, 1985). Most of these substances diffuse freely through the yeast plasma membrane by simple (e.g. organic acids) or catalyzed diffusion (e.g. sugars). Other components like amino acids are taken up by active transport in the expense of ATP (Hough, 1985). During this active phase, the yeast metabolize glucose and fructose within the cell, followed by maltose and maltotriose, to produce an ample amount of glucose substrates for the initiation of glycolysis as an energy requirement for cell's growth and reproduction. The glycolytic pathway is represented in Fig 2.5. The net output of glycolysis per glucose molecule used is two ATP molecules with the production of two pyruvate molecules (Lenhinger *et al*, 1993).

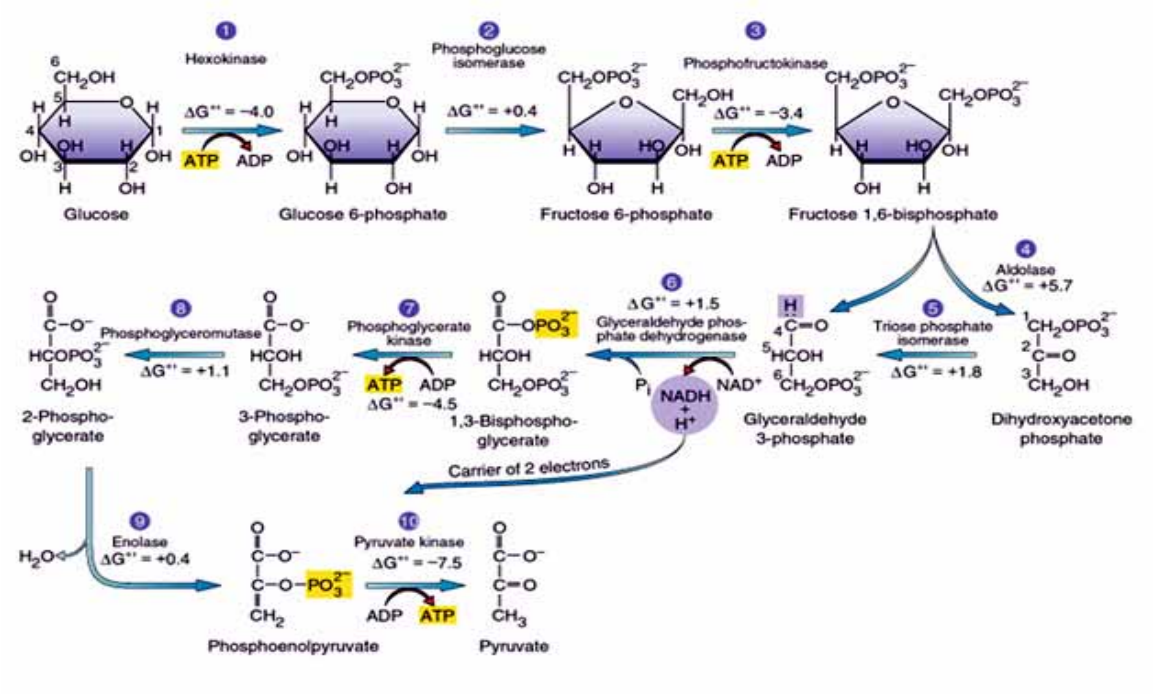


Fig 2.5: Glycolysis pathway

(http://www.fig.cox.miami.edu/cmallery/255/255atp/glycolysis_pathway.jpg).

During the aerobic / lag phase, the yeast uses all the available oxygen to break down wort sugars and stored glycogen into glucose for the cell's reproduction. Once all the oxygen is depleted, the yeast cell switches to an anaerobic process in which the majority of the wort sugars are converted to ethanol and carbon dioxide (Fig 2.6).

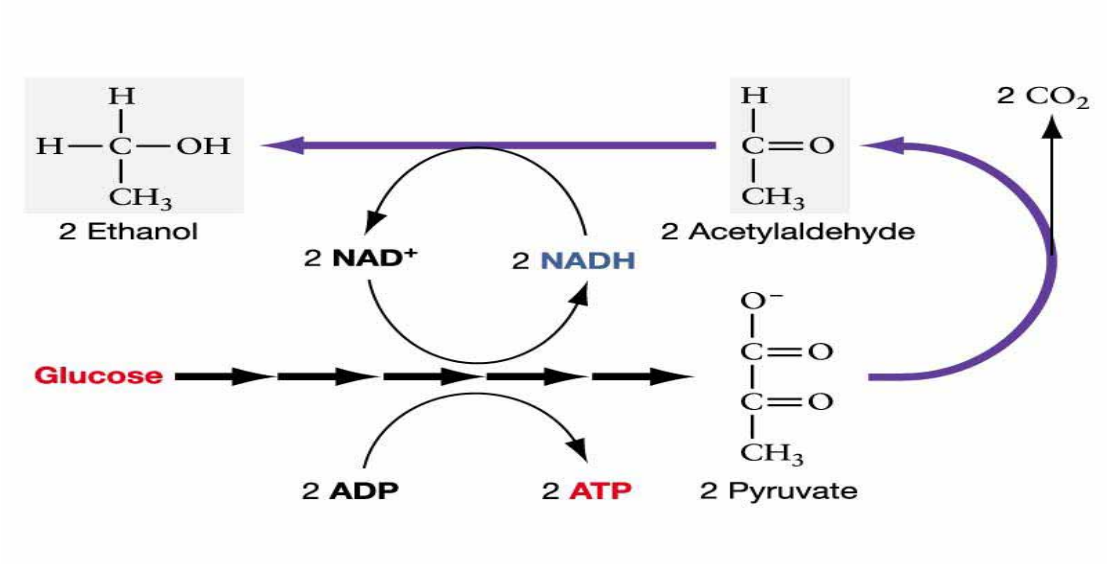


Fig 2.6: Fermentation reaction for the production of ethanol and carbon dioxide.

This phase is also characterized by a significant drop in pH during the active phase of fermentation due to the formation of some acids, with the yeast in suspension growing exponentially till attenuation (the time at which all assimilable carbon sources have been utilized in fermentation) is reached. Once most of the wort sugars have been utilized, the yeast cells enter a stationary phase in which a spontaneous aggregation is triggered, causing sedimentation and subsequent flocculation of the yeast cells to the bottom of the vessel (Bowen and Ventham, 1994).

2.3 Formation of esters during brewing

Ester synthesis during brewing fermentation is important for the contribution of the finished product's aroma and flavour (Bardi *et al*, 1993). Esters are synthesized within the yeast cells and depending on their chain length, they diffuse into the surrounding medium (Peddie, 1990). The most valuable esters are ethyl acetate, isoamyl acetate, isobutyl acetate and β-phenylethyl acetate (Stewart *et al*, 1999). Esters are formed via a biochemical pathway involving the compound acetyl-CoA playing a central role in many other biosynthetic reactions (fatty acids, amino acids and TCA cycle) (Peddie, 1990).

There are several reasons why ester production is beneficial to the yeast cell (Stewart *et al*, 1999):

- In the regulation of the acetyl charge relative to the cellular concentrations of acetyl-CoA and free CoA.
- As a detoxification process because fatty acids with chain length between C8 and C14 are toxic to the cell.

The enzymes hydrolyzing these esters are the yeast carboxyl esterases, which are more important during high-gravity brewing to limit the ester concentrations during alcoholic fermentation (Bardi *et al*, 1993). Esterase activity has been located in the vacuoles of the yeast cells demonstrating hydrolysis activity towards *p*-nitrophenyl acetate (Schermers *et al*, 1976). Another esterase enzyme, called alcohol acetyl

transferase (AAT) was located in or near the cell envelope and 77% of the total esterase activity towards β-naphthyl acetate was found in the cell wall digest.

2.4 Yeast sugar uptake

It has been observed that the uptake of usable wort carbohydrates by the yeast is an important factor to consider when evaluating the fermentation performance and the final beer quality (Phaweni *et al*, 1992). The different assimilable wort sugars are the monosaccharide glucose and fructose, the disaccharides sucrose and maltose and the trisaccharide maltotriose. Among the main fermentable sugars, maltose represents about 70% of the total fermentable sugars (Fukui *et al*, 1989). During wort fermentation, sucrose is first removed by hydrolysis with the invertase enzyme located in the periplasmic space and the resulting glucose is taken up by facilitated diffusion through multiple hexose transporters (Meneses *et al*, 2002). Glucose and fructose are assimilated for yeast growth early during the fermentation by facilitated diffusion similar as the sucrose uptake. Once the growth of yeast cells stop, maltose and maltotriose are consumed for the conversion into ethanol since maltose uptake is repressed by the presence of the monosaccharide glucose during the growth phase (Meneses *et al*, 2002). This late assimilation of maltose and maltotriose causes a delay in attenuation, with high osmotic pressure and ethanol levels that increase the risks of poor fermentation efficiency towards the end of fermentation. The uptake transport for maltose and maltotriose is an active proton symport transport system at the expense of metabolic energy by the cell, mediated by the enzyme permease across the cell membrane into the cell (Fukui *et al*, 1989). Once inside the cell, maltose and maltotriose are hydrolyzed to glucose by the maltase enzyme or namely α-glucosidase enzyme.

As mentioned earlier, high levels of glucose repress maltose utilization which is known to be a common problem in high-gravity brewing wort when using adjuncts of dextrose and high-maltose syrup. This gives rise to lower fermentation efficiency and a final beer product with a high residual maltose concentration (Phaweni *et al*, 1992).

2.5 Yeast handling processes

2.5.1 Cropping

Once the final gravity and optimum cell count at the end of fermentation are achieved, the non-growing yeast slowly flocculate at the bottom of the fermenter, while the yeast in suspension are forced to flocculate by a temperature decrease and are "cropped" subsequently at the end of fermentation. The yeasts in suspension contribute to the developing beer flavours until the final beer is centrifuged to remove all yeast and transferred by a commonly known process called "racking", into a storage vessel. The racking process is accompanied by a sharp decrease in temperature to reach a storage temperature between 0°C and 2°C (O'Connor Cox, 1997). The time of cropping is critical to the yeast quality / performance since environmental conditions at the bottom of the fermenter cone are mostly unfavourable to the yeast's vitality (measure of the yeast cell activity) such as high cell densities, nutrients deficiency, high ethanol concentrations, high carbon dioxide concentrations, temperature rises and variable hydrostatic pressures (O'Connor Cox, 1997). All of these factors promote a decline in cell viability, and a progressive degradation of their energy reserves like glycogen. These cropping conditions would result in pitching yeast with poor vitality for the initial sugar uptake in the next fermentation, and resulting in a poor fermentation performance.

2.5.2 Storage

In most breweries, yeast is stored for a maximum time of 24h in the period between cropping and re-pitching (Boulton, 1991). It is critical at the time of storage to maintain the optimum physiological condition of the yeast. Storage conditions are optimal when the yeast's metabolic activity is reduced to a minimum and kept constant to prevent any yeast deterioration in terms of viability and storage reserves degradation (O'Connor Cox, 1997). The cropping yeast in the form of slurry is immediately chilled down to temperatures between 0 and 4°C during the transfer between the fermenter to the yeast collection vessel. By lowering the vessel temperature, the yeast's metabolism is slowed down to a minimum to provide a better

resistance of the cell to the extreme ethanol conditions present in the slurry (O'Connor Cox, 1997). The larger percentage of the yeast population is in a non-growing stationary phase which is known to be more resistant to adverse stress conditions, in particularly to cold shock and ethanol stress. Growing active cells are more susceptible to temperature fluctuations (O'Connor Cox, 1997). The most important storage parameters to consider prior to maintaining maximum fermentation performance for subsequent fermentations are the preservation of the energy carbohydrate glycogen concentrations (can start to synthesize lipids for growth, which would lower the next fermentation performance). This preservation is performed by maintaining low temperatures, together with a minimum oxygen exposure to prevent any further biochemical reactions (Sall *et al*, 1988).

2.5.3 Serial repitching

The physiological state of the pitching yeast population is considered to have a large effect on the resulting beer composition and flavour (Edelen *et al*, 1996). During serial re-pitching, the physiological state varies as a result of the different stress conditions that the yeast are exposed to, such as transfer from the active propagation through early stages of fermentation to stationary phases, and finally to storage (Jenkins *et al*, 1999).

The optimization of the pitching rate is crucial to obtain consistent fermentation performance with the same number of viable yeast cells for each pitching (O'Connor Cox, 1998). The recommended pitching rate is one million viable cells per degree Plato per ml of wort (O'Connor Cox, 1997). For high-gravity brewing, the pitching rate accordingly increases to the original wort gravity but certain nutritional and environmental factors need to be optimized. To ferment the additional sugars in wort in the same period of time, the oxygen concentration has to be proportionally regulated since sterol synthesis during yeast growth is directly related to the oxygen availability (Boulton, 1991). At the same time, the levels of usable nitrogen for initial yeast growth are equally important to achieve an optimum fermentation rate.

Direct methods to determine the pitching rate on the basis of yeast counts and viability have been developed. In many breweries, the consistency of the slurry is

determined which automatically accounts for the cell's viability. More accurate but labour intensive methods are conducted by microscopic counting with a haemocytometer and by the methylene blue viability stain. The latter method is inaccurate for viabilities lower than 95% and the viability of the yeast slurry is overestimated (O'Connor Cox *et al*, 1997). A more precise method has been developed to perform accurate and rapid yeast enumeration, called the radio-frequency impendance method (Boulton *et al,* 1999). The principle of this particular method is that viable cells exclude the fluorescent dye $DiBAC_4$, contrary to dead cells that take up the dye and become fluorescent when excited with light of a suitable wavelength.

2.6 Brewing-yeast population profiles

As sugars are exhausted and when attenuation is reached, larger and consequently older cells settle faster in the lower regions of the cylindrical cone. This automatically forms an age gradient (Fig 2.8). Therefore careful selection of a yeast population is required, comprising a good balance of age for optimum fermentation performance when yeast is repitched. Fermentative abilities between different cell ages have been analyzed in the initial stages of fermentation, given that a typical laboratory grown batch culture comprises approximately 50% virgin cells, 25% first-generation cells and 12.5% second-generation cells:

- Newly budded virgin cells in the upper part of the cone (Fig 2.8 fraction A) display a much longer lag phase before they attain the appropriate size to start dividing and increasing biomass. They are slower in converting sugars, and result in a delay in the onset of fermentation (Powell *et al*, 2003).
- Middle-aged mother cells (Fig 2.8 fraction B & C) present in the second part of the cone start to divide more rapidly, thereby reducing the fermentation lag-time, achieving a greater fermentation rate and reaching attenuation faster. This centre-top portion contains the most active yeast cells and is generally removed for subsequent re-pitching. The cells entering the stationary phase at onset of stress

conditions experienced by the high ethanol level at the end of fermentation will be more resistant to the cold stress prior to storage (Powell *et al*, 2003).

- The older, larger aged cells and dead cells accumulate and settle in lower regions of the fermentation cone (Fig 2.8 fraction D). They are poor fermenters and are discarded as waste with protein debris (trub) (Powell *et al*, 2000).

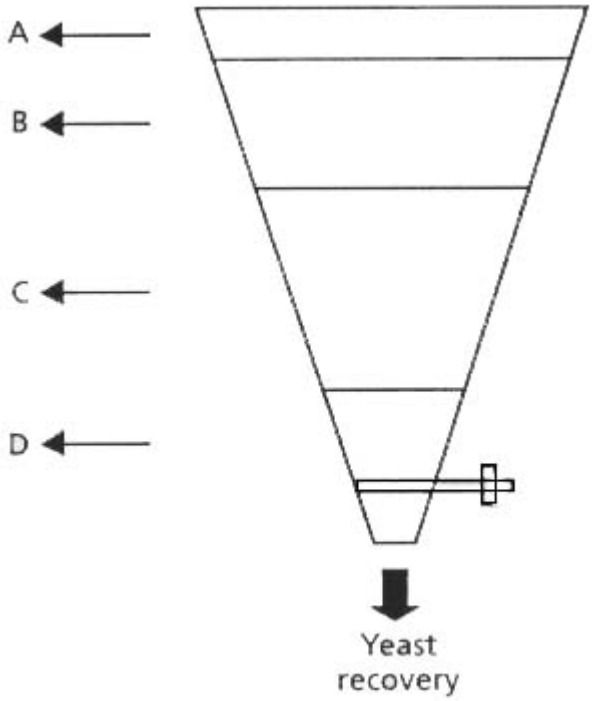


Fig 2.8: Stratification of yeast during sedimentation in the fermenter cone. (A) Youngest cell fraction, (B) the predominantly aged fraction, (C) middle-aged mother cells, and (D) yeast removed from the vessel during cropping (Powell *et al*, 2000).

2.7 Assessment of yeast quality in brewing

The effect of the physiological condition of the pitching yeast on subsequent fermentation performance and on the final beer quality is considerable (Kara *et al*, 1987). The quality of yeast is defined in terms of viability / vitality. Yeast viability is a measure of the number of dead or living cell in a yeast cells population, whereas yeast vitality is a measure of the yeast cell activity, or its physiological status to endure stress and still be able to perform (Imai *et al*, 1999).

The use of a reliable vitality "marker" as an indicator of yeast quality for the prediction of subsequent fermentation performance is an ideal information that has been difficult to achieve (Mochaba *et al*, 1998). The most appropriate vitality test performed as a routine test should be rapid, simple, and inexpensive to be used readily with available laboratory equipment (Boulton and Quain, 2001). In the course of this thesis, various methods to evaluate the yeast vitality shall be discussed. These methods have been largely based on using vital stains and macroscopic counts to measure the yeast viability based on cell replication. However, vital stains are often unreliable and plate counts on solid media are more accurate than staining methods but are too slow to be of practical use in the control of brewing fermentations (O'Connor Cox *et al*, 1997).

2.8 Stress factors imposed on yeast during brewing

2.8.1 Introduction

A number of factors involved present in high-gravity brewing that may impose stress on yeast and affect the yeast's fermentation performance (Smart, 1999):

- Wort composition: Yeast are subjected to higher osmotic pressure together with lower water activity, especially at the beginning of fermentation, due to the increased sugar concentrations with the addition of different adjuncts, which have an inhibitory effect on yeast growth. An optimum growth water activity for growth rate lies around 0.997-0.998 and falls below 0.995 once the yeast has been transferred to an unfavorable physiological environment such as the fermentation wort (Jakobsen *et al*, 1989).
- Nutritional deficiencies: An oxygen and nitrogen limitation in high-gravity wort is a common problem. As a result of a higher pitching rate, there is an increase in sterols and unsaturated fatty acid concentrations that are required for a faster yeast growth at the beginning of fermentation (Casey *et al*, 1984).

- Ethanol toxicity: Elevated ethanol concentrations up to 7-8% have a proportional inhibitory effect on both cell growth and viability.

All of these factors together with hydrostatic pressure and CO_2 are important in causing sluggish or slow fermentations due to poor yeast viability. A number of other stresses imposed during the handling of yeast are described in Table 2.2.

Table 2.2: Stresses that may lead to necrosis during brewery handling (Smart, 1999).

FERMENTATION	CROPPING AND TRANSFER	STORAGE VESSEL	ACID WASHING	PITCHING
• Ethanol • Starvation • Lethal DNA damage (suicide mutations) • Hydrostatic pressure • CO_2	• Cold shock • Shear • Anaerobiosis	• Shear • Cold shock • Starvation • Anaerobiosis	• pH • Cold shock	• Osmotic • Free radicals

2.8.2 Temperature shocks

When yeast cells were exposed to elevated temperatures (between 35°C and 45°C for at least 45 min), they produce an adaptive physiological response to counteract the adverse stress effect by decreasing the membrane fluidity and by changing their fatty acid composition (Amore, 1992). During continuous exposure to heat stress, the cell will trigger a protective response to acquire thermotolerance, by the production and intracellular accumulation of trehalose and heat shock proteins (Arguelles, 1994). Induced thermotolerance can be briefly explained by the following protective actions of particular cell metabolites such as:

(a) Heat shock proteins: It has been found that under severe heat stress, trehalose synthesis is accompanied by the induction of heat shock proteins. In particularly, the induction of Hsp30p, a hydrophobic integral membrane protein, is triggered in association with the yeast plasma membrane (Piper, 1995). Heat shock proteins act in synergy with trehalose by stabilizing membrane proteins, and function as molecular

chaperones through their binding to proteins in order to enhance proper protein refolding and inhibiting protein aggregation (Fig 2.9) (Brosnan, *et al*, 2000).

(b) Trehalose: a non-reducing disaccharide composed of two α- linked glucose units, whose concentration increases as part of the cellular response to elevated temperatures as well as to high ethanol concentrations. Trehalose protects the cell from heat by preventing the denaturation of native proteins and replacing water binding to the polar head groups of phospholipids, thereby preserving the properties of a hydrated membrane (Fig 2.10) (Arguelles, 1994).

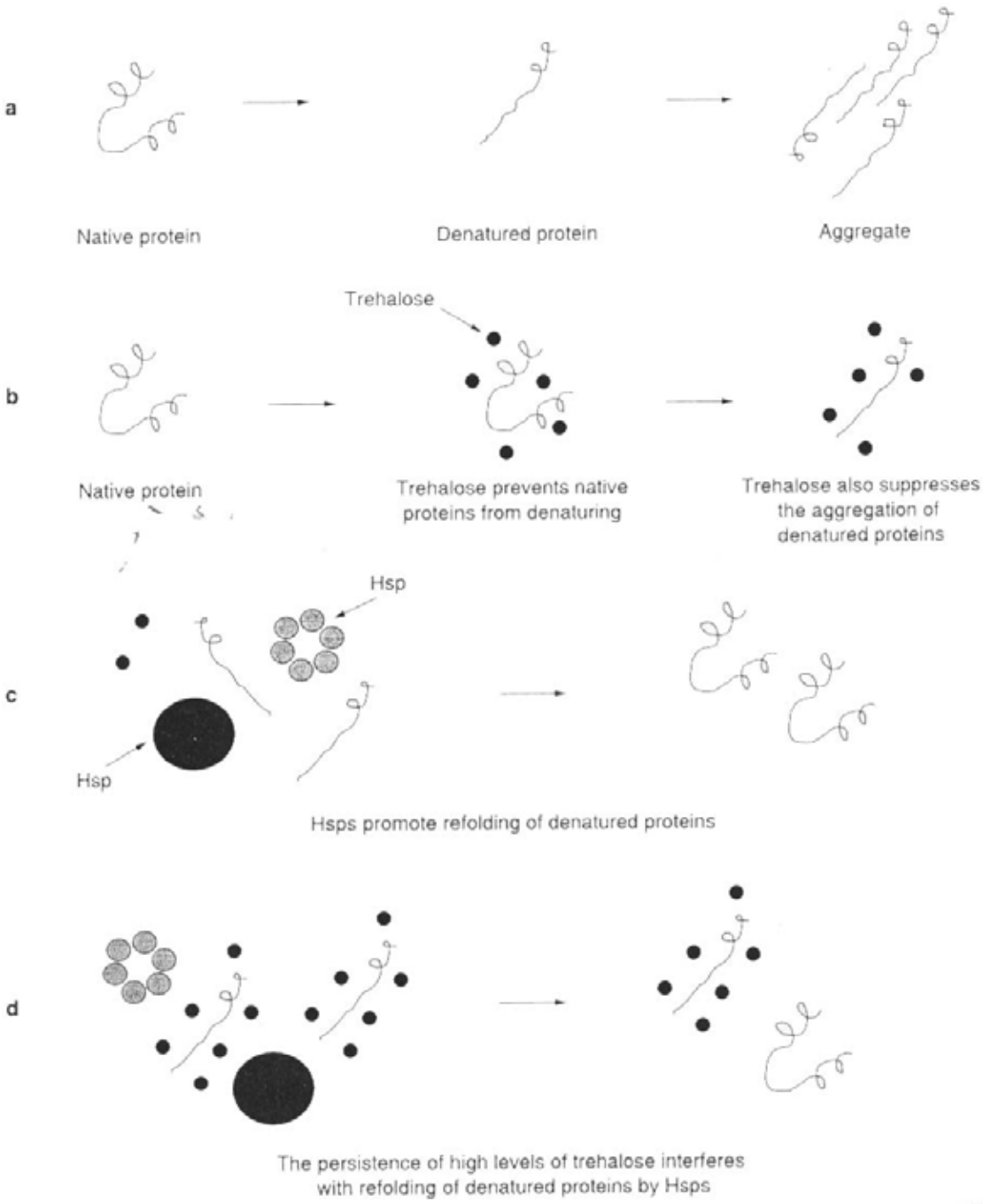


Fig 2.9: Heat protein denaturation re-folding mechanism by heat shock proteins and trehalose. (a) unfolded proteins aggregate, (b) trehalose synthesis, (c) molecular chaperones prevent aggregation and (d) re-folding of denatured proteins by Hsps. (Singer and Lindquist, 1998).

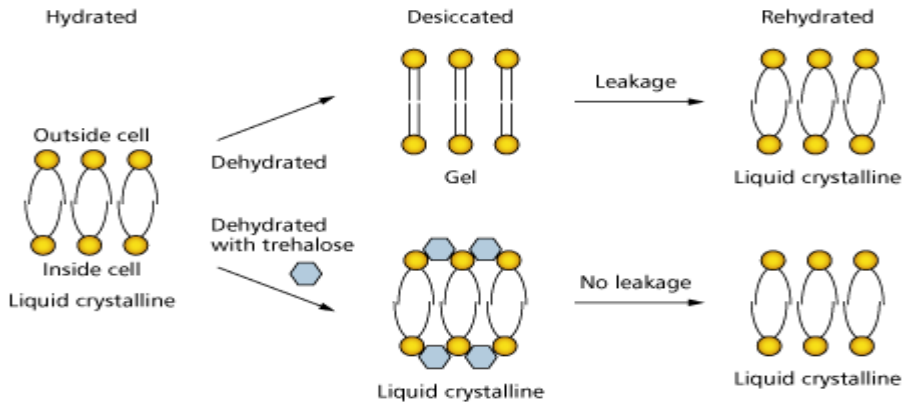


Fig 2.10: Trehalose protective mechanism towards membrane integrity (http://www.chemsoc.org/chembytes/ezine/images/2002/wharton_oct2_img2.gif).

2.8.2.1 Plasma membrane damages

An increase in membrane fluidity in response to elevated temperature occurs through the denaturation of membrane proteins (peripheral and integral) and alterations in the membrane lipid saturation. Furthermore, when yeast cells are exposed to sub-lethal heat stress, the activity of the plasma membrane H^+-ATPase is increased to maintain the electrochemical gradient across the membrane and enhances the proton efflux (Piper, 1995). As a result of this struggle to maintain homeostasis, the increased proton motive force results in an increased demand in energy to survive the heat stress.

2.8.2.2 Heat and ethanol synergy

The major damages caused by heat stress are often enhanced by the presence of ethanol levels above 3% (Piper, 1995). Both heat and ethanol stresses cause an increase in membrane lipid fluidity and denaturation of membrane proteins. High concentrations of ethanol in particularly, trigger the heat shock response of the yeast through the synthesis of very similar protective molecules like heat shock proteins and trehalose. This synergy results in a more severe damage by ethanol at higher temperatures but an ethanol pre-exposure leads to an increased thermotolerance (Piper, 1995).

2.8.2.3 Heat stress-induced gene expression

When yeast cells are exposed to elevated temperatures (37-45°C), the yeast initiates a heat shock response by elevated transcription of their heat shock genes to prevent maximum damage to their cellular components, including protein denaturation and aggregation and membrane damage (Hohmann, 1997). However, a brief initial exposure to severe temperature promotes yeast cells to develop thermotolerance. Through the initial heat shock response, the yeast increases the level of tolerance towards other types of stresses like oxidative or osmotic stress.

In response to heat stress, two regulatory elements are responsible for the induction of heat shock genes: STRE and HSE (heat shock element). The HSE binding sites are composed of at least three copies of the 5 bp repeating sequence nGAAn in alternating orientation, and are the binding sites for the *trans*-acting transcription factor HSF (heat shock factor) (Sorger, 1991). This HSF factor is encoded by the single *HSF1* gene that contains helical DNA-binding domain and a coiled hydrophobic repeat domain that regulates trimerisation of Hsf1p required for DNA association (Estruch, 2000). It has been demonstrated that Hsf1p is bound to HSE both in the absence and the presence of heat shock. Under non-shock conditions (normal growth), Hsf1p is present as a trimer in high concentrations in an inactivated conformation through the interaction of the heat shock protein Hsp70p. In response to heat shock, Hsf1p activation of gene transcription can be described in two steps:

(1) Induction of DNA binding: the chromatin structure of inactivated hsf1p is altered in such a way to facilitate HSF binding to HSE promoters (containing STRE triggered by Msn2p and Msn4p binding) (Sorger, 1991).
(2) Rapid phosphorylation of Hsf1p upon heat shock leading to transcription. The extent of phosphorylation is proportional to the temperature range (Mager and Ferreira, 1993).

2.8.3 Responses of yeast to cold stress

At the end of the fermentation process, flocculated yeast is pumped from the cone of the fermentation vessel through a plate and frame heat exchanger in a single pump motion as quickly as possible down to the lowest temperature to keep yeast metabolism to its absolute minimum (O'Connor Cox, 1998). To minimize the "cold shock" effects by this rapid chilling action, the yeast slurry in a non-growing state is consequently more resistant to mechanical stresses resulting from the heat exchanger. An important aspect in the yeast handling process is to keep the entire crop at a constant low temperature between 2 and 4°C sequentially to maintain the yeast in the same physiological condition. A temperature increase would cause a deterioration of the yeast's reserve carbohydrates (O'Connor Cox, 1998). Rapid cooling that leads to lower external temperature to the internal cell solutes exerts a hyperosmotic stress from a rapid loss of intracellular water (Dumont *et al*, 2003). The cell becomes dehydrated as a result of water diffusing from the cytoplasm into more concentrated external medium, whose effect increases as the temperature decreases (Dumont *et al*, 2004). Determination of the cooling rate is a strong factor to consider because rapid cooling rates can be detrimental to cell viability and cause rupture of the plasma membrane due to differences in hydrogen ions across the plasma membrane in order to maintain turgor pressure and allowing the water outflow. On the other hand, slow cooling rates are more appropriate because intracellular water can flow out of the cell more freely and has more time to establish an osmotic gradient across the plasma membrane (Dumont *et al*, 2004).

2.8.4 Ethanol stress

During high-gravity fermentation, brewer's yeast develops ethanol tolerance mechanisms due to the high concentrations of sugar substrates yielding high ethanol levels at the end of the fermentation. Ethanol toxicity eventually reduces growth and fermentation rates, resulting in a loss of viability (Piper, 1995). Heat-induced and ethanol-induced damages are quite similar and work in combination in such a way that ethanol damages are more severe at higher temperatures (Piper, 1995).

The conversion of sugars to ethanol results in a sharp reduction in water activity (a_w) of the wort medium. This causes a water stress associated with the disruption of the hydrogen bonds of hydrated cell components such as glycolytic enzymes, lipid bilayers and membrane-associated proteins (Hallsworth, 1998). The small, hydrophilic ethanol molecule enters the hydrated lipid bilayer of the plasma membrane, disrupts the hydrogen bonding network of membrane lipids, and lowers the transition temperature, finally resulting in an increase in membrane fluidity that may cause cell lysis (Hallsworth, 1998).

The production of compatible solutes such as glycerol and trehalose has a vital role for the adaptation of yeast cells to ethanol stress. Glycerol is important for the adaptation of yeast cells to low a_w and its synthesis is stimulated by the presence of high sugar concentrations that initiate hyperosmotic conditions. Trehalose on the other hand, reduces membrane permeability in the presence of ethanol to maintain the function and integrity of enzymes and lipid bilayers (Hallsworth, 1998).

2.8.4.1 Responses of yeast to ethanol and water stress

There exist a strong attraction between "free water" in contact with solute molecules and extracellular water, which is more polar. The strength of this attraction determines how available water is to the cell, named "water availability" (Hallsworth, 1998). Ethanol being a hydrophilic molecule is small enough to enter the hydration layer of membranes by disrupting the lipid-lipid interactions. This affects the hydrogen-bonded water network, resulting in increased membrane fluidity and cell lysis (Hallsworth, 1998). The plasma membrane is the main target of ethanol and is known to damage membrane-associated cell structures like the lipid bilayers, membrane-associated proteins and glycolytic enzymes. As ethanol concentration increases, there is a severe reduction in water availability, affecting protein conformation that can lead to the inhibition of enzymes activity (e.g.: glycolytic enzymes), disruption of vacuolar membranes and release of proteases into the cytoplasm. Concentrations more than 5% ethanol cause disruption of the hydrophilic regions of cell components and inhibit yeast growth by reducing a_w (Hallsworth, 1998).

2.8.4.2 Plasma membrane damage

The plasma membrane is the primary target of ethanol toxicity, resulting in an altered membrane organization and permeability. Ethanol with heat stress is together toxic in a synergistic way that helps in the denaturation of membrane proteins (Alexandre *et al*, 1993). The following changes occur:

- Dissipation of the electrochemical gradient maintained across the plasma membrane by H^+-ATPase activity, which is responsible for the active uptake of glucose, maltose, ammonium and amino acids, as well as for the leakage of potassium, amino acids and nucleotides into the extracellular medium and the regulation of the intracellular pH (Piper, 1995). Exposure to sub-lethal heat shock and ethanol levels stimulates the activation of H^+-ATPase and causes an enhanced proton efflux. A decreased proton motive force also occurs that decreases intracellular pH and eventually results in an inhibition of fermentation rate (Alexandre *et al*, 1993).
- Changes in the lipid composition: A decrease in the unsaturation of the fatty acids of phospholipids that interferes with the tight hydrophobic interior of the membrane bilayers and results in less stable aggregates is the primary response to ethanol tolerance. Furthermore, sterol synthesis is reduced and alterations in the sterol type present in the cell modify the membrane fluidity (Alexandre *et al*, 1993).

Other possible target sites for ethanol toxicity are shown in Fig 2.11. Other inhibitory mechanisms of ethanol have been proposed, including denaturation and inhibition of glycolytic enzymes and inhibition of the transport of glucose, maltose, ammonium ions and amino acids (Stewart *et al*, 1988).

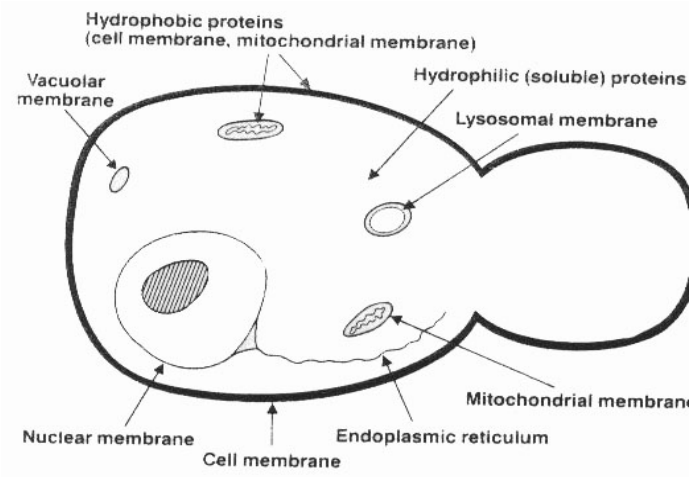


Fig 2.11: Possible targets of ethanol toxicity in yeast cells (Stewart *et al*, 1988).

2.8.4.3 Production of compatible solutes

Under water stress, the cell synthesizes compounds that protect the structure and functions of hydrated cell components (Hallsworth, 1998). The two most efficient compounds are glycerol and trehalose which both act as membrane protectants during water stress (Sharma, 1997). Glycerol yield is generally proportional to ethanol yield and as a result of a reduced a_w acting as a protective effect against a water stress (Jones and Greenfield, 1987). Trehalose has a protective effect on lipid bilayers, membrane-associated proteins and hydrophilic enzymes by replacing water to the dehydrated cell components like membranes.

2.8.4.4 Effect of ethanol on gene expression

It has been shown that 6% ethanol induces stress response element sequences that are also involved in the heat shock response (all part of the general stress response). Genes that are up-regulated by ethanol are mainly involved in energetic metabolism, protein destination, ionic homeostasis, and the stress response, all as part of the environmental stress response (Alexander *et al*, 2001). There is strong support for the conclusion that the production of trehalose and heat shock proteins are interlinked, since a group of highly responsive heat shock proteins, together with genes involved in trehalose synthesis are both being induced during ethanol stress, confirming their

role in protecting against protein denaturation and aggregation (Alexander *et al*, 2001).

2.8.5 Osmotic stress

Osmotic pressure is defined as the force present when two solutions of different molar concentrations are separated by a semi-permeable membrane (Hammond *et al*, 1999). The yeast suspended in wort is subjected to an osmotic stress due to the difference in concentrations of intracellular yeast components and wort sugars. In high-gravity brewing, the yeast needs to overcome the osmotic stress of the high initial sugar concentrations in wort, together with the toxic effects of ethanol (Jakobsen and Piper, 1989). In the first 8h of fermentation, the high osmotic pressure of the wort would have an inhibitory effect on yeast viability, growth and fermentation performance (Pratt *et al*, 2003). This hyperosmotic shock results in a loss of cell turgor pressure, and subsequently causes a rapid outflow of the cytoplasm water content and cell volume, and hence shrinkage of the cell, followed by growth arrest and eventually cell death (Fig 2.12) (Pratt *et al*, 2003). This immediately results in an increase in the internal solutes concentrations, damage to cell membrane with the influx of toxic solutes (e.g.: Na^+) and the loss of cytoskeleton components (actin proteins). All directly affect cellular processes like nutrient uptake, protein biosynthesis and enzyme activities (Mager and Varela, 1993).

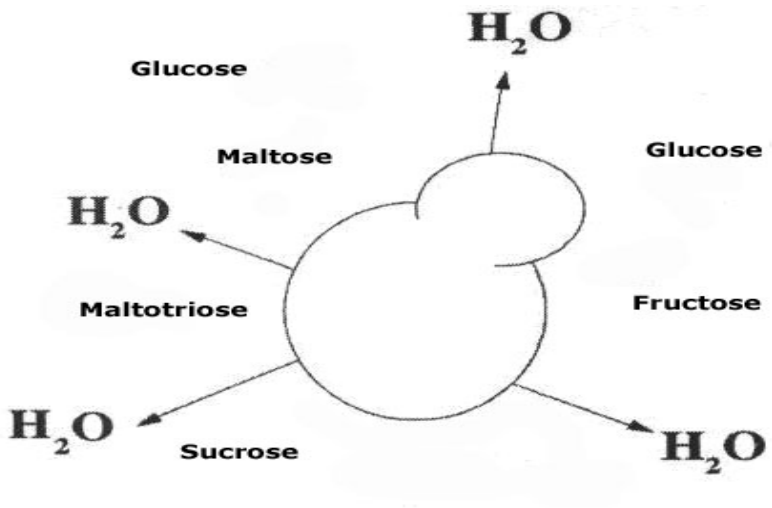


Fig 2.12: Initial response of the cell to hyperosmotic stress by cell shrinkage (adapted from Blomberg, 2000).

2.8.5.1 Production of polyols

Glycerol is the main solute produced by *S. cerevisiae* in response to osmotic stress (Carvalheiro *et* al, 1999). Osmotically stressed cells enhance the production on intracellular glycerol to balance the osmotic pressure (Albertyn *et al*, 1994). Glycerol is synthesized in the cytosol in two steps from the glycolytic intermediate dihydroxyacetone phosphate, which is catalyzed by an NADH-dependent glycerol-3-phosphate dehydrogenase (GPDH) and glycerol-3-phosphatase (GPP) (Hohmann, 1997). The NADH-dependent glycerol-3-phosphate dehydrogenase activity coded by the gene GPD1, is increased several times under osmotic stress (Albertyn *et al*, 1994). Glycerol production through the biosynthetic pathway via GPDH and GPP converts NADH to NAD, whereas glycerol dehydrogenase (GCY1p) and dihydroxyacetone kinase (DAK1p) convert glycerol back to dihydroxyacetone phosphate by reducing NADP to NADPH, since greater amounts of NADPH are required under stress conditions (Hohmann, 1997).

By the accumulation of glycerol, the cell regains turgor by the re-entry of the lost water. This increases the internal osmolarity and turgor to re-establish the surface tension. It allows the cell to retrieve water more efficiently from the medium and also stabilizes the water around macromolecules (Fig 2.13) (Hohmann, 1997). A glycerol-specific channel, Fps1p, has been identified and characterized as a glycerol facilitator that regulates the intracellular glycerol concentration (Hohmann *et al*, 2000). When the critical cell size is attained after recovery from hyperosmotic stress, growth resumes and the cells continue to divide (Mager and Siderius, 2002).

It has also been suggested that the yeast plasma membrane is able to respond to an increase in external osmolarity through processes such as a disturbance of the ionic gradients (e.g.: Na^+, K^+, H^+). This contributes to turgor regulation, increases ATPase activities involved in osmoregulation and triggers a protein kinase cascade that leads to the modification of enzyme activities and changes in gene expression (Mager and Varela, 1993).

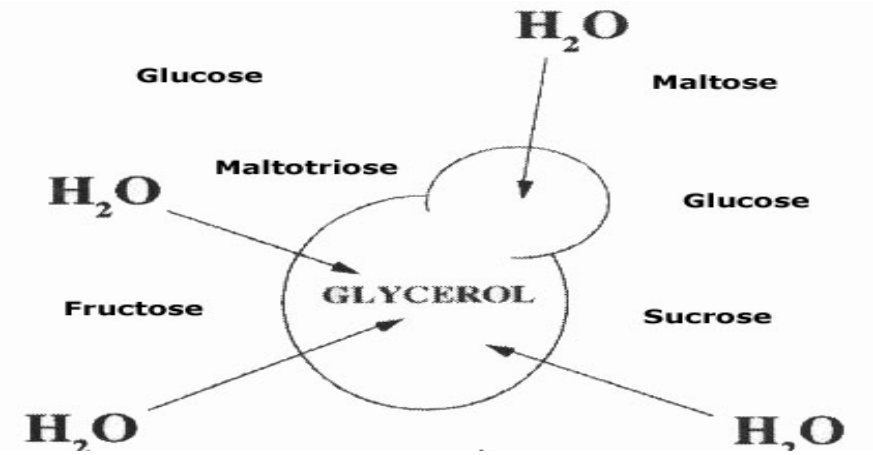


Fig 2.13: Increased production and intracellular accumulation of glycerol to regain lost water (adapted from Blomberg, 2000).

2.8.6. High pressure and mechanical stresses

In the brewing industry, cells are subject to high hydrostatic pressures encountered in tall cylindroconical fermenters vessels, and due to elevated CO_2 levels during fermentation (Heggart *et al*, 1999). The flow of yeast slurry through pipe fittings, pumps, heat exchangers or agitated vessels cause changes in the flow pattern of yeast slurries that generate hydrodynamic shear. The effect of 'shear' is the action of a force on and parallel to the interface between the cell surface and its fluid environment. Neither of the above mechanical treatments has shown to contribute to a reduction in yeast quality through changes in metabolic rates and cell death (Heggart *et al*, 1999).

2.9 Yeast stress factors

2.9.1 General stress response by yeast

Under the exposure of various stresses in their environment, yeast cells must adapt in such a way to maintain an optimum growth rate and to develop different cellular stress responses that are often linked and known as the "general stress response" (Hohmann, 1997). Yeast cells through this general stress response have been shown to be able to withstand more severe stress after being previously exposed to a lower level

of that same stress as well as to other types of stresses. This phenomenon is called "cross-protection" (Estruch, 2000). It consists of an integrating system that triggers and regulates the induction of stress genes through a common *cis* element in their promoter, known as the stress response element (STRE) (Estruch, 2000). Tolerance to various stress conditions is obtained through the transcriptional activation of the STRE element that mediates the expression of a set of stress-responsive genes. For example, cells pre-treated with a mild osmotic shock develop an acquired resistance to heat shock. Exposure to high ethanol concentrations or weak acids also confers thermotolerance (Hohmann, 1997). These stress-responsive genes encode proteins that protect the yeast cell against stress damages such as denaturation of proteins, membrane disordering or DNA damage.

Some of the most important general stress-responsive genes are (Attfield, 1997 and Estruch, 2000):

- Heat shock proteins genes (*HSP*s) functioning as molecular chaperones to prevent protein denaturation under various types of stresses conditions.
- The ubiquitin gene *UBI4* involved in the nonlysosomal proteolysis of proteins.
- The *DDR2* gene responsive to DNA damage or heat shock.
- Trehalose biosynthesis genes that are expressed upon all kinds of stresses (*TPS1*, *TPS2*, *TPS3* and *TSL1*).
- Catalase T gene (*CTT1*) that is induced by heat shock, high osmotic shock and oxidative stress by eliminating reactive oxygen intermediates.

2.9.2 Role of the *trans*-acting transcriptional factors

Two *trans*-acting transcriptional factors, Msn2p and Msn4p, encoding a zinc protein, are involved in the STRE-mediated gene expression by translocation to the nucleus and binding to the promoters of the different stress-responsive genes (e.g: *HSP12* and *CTT1*) (Estruch, 2000). More than 90% of STRE-containing genes were shown to be dependent on the overexpression of Msn2p and Msn4p in response to heat shock or exposure to H_2O_2 stress conditions (Gasch *et al*, 2000). A msn2 and msn4 double deletion strain did not show any significant induction of the transcription of the *HSP26*, *HSP12*, *CTT1* and *DDR2* genes after a carbon-source starvation, heat shock

treatment, high osmotic shock or high ethanol concentrations under severe stress (Hohmann, 1997). Indirect induction effects of Msn2/4p overexpression supports the hypothesis that in response to stressful environmental changes, the dependence of gene induction on Msn2/4p or any other transcription factors (Msn1p and Yap1p) is condition specific, and the deletion of *MSN2/4* did not affect the expression of genes containing no STRE promoter element (Gasch *et al*, 2000).

2.10 Indicators of stress in yeast

2.10.1 Physiological markers

2.10.1.1 Glycerol

The functions of glycerol in cell metabolism

(a) Osmoregulation: A general response of yeast cells as a result of an increase in external osmolarity is the rapid efflux of cellular water into the medium that cause cell shrinkage and growth arrest action. To compensate for this loss of turgor pressure, there is an enhanced intracellular production of glycerol as a compatible solute to balance the osmotic pressure of the cytosol in relation to that of the medium (Hohmann and Prior, 1997).
(b) Redox balancing: Glycerol is produced as a by-product during fermentation of glucose to ethanol in order to maintain the intracellular redox balance (Michnick *et al*, 1997). Through ethanol production, a surplus of NADH is formed during the oxidation of glyceraldehyde-3-phosphate and is re-oxidized during glycerol formation, thereby compensating for cellular reactions which produce NADH (Nevoigt and Stahl, 1997).

Glycerol synthesis

In *S. cerevisiae*, glycerol is produced by a reduction of dihydroxyacetonephosphate to glycerol-3-phosphate (G-3-P) catalyzed by a cytosolic NAD^+-dependent glycerol-3-phosphate dehydrogenase for which two isoenzymes are encoded by *GPD1* and *GPD2* (Hohmann, 1997). G-3-P is then dephosphorylated by glycerol-3-phosphatase encoded by two homologous genes *GPP1* and *GPP2,* to yield glycerol (Fig 2.14). The

substrate dihydroxyacetone phosphate is provided either by the glycolytic degradation of sugars, or by the gluconeogenic pathway when non-fermentable sugars are used (Nevoigt and Stahl, 1997). Both *GPD1* and *GPD2* expression show a different role for these genes in yeast: *GPD1* is induced by osmotic stress whereas *GPD2* expression increases in the absence of oxygen (anaerobic conditions). This shows that *GPD1* is more active in the osmoregulation of glycerol synthesis whereas *GPD2* is more important in redox balancing (Estruch, 2000). The transcription of *GPP2* is induced by high osmotic stress together with *GPD1*. Expression is rapidly increased by up to 50-fold in a high osmolarity medium but the reputation of mRNA induction is not fully understood (Hohmann, 1997).

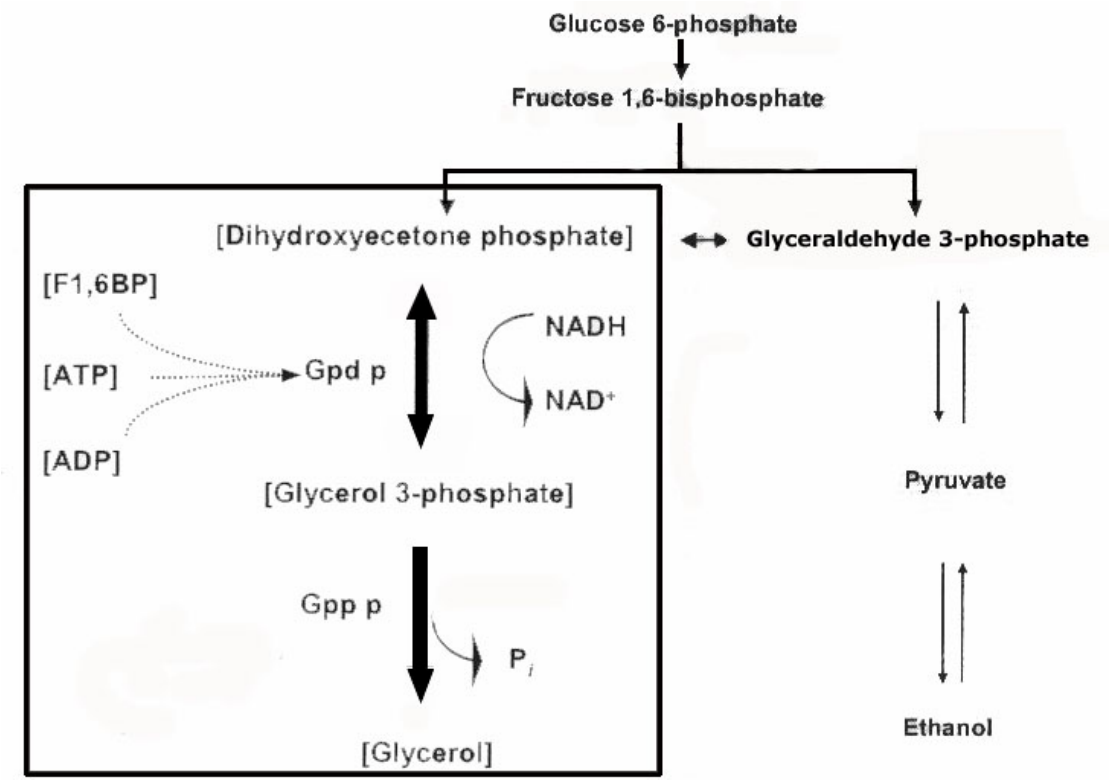


Fig 2.14: Glycerol synthesis pathway in *S. cerevisiae* (Cronwright *et al*, 2002).

Signal transduction in response to hyperosmotic stress

The HOG (high osmolarity glycerol) pathway mediates the response of yeast cells to adapt to a hyperosmotic shock in order to stimulate glycerol production (Tamás *et al*, 2000). This HOG pathway is a signal transduction system through the activation of protein kinases of the mitogen-activated protein kinase (MAPK) family (Fig 2.15)

(Westfall *et al*, 2004). Two distinctive plasma membrane proteins, Sho1p and Sln1p, function as upstream osmosensors to regulate the MAPK pathway (Tamás *et al*, 2000). The first protein, Sho1p, is activated in response to strong hyperosmotic conditions (e.g: 0.5 to 1.0M NaCl), which physically interacts with Pbs2p via its SH3 domain (Mager and Siderius, 2002). The Sho1p branch uses proteins as a part of the MAPK pathway that includes Ste20, Ste50 and MAPKKK Ste11. Ste20 activates Ste11 by phosphorylation with Ste50 being a cofactor for Ste11 because these proteins form a complex through interactions of their SAM domains (O'Rourke *et* al, 2002). This mechanism results in Hog1p activation. The second branch resulting in phosphorylation and activation of pbs2p occurs during less severe hyperosmotic conditions (0.125 to 0.25M NaCl) (Westfall *et al*, 2004). In the absence of osmostress the transmembrane protein Sln1 is autophosphorylated at an internal histidine residue, where phosphate is transferred to an aspartic acid residue, and then to a histidine residue in Ypd1p (Mager and Siderius, 2002). Subsequently aspartic residue in the "response regulator" protein Ssk1p is phosphorylated thereby preventing activation of the HOG pathway. Upon osmostress, the phosphorylation of Ssk1p ceases and the non-phosphorylated form activates the MAP kinase kinase kinases (MAPKKKs) Ssk2/Ssk22p. These MAPKKKs in turn activates MAP kinase kinase Pbs2p, which subsequently phosphorylates and activates Hog1p (Mager and Siderius, 2002). Phosphorylation of Hog1p triggers its transport to the nucleus, which stimulates the induction of *GPD1* and *GPP2* for immediate glycerol production. This above dual phosphorylation of Hog1p takes place within minutes after exposure to hyperosmotic conditions (Westfall *et al*, 2004).

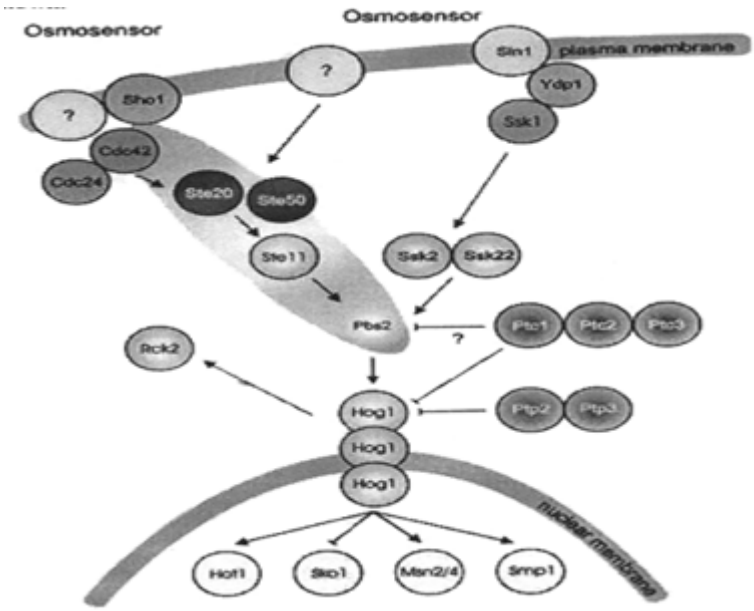


Fig 2.15: A model of high osmolarity signal transduction pathway in *S. cerevisiae* (Hohmann, 1997).

Intracellular glycerol retention

The membrane glycerol permeation occurs in two ways: passive diffusion in both directions of the membrane or by a channel protein Fps1p exclusively for glycerol export (Wang *et al*, 2001). Fsp1p belongs to the MIP (major intrinsic protein) family of channel proteins (Tamas *et al*, 2003). They are also commonly called ”aquaglyceroporins”, since this channel is responsible for glycerol export and remains open to allow glycerol to freely permeate from the cell especially under fermentative conditions. Under hyperosmotic stress the channel closes preventing glycerol leakage and thereby allowing the cell to accumulate glycerol (Wang *et al*, 2001).

Measurement of glycerol

The determination of glycerol can be carried out by using gas chromatography techniques by direct injections of glycerol onto packed columns or by HPLC or enzymatically. The gas chromatographic method is rather complex (Sánchez *et al*, 1992). An established ion-exchange chromatography method using either cation or

anion-exchange resins are generally used for a reasonably fast and efficient determination (Calull *et al*, 1992). This HPLC method was found to give more reproducible results in comparison to the enzymatic method (Sánchez *et al*, 1992).

2.10.1.2 Trehalose

Trehalose as a vitality marker

The non-reducing dissacharide trehalose (α-D-glucopyranosyl (1-1)-α-D-glucopyranoside) is widely distributed among many organisms such as bacteria, fungi, plants and insects (Plourde-Owobi *et al*, 2000). In the yeast *S. cerevisiae*, trehalose has many functions such as: (a) storage carbohydrate that accumulates in the cell at the beginning of the stationary phase, and is mobilized as a carbon source in periods of starvation and in growth initiation (Hounsa *et al*, 1998). (b) Important environmental stress-protectant in response to any physiological stress such as osmotic stress, nutrient depletion and during heat or ethanol shock (Odumeru *et al*, 1993). A particular function mainly related to trehalose, is the preservation of the plasma membrane integrity and protein under stress conditions, especially during high-gravity brewing in the presence of high ethanol concentrations. It has been observed, as the wort gravity increases, the intracellular trehalose concentrations also increases (Majara *et al*, 1996).

Trehalose stabilization effects on the membrane are: (1) the substitution of water and binding to the polar heads of phospholipids (Arguelles, 1994) (2) stabilization of proteins in their native state to prevent protein inactivation through denaturation and (3) prevention of the aggregation of denatured proteins by maintaining them in a partially folded state (Lucero *et al*, 2000). An increase in the synthesis of unsaturated fatty acids, as well as in the total lipid content in the cell proportionally with the oxygen depletion, are both requirements for promoting a more structural integrity against ethanol stress (Majara *et* al, 1996). Other stress condition that leads to an impaired fermentation performance is osmotic shock from high substrate concentrations. Trehalose helps in the osmoregulation and water activity in parallel with glycerol production. Trehalose has been shown to accumulate in *S. cerevisiae*

during the brewing process in such conditions: (a) reduced growth rate (resting cells) such under high osmotic pressure because of high sugars concentration, (b) during nutrient starvation periods at the beginning of the stationary phase associated with ethanol toxicity, (c) at time of cropping as a result of depletion of sterols and unsaturated fatty acids and (d) mostly during storage periods at low temperatures to preserve membrane integrity from ethanol exposure and dehydration (Majara *et al*, 1996). The initial trehalose content of pitching yeast has been proposed as a potential vitality indicator for the prediction of the fermentation performance in subsequent fermentations (Guldfeldt and Arneborg, 1998).

Trehalose biosynthesis and catabolism

Trehalose (Fig 2.16) is synthesized in a two-step process from the nucleotide UDP which donates glucose, and which reacts with glucose-6-phosphate by a two-step reaction: (1) trehalose-6-phosphate synthase (coded by *TPS1* gene) converts UDPG and glucose-6-P to yield trehalose-6-phosphate (2) trehalose-6-phosphohydrolase (coded by *TPS2* gene) cleaves off phosphate to yield trehalose (Hottiger *et al*, 1987). These two enzymes are commonly called the trehalose synthase enzyme complex.

Trehalose hydrolysis is mediated by two distinct trehalases differentiated by their pH optima: an acid trehalase situated in the vacuole with a pH optimum of 4.5, and a neutral trehalase in the cytosol with a pH optimum between 6 and 7.5, whose activation is regulated by cyclic AMP-dependent phosphorylation (Hottiger *et al*, 1987). The trehalose levels are controlled by a balance of the synthesizing and hydrolyzing enzymes, in which the neutral trehalase activity is most active when intracellular trehalose levels are the lowest during the exponential growth on glucose, and drops drastically before the cells enter the stationary phase (Hounsa *et al*, 1998).

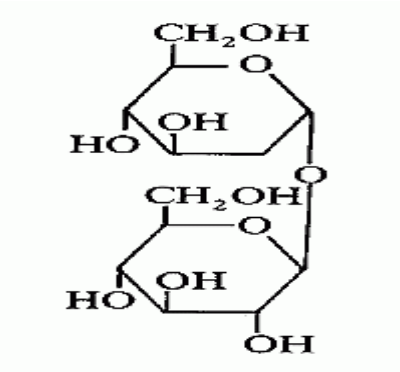


Fig 2.16: Trehalose chemical structure (http://www.benbest.com/cryonics/trehalose.gif).

Thermotolerance related to trehalose accumulation

A correlation between intracellular trehalose content and thermotolerance has been observed, in which trehalose accumulation can be directly related to the increased activities of two key enzymes: trehalose-6-synthase and neutral trehalase (Neves and Francois, 1992). This accumulation results from heat-induced changes in the activity of the two subunits of the trehalose-6-synthase, or/and the activation of neutral trehalase through an increase in the cAMP levels (Panek, 1990).

Measurement of trehalose

Trehalose can be extracted from yeast cells by an alkali treatment in boiling 0.25 M sodium carbonate, which quantitatively extracts both glycogen and trehalose (Parrou and Francois, 1997). The mixture is brought to pH 5.2 and incubated with trehalase at 37°C for 4h in which trehalose is hydrolyzed to glucose. The resulting glucose is then measured by the use of an enzymatic kit based on the glucose oxidase-peroxidase reaction. This method was found to be time-consuming, laborious and includes too many steps that might decrease the precision of the method (Schulze *et al*, 1995). A chromatographic method based on the HPLC with an anion-exchange column is found to be more precise and reliable. The HPLC method was found in comparison to the enzymatic method to be more simple and accurate for routine analysis of cell extracts (Ferreira *et al*, 1997).

2.10.1.3 Glycogen

Glycogen as a vitality marker

The carbohydrate storage glycogen, composed of numerous α (1→4)-linked D-glucose residues, is an important intracellular energy reserve and carbon source during periods of yeast starvation. Glycogen accumulates in *S. cerevisiae* at the end of the growth phase and at the beginning of stationary phase (Heggart *et al*, 1999). During storage periods, glycogen can be considered as a vitality marker from its major role as an energy source during storage periods prior to pitching. The brewer's yeast can use up to 30-40% of its glycogen levels during storage (Heggart *et al*, 1999). During the initial hours of fermentation, glycogen is the main source of metabolic energy for the synthesis of membrane sterols and fatty acids in the presence of oxygen (Cahill *et al*, 2000). Low glycogen levels in pitching yeast as a result of glycogen depletion during storage (from oxygen exposure or extended storage periods), will give rise to an extended lag phase at the start of fermentation in correlation with poor lipid synthesis, slow growth and decreased fermentation performance (Heggart *et al*, 1999). A decrease in cellular glycogen content is an indication of yeast deterioration, which can have an impact on the subsequent fermentations performance.

Glycogen synthesis and regulation

The synthesis of glycogen starts with the conversion of glucose-6-phosphate to glucose-1-phosphate catalyzed by phosphoglucomutase (Ryder and Masschelein, 1993). Glucose-1-phosphate is then converted to UDPG-glucose by UDPG-glucose pyrophosphorylase with the consumption of the triphosphate UTP in a key reaction in glycogen biosynthesis (Lenhinger *et al*, 1993). The resulting UDP-glucose is the glucose donor in the enzymatic formation of glycogen by glycogen synthase, which catalyzes the transfer of the glucosyl residue to a non-reducing end of the branched glycogen molecule (Fig 2.17). By increasing the number of reducing ends, it renders the glycogen molecule more soluble and more reactive to both glycogen phosphorylase and glycogen synthase (Lehninger *et al*, 1993). Glycogen degradation or commonly called glycogenolysis is catalyzed by the enzyme phosphorylase, which transfers a glucose residue from the non-reducing end of the chain to inorganic

phosphate. This in turn yields glucose-1-phosphate that isomerizes back to glucose-6-phosphate and recycled through the glycolytic pathway (Ryder and Masschelein, 1993).

The regulation of glycogen is mostly controlled by the reversible phosphorylation in the activities of glycogen synthase (Gsy2p) and glycogen phosphorylase (Gph1p) (Ni and Laporte, 1995).

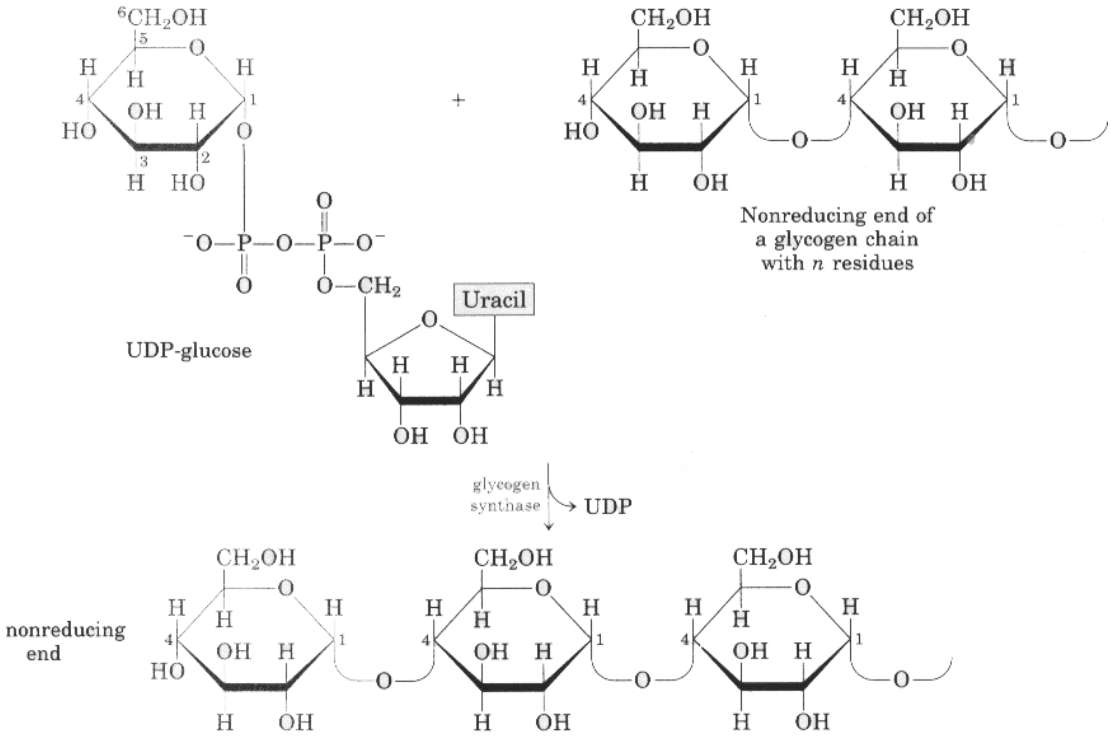


Fig 2.17: Glycogen synthesis pathway (Lehninger *et al*, 1993).

Glycogen determination methods

Few methods have been established in the brewing industry for the determination of glycogen, which can de classified based on the accuracy and the practicability of the method:

(a) Iodine staining: This method quantifies glycogen by a rapid spectrophotometric assay at an optical density of 660 nm of yeast cells mixture stained with an I_2: KI solution (Cahill *et al*, 2000). An increasing

staining intensity of yeast cells from low to high glycogen levels develops from light yellow to a dark brown colour. This method is rapid and easy, but can only determine approximate glycogen content, making it inaccurate and difficult to quantify the metabolite.

(b) Alkali extraction of glycogen in 0.25 M Na_2CO_3 at 90-95ºC for 4h, after which the mixture is incubated with the enzyme amyloglucosidase to degrade glycogen into glucose, which can be determined enzymatically or by HPLC procedures (Quain, 1981). This technique is more labour intensive and time consuming, but is considered to be more accurate in order to quantify glycogen.

(c) On a more routine basis in the brewing industry, the near infrared reflectance spectrometry (NIR) method is mostly used for rapid glycogen determination. This technique is more expensive than the previous two and, a skilled operator is required (O'Connor Cox, 1998).

2.10.2 Molecular markers

Heat shock proteins

The stress response

Under exposure of elevated temperatures, cells trigger the synthesis of a specific group of small proteins called heat shock proteins. These proteins are directly related to an induced tolerance to extreme temperatures, but are also induced by variety of other stress agents like ethanol or heavy metals (Sanchez *et al*, 1992). As discussed previously, ethanol and heat shock both cause membrane disordering and protein denaturation. Heat shock proteins act as molecular chaperones by binding to unfolded or nascent polypeptides in order to promote protein folding and to prevent protein aggregation. Heat shock proteins perform the following functions (Becker and Craig, 1994):

- They stabilize unfolded precursor proteins prior to their assembly into the cytosol or translocation into the endoplasmic reticulum (ER) or mitochondria.
- They are involved in the rearrangement of protein oligomers and in the resolution of protein aggregates.
- They bind unfolded polypeptides and assemble them in higher-order protein structures.
- They regulate the function of folded proteins by binding to them through an ATP-dependent mechanism.

The heat shock response: As a result of a transfer to higher temperatures, the heat shock transcription factor (HSF), binds to a heat shock promoter element (HSE), allowing transcription of the heat shock genes. This increased transcription leads to an increase in the concentration of heat shock proteins in the cell (Craig and Gross, 1991). As part of the heat shock response, a rapid accumulation of the disaccharide trehalose shows a positive correlation between the extent of induced thermotolerance and the induction of heat shock proteins (Gross and Watson, 1996).

At the later stages of fermentation where the environmental conditions are the most stressful as a result of a lack of nutrients, and high ethanol concentrations, the induction of heat shock proteins (mostly Hsp104p) is repressed due to an inhibition in transcription or RNA degradation. Such repression may be due to the combined toxic effects of heat and high ethanol content or high pressure experienced in late fermentation (Brosnan *et al*, 2000). Such toxic effect enhance cell death at the end of fermentation, and causes a reduction in ethanol yield as the yeast is re-used in subsequent brewing fermentations.

Heat shock proteins families

(a) The Hsp70p family: the yeast Hsp70p is the most studied and most abundant heat shock family system in yeast. Hsp70p genes encode heat-inducible 70-kDa Hsps as a multigene family (Becker and Craig, 1994). It consists of nine family members varying in cellular localization such as the mitochondrion, the nucleus, the cytosol and the endoplasmic reticulum (Schlesinger, 1990). They are mostly needed for import of

proteins into other eukaryotic cell organelles including the endoplasmic reticulum and the chloroplast. Hsp70p binds to unfolded proteins regardless of their cellular location through an ATP-dependent manner to facilitate binding to the substrate (Craig and Gross, 1991). For the release of the bounded folded protein, Hsp70p undergoes a conformational change by partial proteolysis, which is dependent on ATP hydrolysis (Becker and Craig, 1994).

(b) The Hsp60p family: These 60-kDa Hsps are commonly referred as the chaperonins, since they participate in the folding of many different proteins and in the assembly of polypeptides. They work together with Hsp70p proteins by binding to the same substrates, by binding in an ATPase dependent manner to the partially folded proteins, whose backbone of unfolded polypeptides has already been bound by Hsp70p (Becker and Craig, 1994).

(c) The Hsp90p family: These heat shock genes is the third major highly conserved group of stress proteins, localized in the cytoplasm, which complexes with a variety of normal cellular proteins (Schlesinger, 1990). Their proper functions are still unclear, but it is believed that they have a role similar to Hsp60p and Hsp70p proteins to bind to nascent polypeptides and escorting them to their proper cellular location (Ang *et al*, 1991).

Hsp12p as a vitality marker

The 12-kDa-heat shock protein was found to act similarly to trehalose in order to protect the plasma membrane integrity against desiccation in the presence of ethanol, as well as under heat and osmotic shock (Sales *et al*. 2000). The Hsp12p has a cytoplasmic location in close proximity to the plasma membrane and is able to interact electrostatically with charged groups present on the membrane surface. Membrane stabilization during dehydration or desiccation is conserved by Hsp12p electrostatically interacting by hydrogen bonding with membrane proteins or glycolipids on the membrane surface. This builds a more hydrophilic membrane and prevents membrane fusion (Sales *et al*, 2000). Hsp12p gene expression in *S. cerevisiae* has been observed to increase in the early stages of the stationary phase as a result of elevated ethanol concentrations and nutrient depletion present at the end of fermentation, and during growth on non-fermentable carbon sources (DeGroot *et al*, 2000).

The transcriptional regulators Msn2p and Msn4p are involved in the activation of Hsp12p expression during stress conditions. The promoter of the Hsp12p gene contains five stress-responsive elements (STREs), to which these factors bind under different stress conditions. Hsp12p gene expression has been shown to be repressed under high glucose concentrations, in particular at the beginning of fermentation (DeGroot *et al*, 2000).

Hsp12p gene expression as a vitality marker is a novel approach to evaluate the fermentation performance of *S. cerevisiae* in the brewing industry. Soluble Hsp12p in a total extract separated on a 20% SDS-PAGE gel, can be identified using immunoblotting techniques such as western blot with anti-Hsp12p antiserum (Motshwene *et al*, 2004). The total cell RNA can also be analyzed by the northern blot technique through blotting onto a nitrocellulose membrane and probed to an Hsp12p probe (Piper *et al*, 1994). These techniques are known to be very sensitive, but do offer numerous technical problems if performed as a routine test due to the costs and time required. Furthermore, these sensitive methods require highly technical skills.

2.10.3 Genes identified in microarray research

DNA microarray technique (cDNA and oligonucleotide) is a recent method for the analysis of gene expression profiles (Duggan *et al*, 1999). The whole-gene expression for a particular organism (whose whole genome has already been sequenced) is tested under certain environmental stress conditions, which can be determined through microarray techniques. In order for the organism to rapidly adapt to the new disruptive environmental conditions, the cell rapidly changes its gene expression levels through induction and repression of certain proteins involved in the stress response (Gasch *et al*, 2000). DNA microarray analyzes changes in transcript abundance in cells responding to various environmental stresses (Gasch *et al*, 2000).

Different uses of microarray can be applied: (a) to define the gene expression patterns during the adaptation to stressful conditions as a common response (called the

"environmental stress response") (b) to compare and contrast the gene expression responses between different stresses (Gasch *et al*, 2000).

Principle of method:

The technique is based on two fluorescently labelled cDNA populations to allow comparison of gene expression between two samples (Lucchini *et al*, 2001). DNA microarray consist of a library of genes or "chips" immobilized on a solid matrix (glass slide or nitrocellulose membrane), where each individual spot contains a known sequence of the oligonucleotide primer derived from a specific gene (Duggan *et al*, 1999). The matrix is incubated with randomly fluorescently labelled cDNA sequences or probes through reverse transcription cycles with an oligo-dT primer that are complementary to the immobilized oligonucleotide primers. The abundance of transcripts formed can be measured by comparing the fluorescent signal generated between a "reference" and a "test" sample. The reference sample would contain known sequences of cDNA, where gene expression has already been determined. The test sample containing cDNA sequences of various amounts is allowed to hybridize to the chip to enable comparison to the reference. After hybridization has occurred, the array slide is excited using an ultraviolet laser to yield emission spectra, which are measured with a scanning laser microscope. The resulting two-coloured hybridization is imported into a computer that interprets each signal intensity in a two-dimensional graph that shows the intensity of each probe in the form of peaks and the names of the identified genes (Duggan *et al*, 1999).

General stress response

The term "environmental stress response" in *S. cerevisiae* refers to the entire set of induced and repressed genes in response to multiple stresses together and represents about 10% of the genome. The genes that are induced have functions in carbohydrate metabolism, cell stress like detoxification of reactive oxygen species, cellular redox reactions, cell wall modification, protein folding and degradation and generation of energy such as fatty acid metabolism and metabolite transport (Gasch *et al*, 1999).

Many of these genes are involved in glycolysis to provide enough ATP required by ATP-dependent molecular chaperones (heat shock proteins for example) in response to cellular stress, and those involved in trehalose and glycogen metabolism necessary for protein stabilization and osmolyte balance. It includes genes encoding the subunits of the enzyme trehalose synthetase (*TPS1*, *TPS2*, *TPS3* and *TSL1*) (Causton *et al*, 2001). The cell stress genes include the heat shock genes (*HSP12*, *HSP26*, *HSP42*, *HSP78*, *HSP104*, *SSA4* and *SSE2*) which promote protein folding to maintain protein conformation. Protein degradation genes (*PHB2*, *RPN5*, *UBC5*, *UBC8*, and *YPS6*) as well are induced in order to prevent the accumulation of protein aggregates (Causton *et al*, 2001). Other genes like those involved in the degradation of oxidative reacting species, are also activated, such as *CTT1* (Catalase T gene).

The repressed genes of the environmental stress response are mainly associated with translation and protein synthesis in a common cluster, in particularly in RNA metabolism (RNA processing and splicing, translation initiation and elongation, tRNA synthesis and processing) (Gasch *et al*, 1999). Environmental stress response genes are known to be regulated by various transcription factors depending on the specific environmental stress applied. The induction of *GPD1*, *HSP12* and *CTT1* genes is regulated by the transcription factors Msn1p, Msn2p, Msn4p or Hot1p depending on the environmental stress. Hsp12p expression is mostly induced as a general STRE-dependent response to cellular stress in particularly to a heat shock, forming a part of the general stress response system (Attfield, 1997). Overexpression of Msn2p / Msn4p that bind to the upstream STRE sequence on the promoter in response to heat shock or hydrogen peroxide exposure causes an induction of the environmental stress response genes (Gasch *et al*, 1999).

Environment-specific responses

These genes consist of 18-38% of the genome of *S. cerevisiae* altered in response to environmental changes. The most common stresses are:

Heat: A temperature shift from 25 to 37°C induces genes functioning in protein folding and transport, such as *EUG1* and *LHS1* that respond to protein unfolding and

denaturation. Most of the heat shock genes are also heavily induces in the heat shock response (Gasch *et al*, 1999).

Hydrogen peroxide: It induces a group of genes involved in the detoxification of both hydrogen peroxide and superoxide such as superoxide dismutase, glutathione peroxidases and thiol-specific antioxidants (Gasch *et al*, 1999).

Hyperosmotic shock: The addition of sodium chloride causes both an osmotic and ionic changes whose gene expressions are fairly similar (Causton *et al*, 2001). Some genes that are super-induced in response to hyperosmotic shock are involved in the synthesis and regulation of osmolytes like glycerol and trehalose (Gasch *et al*, 1999).

Ethanol stress: Under ethanol exposure, the expression of a large number of genes is altered. Repressed genes include those genes involved in protein biosynthesis, cell growth and RNA metabolism associated with cell growth arrest (Alexandre *et al*, 2001). Induced genes are those involved in energetic metabolism, protein translation, ionic homeostasis and the stress response, in very close association with the environmental stress response genes. Some examples of genes that are induced include *HSP12*, *HSP26*, *HSP78*, *HSP104* together with *SSA1*, *SSA2*, *SSA3*, *SSA4*, *SSE1* (Alexandre *et al*, 2001). Trehalose synthesis genes and their precursors including *NTH1*, *TPS1*, *TLS1*, *TPS2*, *UGP1* and *PGM2* are expressed as well to prevent protein denaturation, while *HSP* genes prevent protein aggregation (Alexandre *et al*, 2001). The expression of glycerol synthesis (*GPD1*, *HOR2*) and catabolism genes (*DAK1*) is also increased.

Chapter 3: Experimental procedures

3.1 Fermentation system

3.1.1 Yeast culture

S. cerevisiae SAB5 was obtained from South African Breweries as a frozen vial that was divided into vials in a sterile 75% glycerol stock and maintained at -80°C. The yeast culture was sustained by routine sub-culturing on YPD plates (10 g/l yeast extract, 20 g/l bactopeptone, 20 g/l glucose and 18 g/l agar) by re-streaking a single colony every 4 months, and storing at 4°C.

3.1.2 Propagation of *S. cerevisiae* lager SAB5 strain

Duplicate propagations were performed in defined "Black Label" wort (appendix A) of specific gravity of approximately 15°P kindly supplied by South African Breweries in Newlands, in three successive aerobic stirred propagation stages in a shaking water bath (Scientific Co, Inc., model G76D, Edison, N.J., USA):

1) Two or three pure colonies were inoculated in 20ml autoclaved liquid wort at 20°C in a 100 ml Erlenmeyer flask and shaken at 110 rpm for approximately 40h.
2) The 20 ml wort was transferred to 200 ml autoclaved liquid wort at 18°C in a 1.5 l Erlenmeyer flask and shaken at 110 rpm for approximately 40h.
3) The 200 ml wort was transferred to 800 ml autoclaved liquid wort at 16°C in a 2 l Erlenmeyer flask and shaken at 80 rpm for approximately 40h.

3.1.3 Preparation of fermentation equipment and conditions

3.1.3.1 Fermentation in E.B.C. tubes

Six standard European Brewing Commission (E.B.C) tall glass tubes were made in the workshop of the department of Organic Chemistry, University of Stellenbosch (Fig. 3.1). The tubes were sterilized by autoclaving 2 tubes at a time for 20 min at 121°C, and allowed to cool down to room temperature. The E.B.C. fermentation tubes were suspended in water, in a 300 l stainless steel water tank housed in a walk-in cold room, and maintained at a constant temperature of 18°C and 14°C as two separate trials respectively by a heating circulator (Thermo Haake, model C10, U.S.A) (Fig 3.2). Each fermentation was carried out for approximately 5 days before cropping.

Fig 3.1: E.B.C. fermentation tube. 79 cm long by 8 cm wide.

Fig 3.2: Illustration of fermentation system.

3.1.3.2 Wort transfer

Two litres of "Black Label" wort were autoclaved individually in 2.5 l Schott bottles for 15 min at 121°C. The autoclaved liquid wort was aseptically transferred to the autoclaved E.B.C. tube by bubbling air through a filter system and sterilized silicone tubing (Fig 3.3). The wort was sealed within the fermentation tube by nickel-plated brass clamping screws (Lasec, Cat No: CG45 25 mm) to maintain sterile conditions (Fig 3.1).

Fig 3.3: Illustration of wort transfer equipment.

3.1.4 Wort oxygenation and pitching

Autoclaved liquid wort was oxygenated by bubbling 99.5% pure medical oxygen (Afrox, O_2 IG CYL, 14.2 kg) through a sterile 0.22 µm filter (Cameo Acetate, 25 mm, Cat No: DDA02025SO) for a period of 1h, to a final oxygen concentration of approximately 16 mg/ml.

The pitching yeast was prepared by harvesting the yeast from freshly propagated wort (section 3.1.2), washed twice in sterile deionised water by centrifugation (8000 x g for 15 min at 4°C; Beckman, model J2-21, Galway, Ireland), and re-suspended in 50 ml autoclaved liquid wort. Yeast cells were pitched at 1.57 x 10^7 viable cells/ml per °P,

into 2 l aerated wort (Amore *et al*, 1991). The E.B.C. tube was vigorously shaken by hand to re-suspend the yeast suspension throughout the tube.

3.1.5 Yeast cropping and subsequent re-pitching

At the end of the 5 days fermentation, tubes were removed from the water tank, and the beer was aseptically transferred to duplicate 2 l sterilized Schott bottles. The yeast in the beer suspension was harvested and washed twice with sterile deionised water by centrifugation at 10 000 x g at 4°C for 10 min. The flocculated yeast slurry at the bottom of the tube was also harvested as described above prior to re-pitching, but first re-suspended aseptically in 500 ml sterile phosphate buffer (100 mM phosphate, 150 mM NaCl at pH 7.4).

Both yeast types (suspended yeast and slurry) were re-pitched in 2 l aerated wort in a E.B.C. tube for the fermentation run at 14°C only as described above.

3.1.6 Sampling

Yeast samples were taken at 24h intervals throughout the fermentation from each respective fermentation tube. Samples were withdrawn with the use of a sterile 10 ml glass pipette and collected in sterile McCartney glass bottles that were kept on ice.

3.2 Measurement of fermentation parameters

3.2.1 Yeast count and viability

Methylene Blue 0.02% (w/v) (M & B Laboratory Chemicals, Dagenham, England) was dissolved in sodium phosphate buffer to a final concentration of 0.01% (w/v) at pH4.6. A volume of 100 µl of yeast cells was added to 900 µl of methylene blue staining solution, and incubated at room temperature for 15min. A light microscope (Zeiss, Germany) at 40x magnification was used to count living and dead cells in a haemocytometer chamber (0.1 mm depth, area 1/400 mm^2). Counts were conducted in

four replicates and at least 100 cells were counted (O'Connor-Cox *et al*, 1997). Unstained cells were assumed to be viable, while blue-stained cells dead. Buds were only counted if their size were greater than one half of the parent cell. The viability was reported as the percentage by dividing the number of live cells by the total number of cells and then multiplying by 100.

3.2.2 Wort gravity

The decrease in wort sugars was monitored daily by the measurement of the decrease in specific gravity in Plato degrees (DMA 55 meter, Anton Paar, Austria).

3.2.3 Ethanol production

Ethanol production was monitored daily by a gas chromatograph (Hewlett-Packard HP6890 Series), equipped with a flame-ionization detector and with a high performance capillary column (HP-INNOWax, Cross linked Polyethylene Glycol, 0.25 μm thickness, 30 m length). One-millilitre samples were prepared by centrifugation at 13 000 x g for 10 min at room temperature, and the supernatant was filtered through a 0.45 μm sterile filter (Cameo Acetate, 25 mm, Cat No: DDA04025SO) prior to injection. The concentration in percentage (w/v) was calculated from a standard calibration graph with standards ranging from 0 to 10% (w/v) of chemical absolute ethanol in wort.

3.2.4 Fermentable wort carbohydrates

One millilitre of wort sample was centrifuged as described above for the ethanol determination. The wort sample was first purified to remove the charged components like amino acids through a mixed bed cationic and anionic exchange resin. Positively charged hydrogen ions exchange resins (Amberlite resin IRC-50, Laboratory BDH reagent) and negatively charged chlorine ion exchange resins (Anion resin AG 3-X4, Bio-Rad Laboratories) (soaked in 0.1 M acetic acid for few hours) were both added together and packed into a Perspex micro centrifuge tube. The resins were first washed 5 to 6 times with 500 μl double-distilled water and centrifuged in between (5 min, 13 000 x g).

The wort sample was diluted 100-fold, from which 200 µl was applied to the mixed ion exchange resins, and washed six times with 200 µl of double distilled water. All solutions were collected in an eppendorf tube. The wort sample was diluted a further five times, and filtered through a 0.22 µm syringe filter. The different carbohydrates were separated by using a Dionex series 4500 HPLC, fitted with a Carbopac PA100 column (4 x 250 mm), a Carbopac guard column (2 x 250 mm), a ED40 pulsed electrochemical detector and a GP50 gradient pump delivery module. The mobile phase was prepared by degassing analytical double-distilled water with helium for 10-15 min, and NaOH was added by carefully pipetting to give a concentration of 100 mM NaOH. The flow rate of the mobile phase was 1 ml/min and the injection volume was 500 µl. Injecting a mixture of 100 µM glucose, fructose, sucrose, maltose and maltotriose, from which a serial dilution were performed down to 6.25 µM, standardized the instrument. A standard curve for each sugar was generated by serial dilutions of the standards, and from which the concentration for each carbohydrate in mM was calculated (DIONEX technical note 20 and application note 122).

3.2.5 Dry weight

Two cultures of the *S. cerevisiae* SAB5 strain were grown in wort to late exponential phase at 30°C for 48h. Serial dilutions were performed in sterile wort media, and the OD_{660} of each dilution was subsequently measured. Volumes of 20 ml from each dilution were filtered under vacuum through glass microfibre filters (Scheicher & Schuell Laboratory & Scientific equipment, 25 mm diameter, cat no: FS&5370018). Cells were washed three times with 5 ml double distilled water. The filters were dried to a constant weight in an oven at 80°C for 24h to 48h, cooled and desiccated for 1h before weighing (Guldfeldt and Arneborg, 1998).

The dry weight was determined from the difference in weight between the pre-weight and the dried filter. A standard curve was constructed from the dry weight of filters (mg/ml) and the respective OD_{660}. Yeast dry weight determinations throughout fermentation, were monitored by performing a series of dilutions from 2 to 10-fold in

wort medium, and measuring the absorbance at OD_{660}. The resulting dry weight concentration was calculated from the respective standard curve.

3.3 Measurement of cell metabolites and protein products

3.3.1 Trehalose and glycerol

Duplicate 10 ml wort samples were harvested by washing harvested cells in potassium phosphate buffer (0.1 M pH5.9) at 10 000 x g for 10 min at 4°C. Trehalose (Fluka Biochemica, Cat No: GA 11570) and glycerol (Merck, Cat No: BB101186M) were extracted from cells by incubation in boiling 0.25 M sodium carbonate for 20 min (Memmert, Schutzart, Germany). The supernatant was collected by centrifugation and stored at -20°C until further analysis (Slaughter and Nomura, 1992).

The extract sample was diluted 20-fold with double distilled water and filtered through a 0.22 μm syringe filter. The separation of trehalose and glycerol was achieved using a Dionex series 4500 HPLC, fitted with a Carbopac PA1 column (4 x 250 mm), a Carbopac PA1 guard column (2 x 250 mm) and an ED40 pulsed electrochemical detector (Dionex). The mobile phase was prepared by degassing analytical double distilled water with He for 10-15 min, and NaOH was added by carefully pipetting to give a concentration of 500 mM NaOH. The flow rate of the mobile phase was 0.25 ml/min and the injection volume was 500 μl. The trehalose and glycerol analysis was calibrated from a series of standards ranging from 6.25 μM to 100 μM, from which a calibration standard curve was generated relating peak area to concentration. (Hallsworth and Magan, 1997).

3.3.2 Glycogen

Glycogen was extracted simultaneously in the sodium carbonate extraction with trehalose and glycerol as described previously (section 3.3.1). The extracted glycogen is hydrolyzed to glucose by the enzyme amyloglucosidase (*Aspergillus niger,* E.C 3.2.1.3, Roche, Germany) (Parrou, 1997). A solution reaction containing 10 µl glycogen supernatant, 5 µl 1N acetic acid, and 25 µl 6 U/mg enzyme-buffer mixture in 80 mM sodium acetate, pH4.7, was incubated at 37°C for 2h (Schulze *et al*, 1995). Respective glycogen standards (Sigma, Co., Cat No: G-8876, Type 3 from rabbit liver) concentrations from 0.015 mg/ml to 1 mg/ml were also enzymatically hydrolyzed. The released glucose was estimated by diluting the hydrolyzed sample 25-fold and injected into a Dionex series 4500 HPLC, fitted with a Carbopac PA100 column (4 x 250 mm), a Carbopac guard column and a pulsed electrochemical detector (PED) as described above. The glycogen concentration was expressed as glucose equivalents in mg/ml.

3.3.3 Total protein

Duplicate wort samples (3 ml) were harvested and washed once with double ionized water by centrifugation at 10 000x g for 10 min at 4°C. The cell pellets were extracted by boiling with 3 ml of 1N sodium hydroxide for 10min, and supernatant was collected by centrifugation. The total protein content was determined by the dye binding method (Bradford, 1976), by measuring the absorbance respectively at OD_{595} in a Beckman Coulter DU640 spectrophotometer, with the use of the Bio-Rad protein assay kit (Bio-Rad Laboratories, Cat No: 500-0006, Munich). The final protein content in mg/ml was calculated from a standard curve, using the protein thyroglobulin as the standard.

3.3.4 Neutral trehalase and esterase enzyme activities

3.3.4.1 Crude extract preparation

Ten milliliter duplicate wort samples were harvested by centrifugation as described above, and washed twice with potassium phosphate buffer (0.1 M pH7.4) supplemented with 1 mM dithiothreitol (Cleland's reagent) (Roche, Germany), 0.5 mM phenylmethylsulfonyl fluoride (Fluka Biochemica, Cat No: GA-11568), 1 mM EDTA and 2 mM mercapto-ethanol. Crude extracts were prepared by disrupting the cells by adding an equal volume of acid-washed glass beads (425-600 μm, Sigma-Aldrich, cat no: G8772). The cell suspension was vortexed (Scientific Industries, Inc., Genie 2, model G-560E, U.S.A) 5 times for 1-minute with 1-minute intervals on ice as described by Guldfeldt *et al* (1998). An equal volume of phosphate buffer was added prior to centrifugation to obtain supernatant. The total protein content in the crude extract was determined by the Bradford (1976) method as described previously.

3.3.4.2 Neutral trehalase enzyme assay

The reaction mixture was prepared as follows as described by App and Holzer (1989): 20 μl of the enzyme extract solution, 180 μl of 0.5% trehalose in 50 mM imidazole-HCl, pH7.0 for 10 min at 37°C. The reaction was stopped by adding 500 μl of a solution containing 10 g/l of dinitrosalicyclic acid, 16.8 g/l NaOH, and 300 g/l sodium tartrate. The reactions, including a control with imidazole buffer, were boiled for 5 min at 95°C (Khun *et al*, 1995). The absorbance at 530 nm was measured after equilibration to room temperature, and the glucose concentration in mg/ml was determined from a standard curve. A unit of neutral trehalase activity is defined as nmoles glucose released per min and expressed as U/mg protein.

3.3.4.3 Esterase enzyme assay

Esterase activity in cell free extracts was determined by hydrolysis of *ρ*-nitrophenyl acetate as the substrate and measuring *ρ*-nitrophenol released according to the method of Guldfeldt *et al* (1998). One millilitre of cell free extract was diluted ten times in phosphate buffer (18.0 g of Na_2HPO_4, $2H_2O$ and 6.9 g of NaH_2PO_4, H_2O per litre in distilled water), pH7. One hundred microlitres diluted enzyme extract was added to 200 µl 1.0 mM *ρ*-nitrophenyl acetate solution (Sigma Chemical Co, Cat No: N-8130), 200 µl sodium buffer and 500 µl distilled water. The preparation was incubated for 30 min at 30°C, and the absorbance at 400 nm was immediately measured. For the blank, 100 µl enzyme extract was replaced by 100 µl distilled water. A standard curve was generated by plotting the absorbance against the molar concentration (mM) of *ρ*-nitrophenol. A unit of esterase activity is defined as the release of nmoles of *ρ*-nitrophenol per min.

3.3.5 Hsp12p expression

3.3.5.1 Hsp12p extraction

Ten millilitres duplicate wort samples were harvested and washed twice in PBS (50 mM potassium phosphate buffer, 150 mM NaCl, pH7.4) and centrifuged as described above. The wet weights of the cell pellets were determined and resuspended with an equal volume of PBS. Extraction of Hsp12p was performed by boiling the cell mixture at 95°C for 10 min. The supernatant was collected by centrifugation and stored at -20°C until further analysis. The total protein content of the supernatant was determined by the Bradford (1976) dye-binding method as described previously.

3.3.5.2 Electrophoresis and western blotting

SDS-PAGE was carried out by the method of Laemmli (1970), using 20% separating gels containing 0.5% N, N'-methylenebisacrylamide. About 8 µg of protein per lane were loaded, including a histone marker of a concentration of 1 mg/ml and run at 150-200V at room temperature. One half section of the gel was stained with Coomassie

Brilliant Blue R-250 (USB Chemical Industries, Cleveland, Ohio) and destained in 7% acetic acid and 25% technical ethanol. The other half of the gel was blotted on a nitrocellulose membrane (Amersham, Hybond-C, 0.45 μm, 30 cm x 3 m, Cat No: RPN.303 E) and Hsp12p expression was detected using polyclonal Hsp12p antibodies antiserum prepared as in appendix B (purification performed by Robert Karreman from UCT). The resultants Hsp12p bands intensity in average pixel values from both SDS-PAGE gels were quantified with the UNI-SCAN program (Silk Scientific Corporation, Utah, U.S.A, Gel version 5.1).

3.3.6 Budding scars

The numbers of budding scars on the cell walls of yeast cells pellets from one millilitre of wort sample present in the bottom slurry and in the yeast suspension were both counted manually by overnight incubation with 50 μM calcofluor stain (Sigma, Cat No: F3543-IG) with the use of a 60x objective lens fluorescent microscope (Model Nikon Eclipse E400, D-F-T, Germany). One hundred cells per sample were selected by counting the number of visible budding scars per cell. The average number of budding scars per cell was determined.

3.4 Stress treatments

At the time of cropping (3.1.4), yeast left in suspension were harvested, washed and repitched as described previously. In duplicate consecutive fermentations, the bottom slurry and the yeast in suspension were harvested, washed twice with sterile double-deionized water by centrifugation at 8 000 x g for 20 min at 4°C. Each yeast type was resuspended in sterile PBS (100 mM phosphate, 150 mM NaCl, pH7.4). Duplicate Erlenmeyer flasks containing 50-ml PBS were inoculated at a level of 6 x 10^6 cells/ml and subjected to the following stresses:

- 120 ml/l and 180 ml/l ethanol for 4h at 26°C shaken at 100 rpm
- 120 g/l and 200 g/l sorbitol for 4h at 26°C shaken at 100 rpm
- Heat shock at 40°C for 1h
- Freeze shock at -20°C for 4h

A control in sterile PBS, kept at room temperature, was used for a full analysis of cell metabolites, and yeast count, as described in the previous sections.

3.5 Fermentation ability

3.5.1 Mini-fermentations

Each type of stressed yeast cells, including the PBS control, were pitched at 1.57 x 10^6 viable cells/ml per °P into a 200 ml glass bottle containing 100 ml autoclaved "Black Label" wort. Each bottle was enclosed by a gas-trap, filled with distilled water to facilitate CO_2 release, and incubated at 15°C for 12 days.

3.5.2 Fermentation rate

At 24h intervals for a period of 12 days, each bottle was weighted in order to measure the fermentation rate of the control yeast cells compared to the stressed yeast cells.

Chapter 4: Results and discussion

4.1 Introduction

During successive high-gravity fermentations, brewer's yeast is exposed repeatedly to biological, chemical, physical and yeast handling stresses (Smart, 1999). The subsequent fermentation conditions like high ethanol concentrations, elevated osmotic pressure from concentrated wort, and nutrient starvation promote negative effects on the yeast viability. All of these stressful factors contribute to poor fermentation performance, which will result in low-quality beer. In order to understand and follow the yeast fermentative behaviour during consecutive re-pitching fermentations, selected markers were evaluated on a daily basis over a period of six re-pitching fermentations, in two separate trials, at two different temperatures, 14°C and 18°C. In order to assess the yeast's fermentation activity, biomarkers were investigated to determine the impact of serial re-pitching over successive fermentations on the yeast's vitality to overcome physiological stress (Mochaba *et al*, 1998).

4.2 Evaluation of methods to assess yeast stress

4.2.1 Aldo-keto reductase enzyme activity

An aldose reductase is an enzyme encoded by the *GRE3* gene that uses NADPH as a co-factor, and which was purified from *S. cerevisiae*. Aldose reductase enzyme showed similarities to xylose reductases reported to occur in other yeasts (Kuhn *et al*, 1995; Garay-arroyo and Covarrubias, 1999). The *GRE3* expression is induced mostly under osmotic stress because this enzyme catalyzes the *in vivo* production of sorbitol that promotes aldo-keto reductase enzyme accumulation (Aguilera and Prieto, 2001). This particular enzyme is known to play a role in toxicity removal by catalyzing the reduction of carbonyl compounds such as aldehydes, present in wort during fermentation (Laurent *et al*, 1995). Maximum activity was observed with D,L-glyceraldehyde substrates, as described in Appendix D. The enzyme showed increased K_m values with substrates such as *p*-nitrobenzaldehyde, D-glucose, D-

xylose and L-arabinose (Kuhn *et al*, 1995). The use of aldo-keto reductase enzyme activity as a vitality marker during brewing was investigated. No results are shown because no definite trends in enzyme activity levels, and particularly how the enzyme is regulated under stress conditions, were observed. It is to be noted that this particular enzyme assay was found to be laborious, inconsistent and time-consuming to perform as a routine vitality test.

4.2.2 Acidification power test

In brewing research the acidification power test (APT) is considered as a potential biomarker for the assessment of yeast quality. Measurement of the plasma membrane proton efflux correlates to fermentation performance prior to pitching. Plasma membrane damages caused by stress (Section 2.8.2.1) may result in reduced yeast growth, loss of viability and decrease in metabolic activity because of membrane fluidization (Van Zandycke *et al*, 2003). The principle of the acidification power test is to measure proton movement across the plasma membrane. The yeast is first re-suspended in water for 10 min to set up a fixed ratio between extracellular and intracellular hydrogen ion concentrations (passive proton efflux), from which energy is provided exclusively by the yeast's endogenous reserves like glycogen and trehalose. This first measurement is an indication of the yeast's growth potential, called the "spontaneous acidification power" or WAP, which is subtracted from 6.3 (pH value of water) (ΔpH_{10}) (Kara *et al*, 1988). The fermentable substrate, glucose, is added to the yeast suspension for another 10 min. The proton efflux is induced and becomes dependent on both endogenous and exogenous energy sources. The plasma membrane ATPase action, together with the membrane potential, constitutes the driving force for the uptake of solutes (Van Zandycke *et al*, 1999). This glucose-induced acidification power (ΔpH_{20}), or GAP, represents the fermentative activity of the yeast (Kara *et al*, 1988). The true vitality of a yeast cell population is represented by the glucose-induced proton efflux (GIPE), which can be calculated by subtracting the WAP from the GAP. GIPE has been observed to inversely correspond with intracellular trehalose levels, as an indicator of stressed yeast populations (Van Zandycke *et al*, 1999). Interpretation of both acidification power tests is very simple. The decrease in ΔpH_{10} is relatively small, followed by a large rapid decrease in ΔpH_{20} (Dinsdale and Lloyd, 1995). A decline in WAP is often related to long-term nutrient

starvation because endogenous energy reserves are being exhausted, while a decline in overall GIPE shows membrane damages under exposure to high ethanol concentrations (Van Zandycke *et al*, 2003). Distinctive GIPE values are in the range of 1.4 to 3 or more (Dinsdale and Lloyd, 1995).

No results are shown here for the acidification power test because the values obtained were highly variable and inconsistent with those reported in the literature (Van Zandycke *et al*, 1999; Van Zandycke *et a.*, 2003; Patino *et* al., 1993; Kara *et a.*, 1988; Dinsdale and Lloyd, 1995). Kara *et al* (1995) and Iserentant *et al* (1996) supported that the mean values that were obtained in this study were found to reflect large standard deviations and was considered to lack sensitivity at high AP values, suggesting that the method is unreliable. Therefore, it was decided not to pursue this method further as a stress indicator. The acidification power test is however particularly easy to perform, with simple and affordable equipment.

4.2.3 Adenylate kinase enzyme activity

The enzyme adenylate kinase (called AK), is an intracellular enzyme, which is not secreted by "healthy" cells. AK catalyzes the reaction 2 ADP ↔ ATP + AMP. The enzymatic activity is detected by using a linked assay, in which firefly luciferase enzyme quantifies the ATP produced in excess of purified ADP (Driscoll and Ramsay, 2002). The AK activity level is calculated from an increase in light output during the assay after calibration with an ATP standard. AK extracellular activity is recognized as a good indicator of yeast cell lysis (Cameron-Clarke *et al*, 2003). The release of AK is found to be a good indicator of proteolytic enzyme release into the surrounding medium. The proteolytic enzyme release causes the breakdown of proteins present in the foam and directly affects the quality of beer foam (Driscoll and Ramsay, 2002). Under autolysis conditions, including high ethanol concentrations and extended storage periods at high temperatures, the AK assay is used to sensitively monitor the integrity of brewer's yeast cultures. It can be used to optimize yeast-handling management procedures towards efficient fermentation performances and yeast quality (Driscoll and Ramsay, 2002). Extracellular AK units for "healthy" yeast are below 100 but AK values increase up to 3000 units under conditions favouring autolysis (Driscoll and Ramsay, 2002). Yeasts with higher AK values (as high as

1500 units), reported in older re-pitching fermentations, and are more susceptible to autolysis. No results are reported here for adenylate kinase enzyme activity. Reagents used to perform the assays were faulty, hence the blank values were much too high, and AK activities from the samples could not be correctly determined.

4.3 Assessment of yeast stress during brewing fermentation

4.3.1 External fermentation parameters to assess yeast quality

4.3.1.1 Yeast count

Data on yeast count profiles recorded daily during successive fermentations at 18 and 14°C are illustrated in Figs. 4.1 and 4.2 respectively. A constant pitching rate of 1.5 x 10^7 cells/ml per °P was maintained throughout the experimental procedures. A lower pitching count than 1.5 x 10^7 cells/ml was used for fermentations 3 and 6 because of a smaller number of re-suspended yeast cells available. Shortage of the latter in pitching yeast cells did not appear to affect the initial yeast counts obtained for these particular fermentations. Typical growth curves were observed for each of the re-pitching fermentations, at both temperatures. The starting count was between 1-2 x 10^7 cells/ml, followed by a 2- to 4-fold increase in cell numbers at 48h and a 1- to 3-fold decrease by 96h. These results showed a similar growth pattern curves, but with relatively lower yeast counts compared to the results of McCaig *et al.* (1992). Further experiments with an initial pitching count of over 2 x 10^7 cells/ml reached a maximum count of approximately 5.2 x 10^7 cells/ml at 48h, and decreased to 1.5 x 10^7 cells/ml on the fifth day of fermentation. The fermentation temperature of 18°C was found to stimulate yeast growth and metabolism and resulted in a higher maximum cell count after 48h (Quain and Boulton, 2001). In the RFs 5 and 6 (Fig. 4.2), a 5-fold increase in cell numbers was observed and this suggests that the yeast's replication rate becomes more active in successive re-pitching fermentations. Similar starting yeast counts of between 1-2 x 10^7 cells/ml, followed by a maximum peak count of approximately 4.8 x 10^7 cells/ml, were observed (McCaig *et al*, 1992).

4.3.1.2 Yeast viability

The methylene blue staining procedure is the standard method for the determination of yeast viability recommended by the Institute of Brewing (O'Connor Cox, 1997; Smart *et al*, 1999). The viability percentages for successive re-pitching fermentations at 18°C and 14°C are presented in Figs. 4.1 and 4.2 respectively. The freshly propagated yeasts in the initial fermentation had a viability that was close to 100% at the time of pitching, at both temperatures. This high initial viability originated from optimized propagation conditions at a temperature of 20°C during the final stage of propagation (Quain and Boulton, 1987). The original viability was lower than 90% at the time of pitching for most of the re-pitching fermentations (except for the initial fermentation), in particularly for RFs 2 and 3 (Fig. 4.1). As fermentation progressed, the maximum viability was achieved at approximately 48h for most fermentation. This indicated during this time period the yeast population was at its most reproducible and viable status (Smart *et al*, 1999). The low viability observed in pitching yeast comprised the more physiologically stressed cells because of previous ethanol exposure in the prior fermentation and handling processes such as centrifugation and vortexing (Jenkins *et al*, 2003). Overall, the viability remained stable and generally high (>90%) throughout the six fermentations. The lowest initial viability was observed for RFs 2 and 3.

Yeast viabilities at 14°C were observed to be mostly above 95% for all re-pitching fermentations, except lower initial fermentation viability for RFs 3, 4 and 6 (Fig. 4.2). The viability increased during the later stages of fermentation, around the 48-72h periods. Cunningham and Stewart (1998) measured the change of yeast viability at 13°C by methylene blue staining and obtained comparable results, with a pitching viability of 97.5%. They observe an increase at 72h to 98.5% viability that dropped to 97% after 96h fermentation. The yeast was most likely to be physiologically more "healthy", given that elevated temperatures accelerate yeast metabolism, consuming additional energy reserves to replicate faster and to diminish viability substantially.

Other methods to determine yeast viability such as direct plate counts or slide cultures have been evaluated in the brewing industry (Boulton and Quain, 2001). The plate count method was found to be inappropriate for certain practical reasons: (a) the

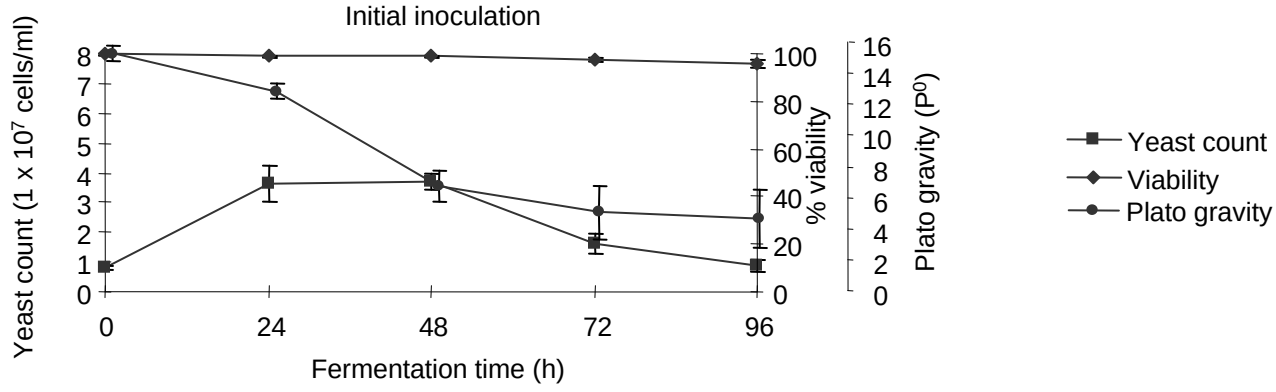
incubation period required for macro colonies to develop and (b) underestimation of viability, since it only takes into account reproductive cells and not all viable cells (Parkkinen *et al.*, 1976).

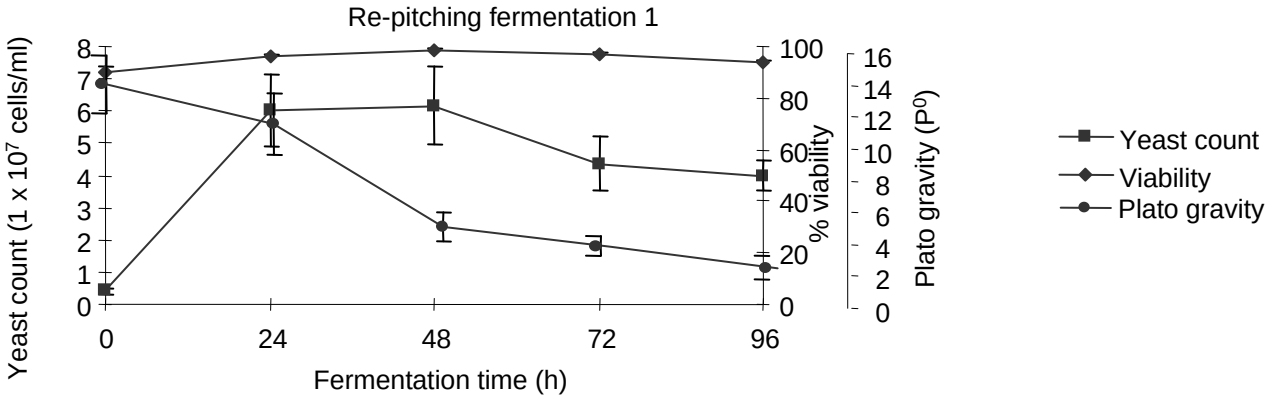
4.3.1.3 Wort attenuation

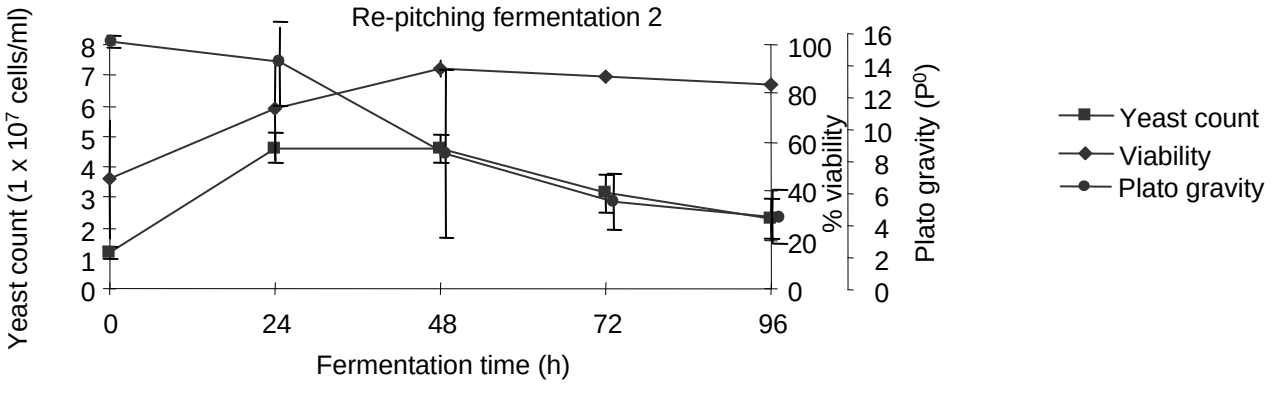
To obtain the desired ethanol level (7-11%) in high gravity brewing, selected fermentation parameters have to be implemented. These include: as an increase in the pitching rate (1-2 x 10^7 cells/ml per Plato gravity), an increase in wort oxygenation (16-18 ppm), supplementation of the wort with an adequate amount of nitrogen (FAN) and yeast food to stimulate maximum cell synthesis (Mc Caig *et al*, 1992). The wort attenuation, in °P, for fermentations at 18°C is shown in Fig. 4.1. The original wort gravity was approximately 15°P and the lowest final attenuations of 2.55, 2.85 and 2.45°P respectively were achieved in RFs 1, 3 and 5. The other re-pitching fermentations (initial fermentation, and RFs 2, 4 and 6) attained final attenuations of between 3.6 and 4.6. This suggests that at slightly lower temperatures, complete wort attenuation (<3°P) would require longer fermentation times of up to 144h to obtain the ethanol yield desired. In each fermentation profile (Figs. 4.1 and 4.2), the largest drop in gravity was generally found in the time period in which yeast cells had reached their maximum size and were actively fermenting (Casey *et al*, 1984). This gravity decrease corresponded with the highest observed yeast count and viability (Fig. 4.1). For example, at 48h for the initial fermentation and RF 1, the maximum yeast counts were 3.7 and 6.2, with an equivalent viability of 99% and 98% respectively. Similar conclusions could be deduced for subsequent fermentations. The 16°P wort reached a final attenuation of around 2.5 at a temperature of 21°C over a fermentation time of 168h (Amore *et al*, 1991).

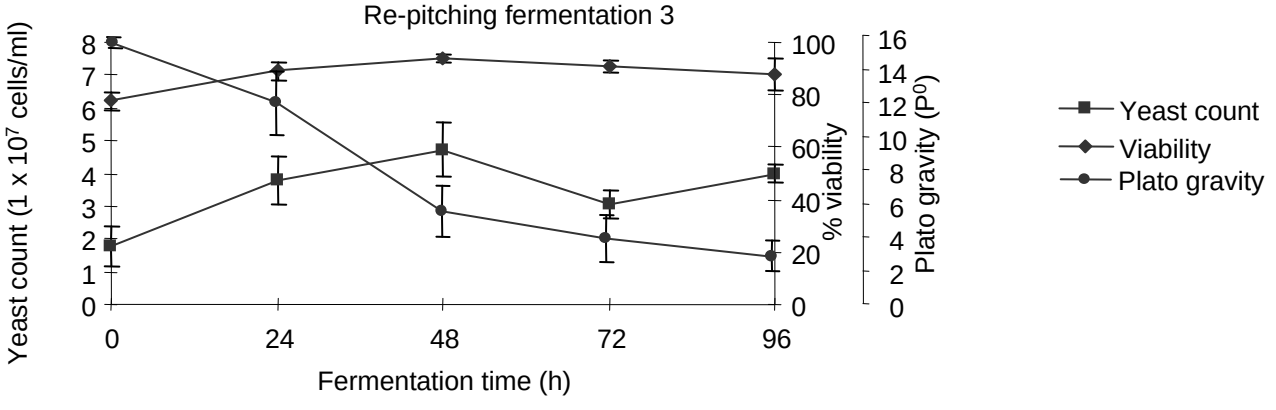
Conventional lager fermentation at 14°C showed incomplete wort attenuation for most re-pitching fermentations, with observed final attenuations between 4.4 and 9.4 at 96h (Fig. 4.2). The decrease in temperature directly affects wort attenuation values because of a decrease in yeast metabolism and fermentation rate (Amore *et al*, 1991). Similarly, a reported attenuation curve at 16°C with an initial wort gravity of 16°P reached an attenuation of 5°P after a period of four days (Fernández *et al,* 1985). This

demonstrates that slower fermentation occurring at lower temperature; attain lower final attenuation values associated with longer fermentation times (Amore, 1992). The cause of incomplete attenuations in certain RFs (3 to 6) might be the result of the required pitching rates that were not attained because of a reduced number of yeast cells available for re-pitching purposes. The maximum decrease in gravity was also associated with a maximum yeast count and viability at 14°C (Fig. 4.2).


Initial inoculation
Yeast count (1 x 10^7 cells/ml)
% viability
Plato gravity (P^0)
Fermentation time (h)
0
24
48
72
96
Yeast count
Viability
Plato gravity


Re-pitching fermentation 1
Yeast count (1 x 10^7 cells/ml)
% viability
Plato gravity (P^0)
Fermentation time (h)
0
24
48
72
96
Yeast count
Viability
Plato gravity


Re-pitching fermentation 2
Yeast count (1 x 10^7 cells/ml)
% viability
Plato gravity (P^0)
Fermentation time (h)
0
24
48
72
96
Yeast count
Viability
Plato gravity


Re-pitching fermentation 3
Yeast count (1 x 10^7 cells/ml)
% viability
Plato gravity (P^0)
Fermentation time (h)
0
24
48
72
96
Yeast count
Viability
Plato gravity

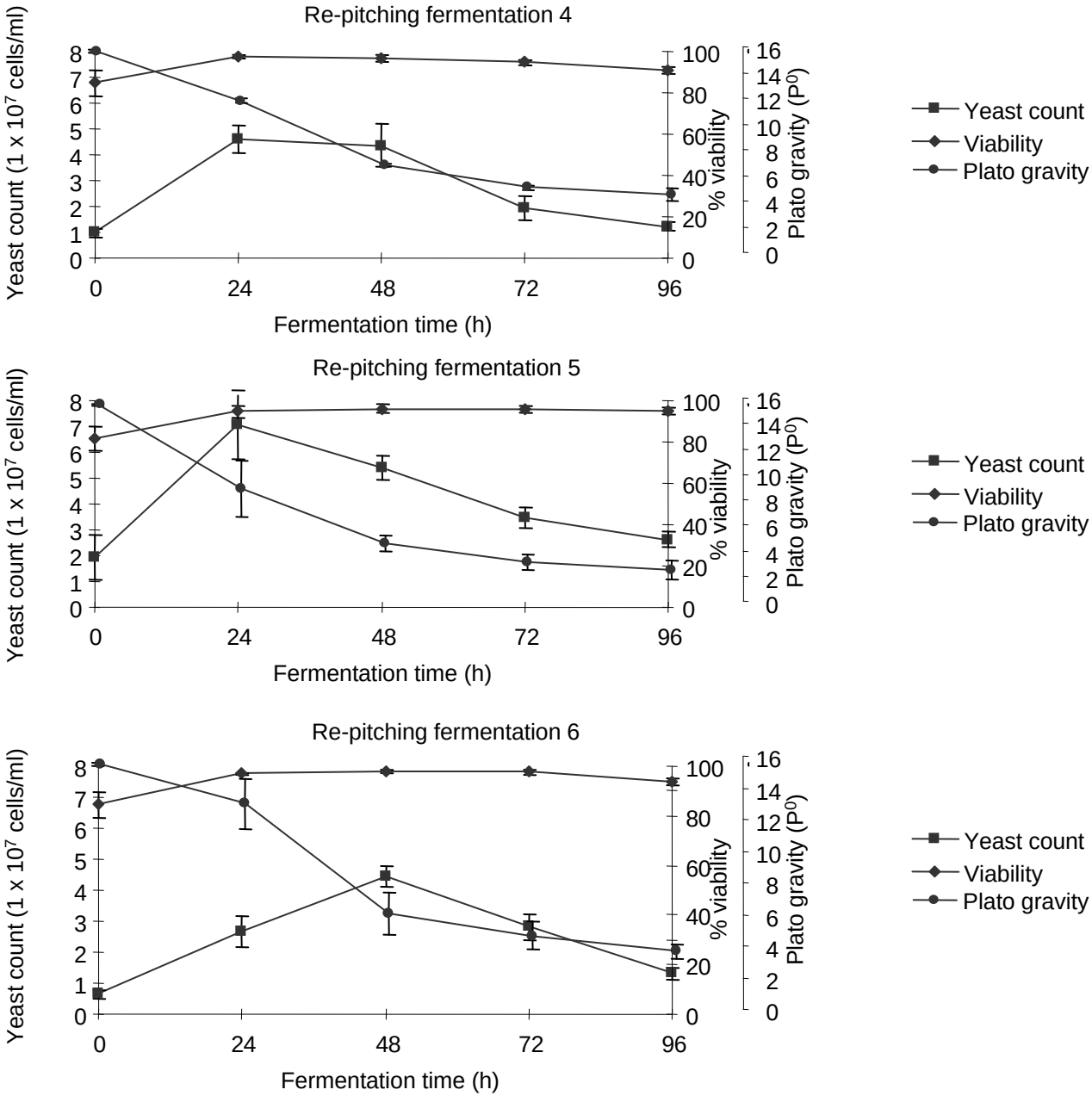
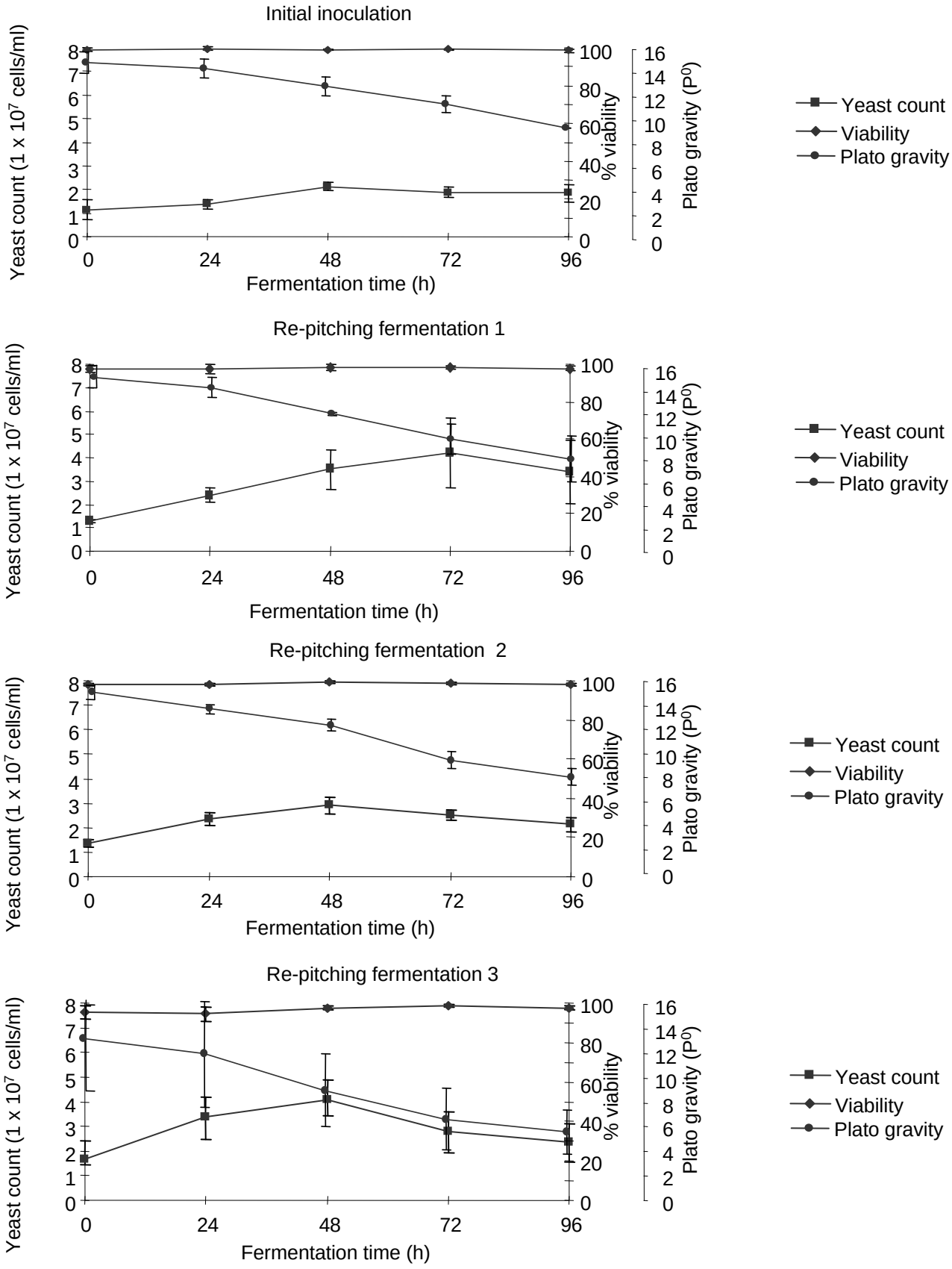


Fig 4.1: Yeast count, viability and wort attenuation for six re-pitching fermentations at a temperature of 18°C. The errors bars represent the standard deviation for three triplicate determinations.


Initial inoculation
Yeast count (1 x 10^7 cells/ml)
% viability
Plato gravity (P^0)
Fermentation time (h)
Yeast count
Viability
Plato gravity
Re-pitching fermentation 1
Yeast count (1 x 10^7 cells/ml)
% viability
Plato gravity (P^0)
Fermentation time (h)
Yeast count
Viability
Plato gravity
Re-pitching fermentation 2
Yeast count (1 x 10^7 cells/ml)
% viability
Plato gravity (P^0)
Fermentation time (h)
Yeast count
Viability
Plato gravity
Re-pitching fermentation 3
Yeast count (1 x 10^7 cells/ml)
% viability
Plato gravity (P^0)
Fermentation time (h)
Yeast count
Viability
Plato gravity

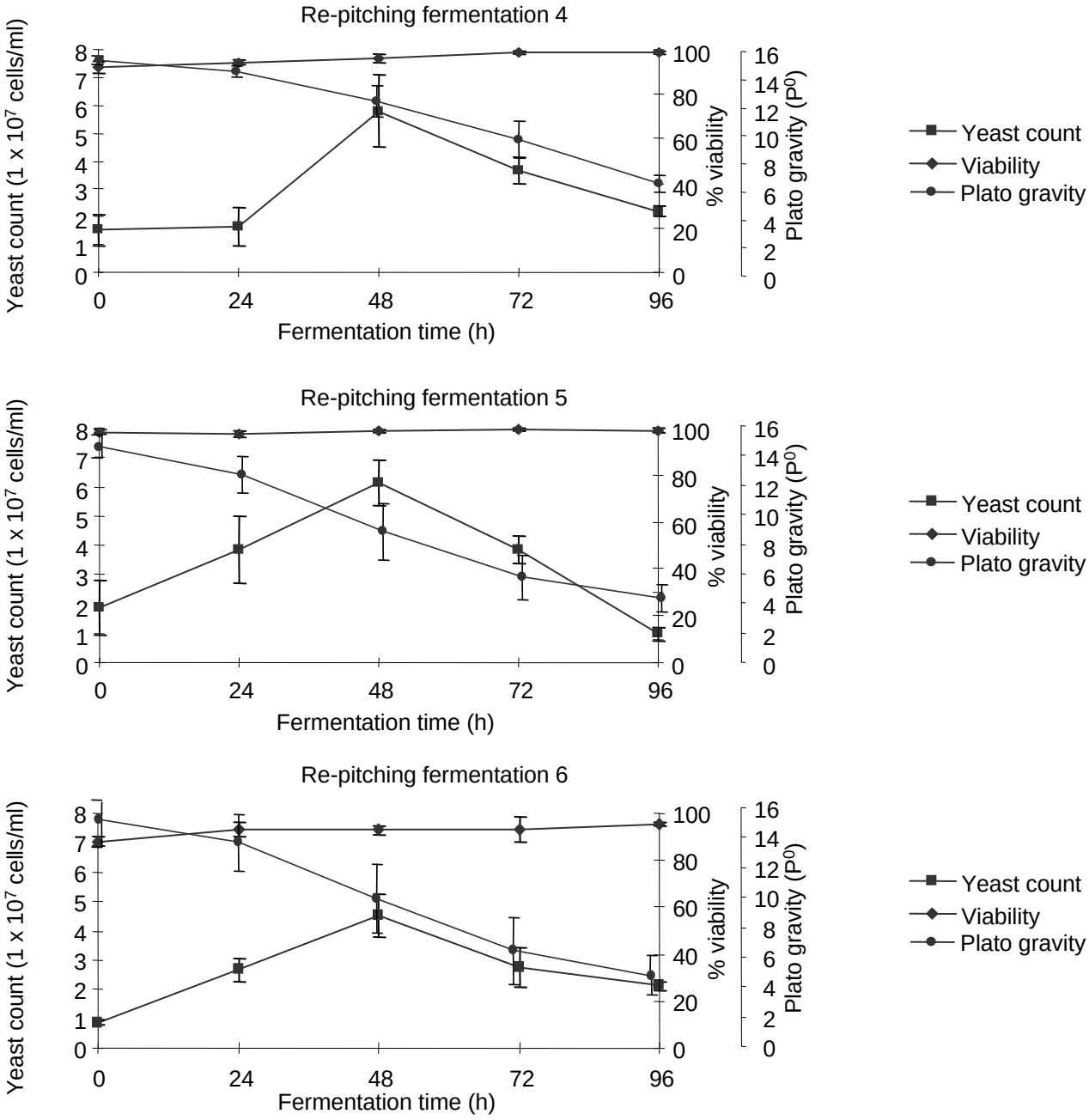
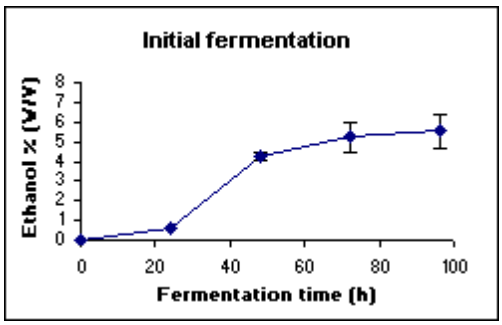


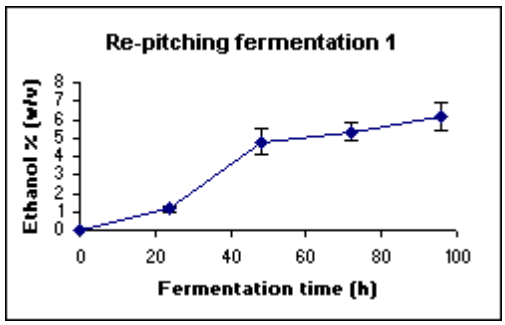
Fig 4.2: Yeast count, viability and wort attenuation for six re-pitching fermentations at a temperature of 14°C. The errors bars represent the standard deviation for three triplicate determinations.

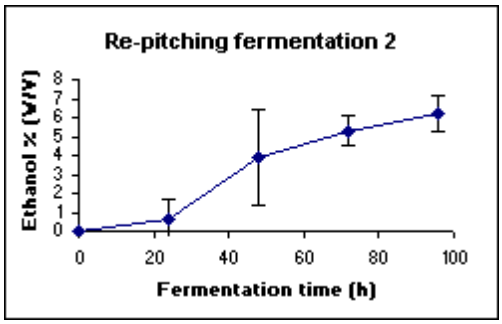
4.3.1.4 Ethanol production

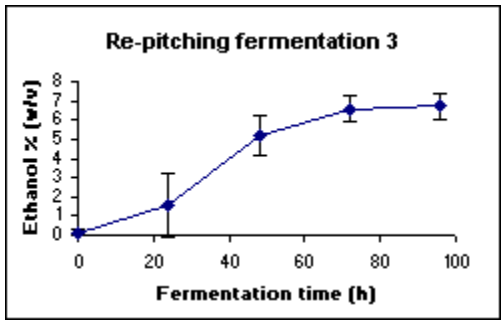
The fermentation of high-gravity worts generally produces beer with ethanol concentrations >6% (w/v) (Fernandez *et al*, 1985). These concentrations should not limit fermentation since brewer's yeast is moderately tolerant to ethanol concentrations up to 10% (Heggart *et al*, 1999). However, during fermentation conditions the inhibition of cell growth and viability proportionally increase with increasing ethanol concentrations, but the fermentative ability remains high, and is only inhibited at very high ethanol concentrations (>12 % v/v) (Amore, 1991).

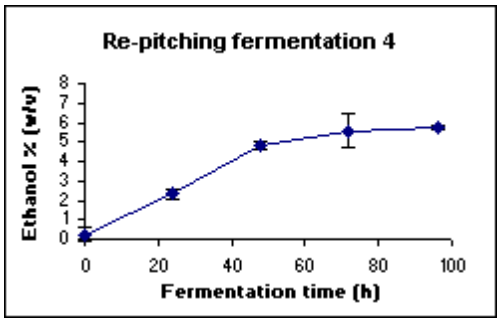
The ethanol concentration profiles for all re-pitching fermentations at 18°C showed increasing initial ethanol concentrations during the first 48h of fermentation, during which time the yeast growth rate and viability rapidly increased without apparent ethanol inhibition effects (Figs. 4.1 and 4.2). At approximately 72h, when the ethanol reached concentrations above 5% (w/v), a decrease in the growth rate and viability in all re-pitching fermentations was observed. Final ethanol concentrations ranging from 5.5 to 6.9% were expected for the 15°P gravity wort (Fernandez *et al*, 1985; Amore *et al,* 1991). RF 5 produced the highest ethanol concentration of 6.9% (w/v) (Fig. 4.3).

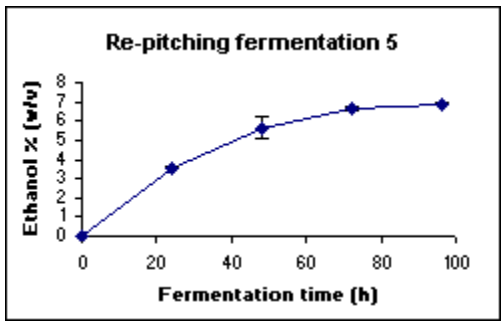












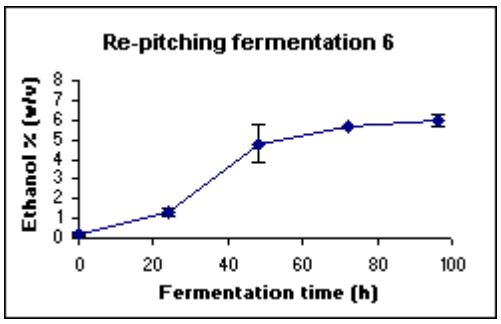
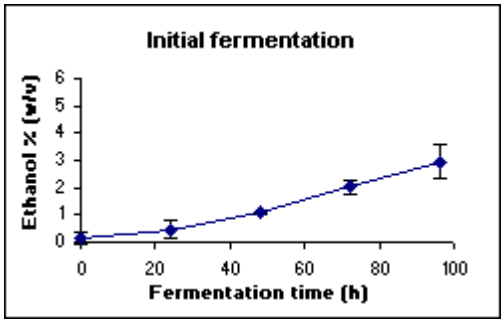
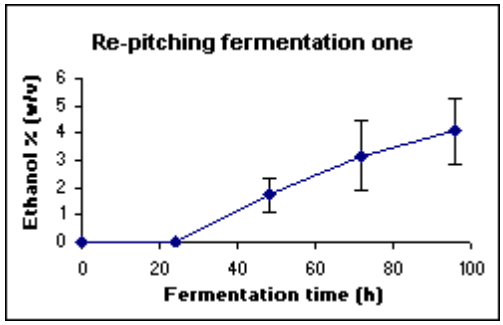
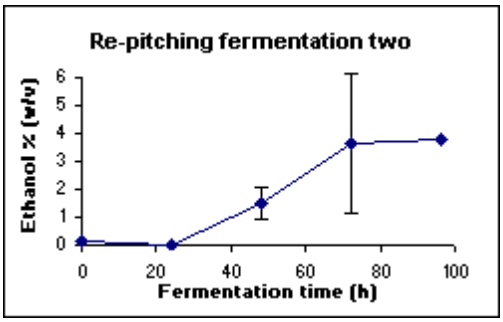


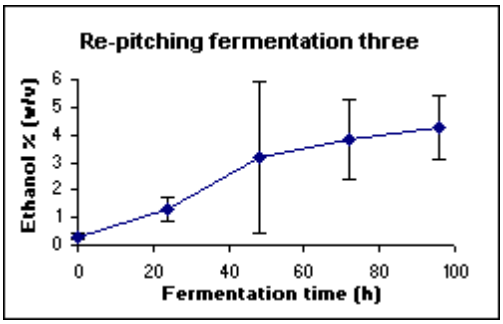
Fig. 4.3: Ethanol production over a period of four days for six re-pitching fermentations at a temperature of 18°C. (Each data point represents the average of duplicate fermentations with errors bars representing the range of the data between the two points.)

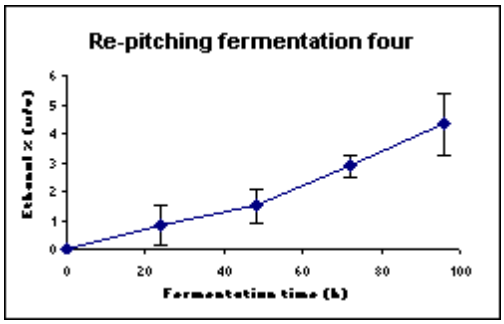
Fig. 4.4 represents the ethanol concentrations produced at 14°C for all re-pitching fermentations. As discussed in the previous section, complete wort attenuation was not achieved after 96h and, subsequently, the final amount of ethanol was reduced (Amore *et al*, 1991). At 14°C the yeast fermented at a slower rate than at 18°C, the ethanol concentration increased at a similar slower rate. However, ethanol was still being produced after 72h, and reached its highest concentration at 96h. If the fermentation was allowed to continue up to 144-160h, complete attenuation might have been attained producing ethanol concentrations of 6-7%, equivalent to ethanol concentrations produced at 18°C (Fig. 4.3). Higher yeast growth rates and viability were achieved due to minimum ethanol inhibitory effects on yeast metabolism because of lower ethanol concentrations at 14°C (Stewart *et al*, 1988). RF 5, followed by RF 6, 4, 3, 1, 2 and the initial fermentation, produced the highest ethanol concentration.

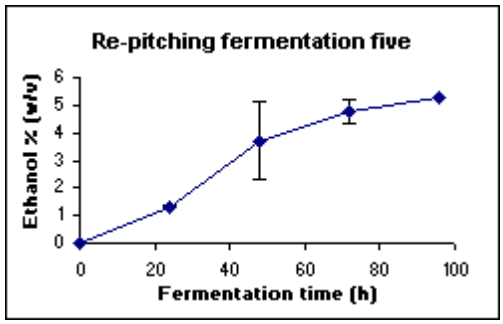












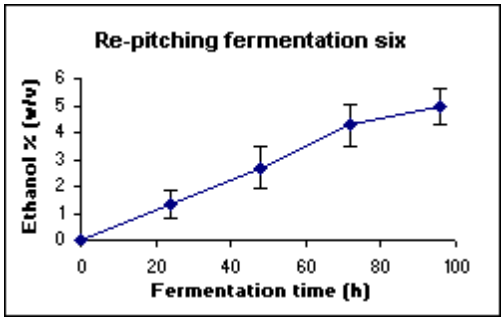


Fig. 4.4: Ethanol production over a period of four days for six re-pitching fermentations at a temperature of 14°C. (Each data point represents the average of duplicate fermentations with errors bars representing the range of the two points.)

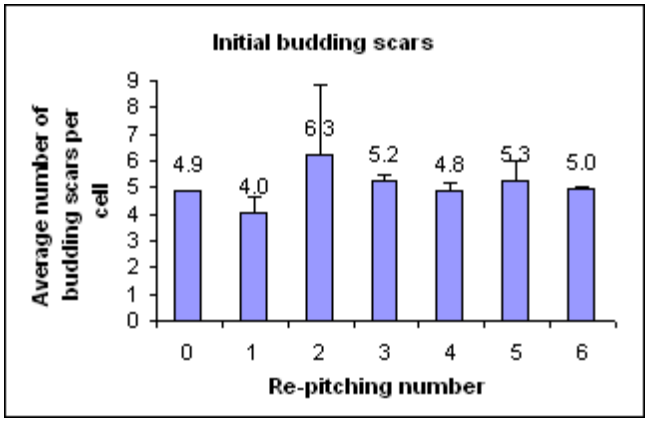
4.3.1.5 Budding scars formation

S. cerevisiae cells are known to divide up to 30-50 times during their entire lifespan prior to cell death (Powell *et al*, 2003). The determination of the replicative age by budding scars enumeration on the cell wall within the yeast slurry gives significant information on the subsequent fermentation performance (Powell *et al*, 2000). As yeast cells divide, distinctive morphological, physiological and metabolic changes take place within the cell (Powell *et al*, 2003). Such modifications include an increase in cell size, causing a decrease in the availability of the surface area for budding and nutrient exchange with the environment. This results in a slower cell division rate and reduced fermentation performance (Powell *et al*, 2000).

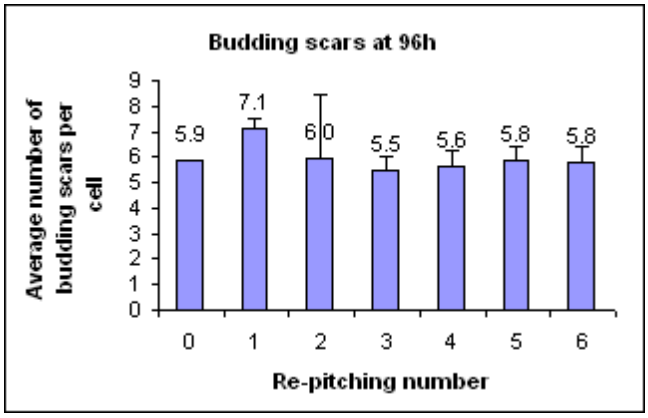
Figs. 4.5 and 4.6 represent the average number of budding scars per cell for re-pitching fermentations, measured by calcofluor staining as described in the experimental procedures (Section 3.3.6). Fig. 4.6 only includes data for the first two re-pitching fermentations. The accuracy of the fluorescent measurements could have been influenced by the fluorescent scattering emitted by older cells (Deans *et al*, 1997). A fluorescent cell, showing the budding scars on either side, is shown in Fig. 4.7. Data presented in Fig. 4.5 and 4.6 reveal little information concerning the number of times cells divide throughout the entire fermentation. A very small increase or no increase at all, in the number of budding scar between the start of fermentation and the time of cropping can be seen. Furthermore, no particular trend in number of budding scars per cell can be seen as the number of re-pitching fermentations increases or as the cells aged. Both fermentation temperatures gave an approximate number of budding scars in the range between three and six.

The results of this study were in contrast to results reported by Powell *et al* (2000), who showed that the number of bud scars increase with age during fermentation and with the number of re-pitching fermentations. In the present study, samples were taken from actively fermenting yeast in the middle of the fermentation tube, whereas the samples of Powell *et al* (2000; 2003) were flocculated yeast from the cone of the fermentation tube. Therefore it appears that the flocculated yeast might have

contained more bud scars per cell compared with cells actively fermenting. However, this requires confirmation.

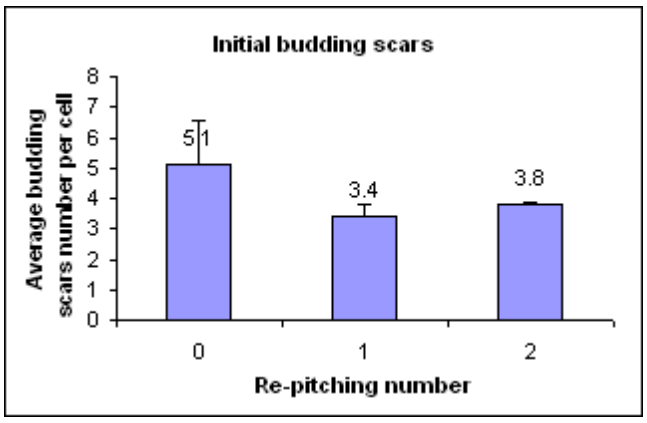


(a)

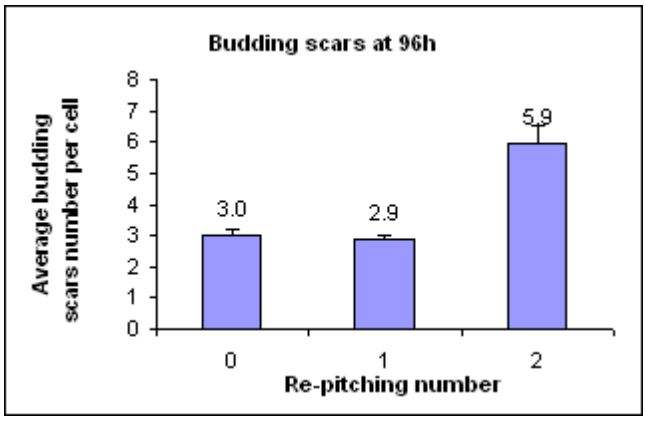


(b)

Fig. 4.5: The average number of budding scars per cell (bars represent the range of duplicate determinations) for each re-pitching fermentation at a temperature of 18°C, measured at: (a) 0h and (b) 96h.



(a)



(b)

Fig. 4.6: The average number of budding scars per cell (bars represent the range of duplicate determinations) for each re-pitching fermentation at a temperature of 14°C, measured at: (a) 0h and (b) 96h.

Fig. 4.7: Fluorescent yeast cell with budding scars on either poles of the cell. (600X enlargement).

4.3.1.6 Wort carbohydrates profiles

The consumption of fermentable sugars at 18°C and 14°C is represented in Figs. 4.8 and 4.9. The wort used in this study consisted of dextrose syrup containing 70% maltose, 27% glucose, and 4% maltotriose, described as high glucose syrup (Appendix A). The sugar composition was comparable to maltose syrup added to similar wort and contains 50-55% maltose, 20-25% glucose, 10% maltotriose and the remainder dextrins (Phaweni *et al*, 1993). The fermentable sugar consumption at 14°C (Fig. 4.9) was much slower compared to the fermentation at 18°C (Fig. 4.8) and significant amount of each sugar were still present at the end of fermentation at 14°C. These observations correlated with the slow decrease in Plato gravity at 14°C, shown in Fig. 4.2.

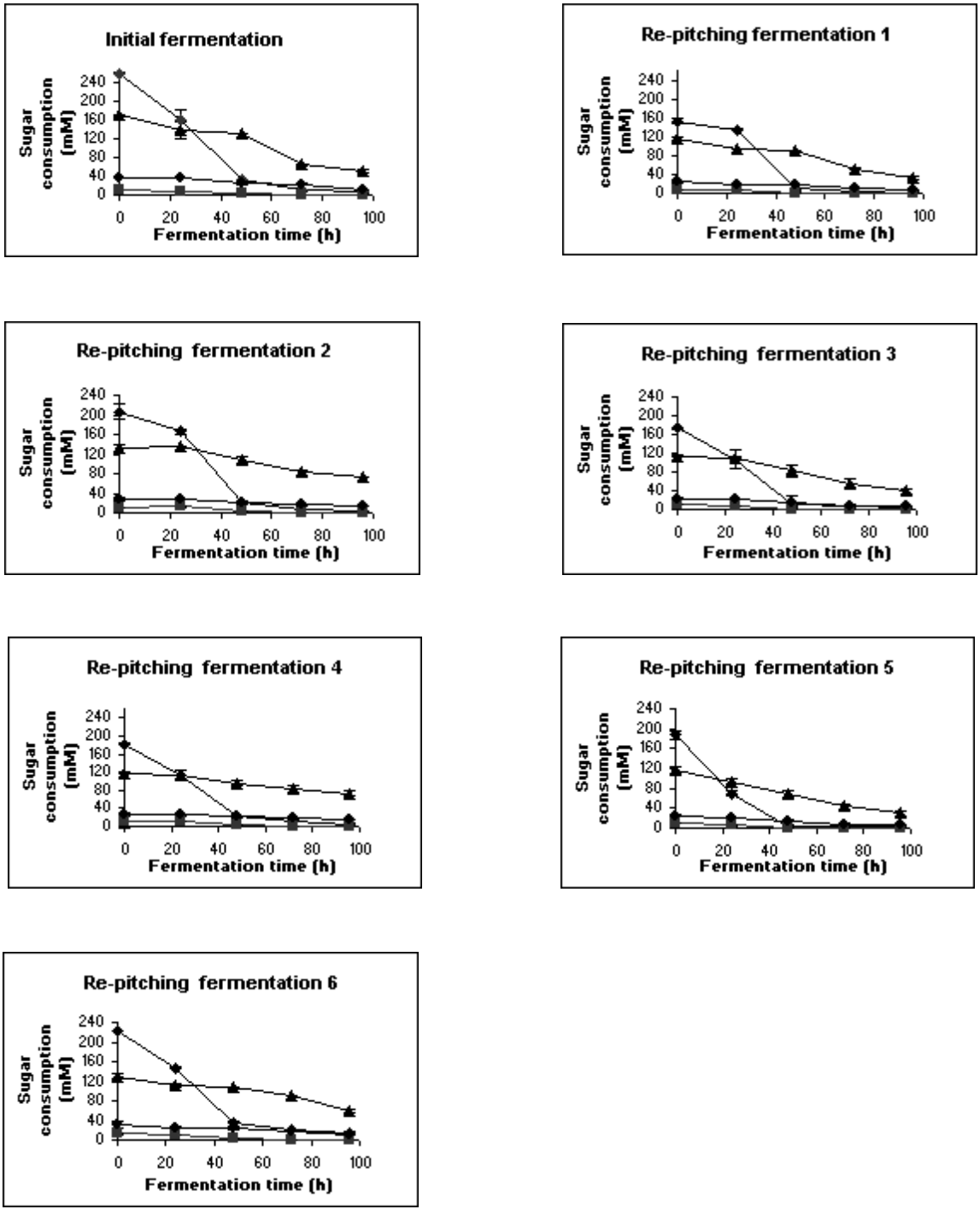


Fig. 4.8: Mean carbohydrates profiles of six successive re-pitchings at a fermentation temperature of 18°C. (Bars indicate the standard deviation of the triplicate determinations.) Glucose (♦), fructose (■), maltose (▲) and maltotriose (●).

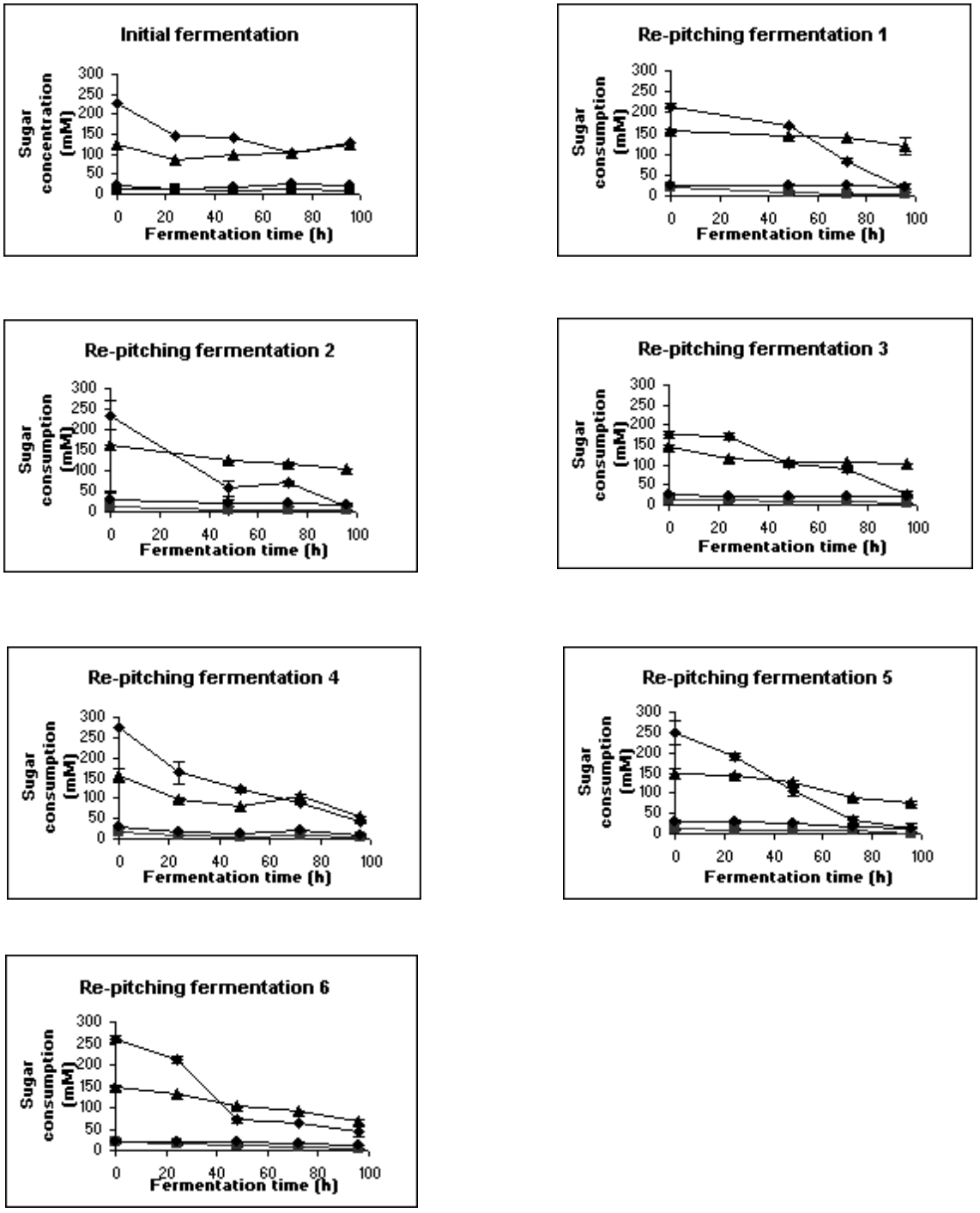


Fig. 4.9: Mean carbohydrates profiles of six successive re-pitchings at a fermentation temperature of 14°C. (Bars indicate the standard deviation of the triplicate determinations.) Glucose (♦), fructose (■), maltose (▲) and maltotriose (●).

Glucose

Most South African breweries add up to 35% dextrose in the wort as an adjunct (Phaweni *et al*, 1991). Dextrose contains mostly glucose as the primary sugar. Glucose is preferred to fructose by the yeast as an energy reserve for growth. However, glucose represses the uptake of maltose and maltotriose during fermentation (Amore *et al*, 1989). In the present study, glucose was immediately used upon inoculation, with the most rapid consumption occurring between 24h and 48h at 18°C (Fig. 4.8). The initial glucose concentration appeared to regulate the use of remaining sugars. However, after 24h, the glucose concentrations did not seem to affect the maltose and maltotriose assimilation. A small glucose concentration, between 4 and 33mM, remained at the end of fermentation, suggesting that the yeast was unable to utilize low glucose concentrations present in high-gravity wort, and it might require a longer fermentation time to utilize glucose completely (Fig. 4.8) (Phaweni *et al*, 1992). In fermentations conducted at 14ºC, the initial glucose concentrations were rapidly used and a 1.2- to 1.7-fold decrease was observed for most re-pitching fermentations by 24 - 48h, except for RF 2, which exhibited a 4.2-fold decrease by 48h (Fig. 4.9). After 48h, glucose utilization slowed down and high residual sugar concentrations of between 15 and 65 mM remained. However, it was difficult to assess the ability of the yeast to efficiently catalyze glucose since the initial glucose concentrations varied between the worts used in the different re-pitching fermentations. No constant assimilation was observed. Phaweni *et al* (1992) reported that glucose consumption, with a 35% glucose adjunct, decreased from 7 to 2 g/100ml between 0 to 144h. High remaining glucose concentrations in the wort were found to inhibit uptake of other sugars (especially maltose and maltotriose), thereby reducing fermentation efficiency (Verstrepen *et al*, 2004).

Fructose

Initial fructose concentrations in wort are much lower than glucose concentrations and fructose is utilized at a much slower rate than glucose at 18°C (Fig. 4.8). For most re-pitching fermentations (except RFs 4 and 6) fructose was completely utilized by 72h, when the glucose concentrations were at their minimum (Fig. 4.8). Complete fructose utilization is normally suppressed by high glucose concentrations (Amore *et al*, 1991).

From the amount of fructose utilized between the different re-pitching fermentations, RFs 3 and 5 utilized fructose at a faster rate, followed by RFs 1 and 2, which had similar utilization rates. In fermentations conducted at 14ºC, the fructose was incompletely utilized by the end of fermentation (Fig. 4.9). An average of 2-8 mM fructose remained in the beer and sweet, off-flavour compounds, such as acetaldehydes, were produced (Amore *et al*, 1989). As with glucose utilization, the sequential re-pitching of the yeast did not appear to affect the ability of the yeast to utilize fructose.

Maltose and maltotriose

Maltose and maltotriose are two sugars used by yeast for the "maintenance phase" of non-growing cells to convert sugars into ethanol (Searle and Kirsop, 1979). At the start of fermentation the utilization of the complex fermentable sugars, maltose and maltotriose, is repressed in brewing yeast in elevated glucose concentrations (Phaweni *et al*, 1993). The most rapid maltose utilization for all re-pitching fermentations was found to occur between 48h and 72h at the point where glucose and fructose concentrations are at a concentration where the repression effect is removed (Meneses *et al,* 2002). High maltose concentrations for all re-pitching fermentations remained on the last day of fermentation, in particularly for RFs 2, 4 and 6. RFs 4 to 6 at 14°C (Fig. 4.9) utilized maltose and maltotriose more rapidly, and demonstrated an enhanced glucose and fructose utilization. Surprisingly, yeast cultures from RFs 4 to 6 had faster fermentation rates in comparison to the newly propagated yeast cultures from the initial fermentation to RF 3 (Figs. 4.8 and 4.9) (Hammond and Wenn, 1985). Maltose and maltotriose concentrations decrease by an average of 1.4-fold from initial fermentation to RF 3, and by an average of 2.4-fold decrease between RFs 4 to 6. Most maltose and maltotriose were fermented in the first 48h and this delay in utilization during later stages of fermentation might be caused by ethanol toxicity (Meneses *et al,* 2002). The pattern of utilizations is important in assessing fermentation performance (Stewart *et al*, 1995). A common finding is that the initiation of maltose and maltotriose utilization is coordinated with glucose depletion when concentrations fall in the range between 4 and 33 mM (Fig. 4.8) (Meneses *et al*, 2002). Maltotriose was apparently consumed to lesser degree than maltose because

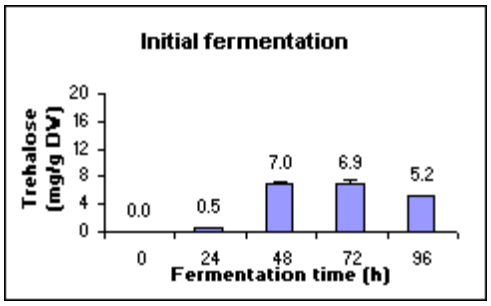
both sugars compete for the same permease transporter across the cell membrane and are hydrolyzed to glucose by the α-glucosidase enzyme within the cell (Fukui *et al*, 1989).

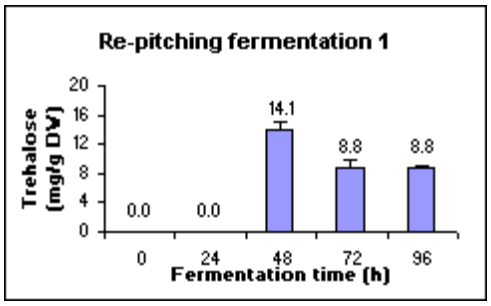
4.3.2 Intracellular metabolites as vitality markers

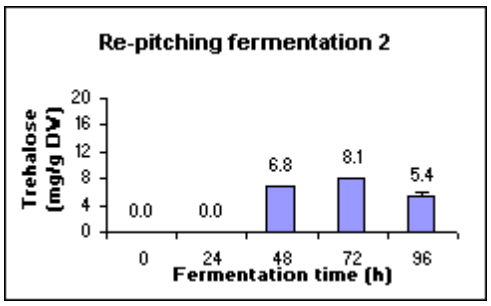
4.3.2.1 Trehalose

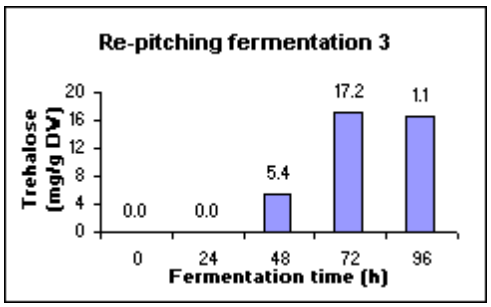
In the present study, changes in intracellular trehalose concentrations were used as a general stress indicator to evaluate fermentation performance and to assess the yeast's vitality in high gravity worts over six re-pitching fermentations. Fig. 4.10 illustrates intracellular trehalose concentrations for each re-pitching fermentation at 18°C. No trehalose was produced during the initial phase of fermentation at pitching and at 24h. The trehalose absence during the growth phase is mostly due to repression by high glucose concentrations of trehalose synthesis (Majara *et al*, 1996). In the initial fermentation and RFs 1 and 2, the highest trehalose production occurred at 48h, followed by a slight decrease towards the end of fermentation, with RF 1 producing the highest trehalose concentration. Early trehalose synthesis shown to be repressed by glucose in exponential growing cells, but was fully active in stationary phase cells in the presence of higher maltose concentrations when most of the glucose was depleted (Majara *et al*, 1996). In most re-pitching fermentations trehalose synthesis commenced at 48h and maximum trehalose concentrations were found at the end of fermentation with highest values for RFs 1, 3 and 6. Since trehalose is mobilized during environmental stresses, such as ethanol toxicity and nutrient starvation, the production of trehalose in non-growing cells at 48h indicated that the yeast is being exposed to stressful conditions (Boulton and Quain, 2001). Trehalose accumulation is thought to increase in the stationary phase to serve as a carbon source and a general stress protectant, such as during periods of nutrient starvation and ethanol toxicity (Hounsa *et al*, 1998). In this study, intracellular trehalose concentration increased with serial re-pitching possibly due to repeated exposure to stress as observed in RFs 5 and 6 (Jenkins *et al*, 2003). All of these factors suggest that yeast needs trehalose to adapt to the "new" stress environment in order to help protect the membrane and proteins from ethanol toxicity and high osmotic pressure (Boulton and Quain, 2001). Hounsa *et al* (1998) reported trehalose concentration values as low as 1.6 mg/g dry weight

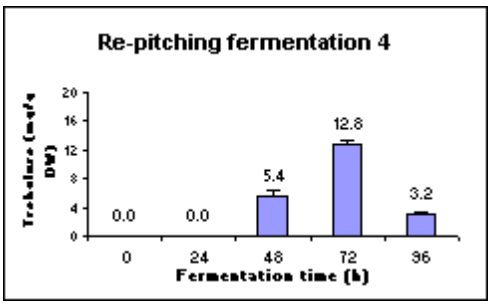
after 48h whereas much higher trehalose concentrations were found in yeasts from these fermentations. However, higher trehalose concentrations are found to be present in brewer's yeast and baker's yeast compared to laboratory yeast strain. For example, Amore *et al* (1991) reported intracellular trehalose concentrations ranging between 2 and 11 mg/g dry weights for a brewer's yeast strain of *S. cerevisiae* cultivated in a medium containing 10% glucose at 30°C. This suggests that the greater tolerance of brewer's yeast to stress than laboratory yeasts might be related to its greater trehalose concentrations.

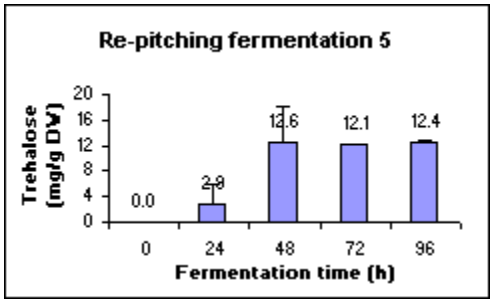












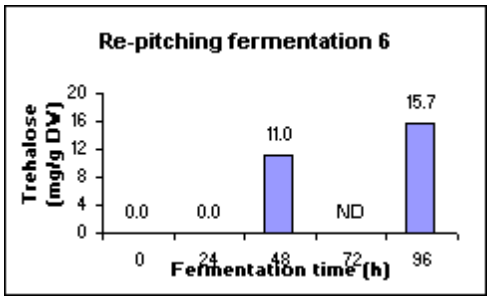


Fig. 4.10: Mean intracellular trehalose concentration over six successive re-pitching fermentations at a fermentation temperature of 18°C. (Bars indicate the range or standard deviations of triplicate determinations. ND indicates not determined.)

Fig. 4.11 illustrates intracellular trehalose concentrations for each of the re-pitching fermentations at 14°C. Lower trehalose concentrations were observed when the yeast was exposed to a lower fermentation temperatures and this might be associated with the fact that yeast is more ethanol tolerant as the temperature decreases, producing less trehalose throughout fermentation (Sharma, 1997). A notable difference in the trehalose concentrations in yeast pitched at 14°C and 18°C is that low trehalose concentrations from approximately 2 to 18 mg/g dry weight are found at pitching at 14°C (Fig. 4.11). This difference in the pitching trehalose concentration could be as a result of yeast being exposed to stress during the handling conditions between re-pitching transfers. Trehalose production under various different conditions such as storage or pitching is as results of stress applied to the yeast (Boulton and Quain, 2001). Fig. 4.11 shows that the pitching yeast for RFs 3 and 6 might be more stressed in comparison to yeast used in the other re-pitching fermentations. In the older re-pitching fermentations, the trehalose concentration gradually increased, for example up to 18 mg/g dry weight in RF 6. In younger re-pitching fermentations, (the initial to RF 3), the trehalose concentration was below 4 mg/g dry weight, in particular towards the end of fermentation when the ethanol concentrations increased. In RF 4 revealed higher trehalose concentrations at 24h followed by a small decrease and a sudden increase at the end of the fermentation. These results correlated with those of older re-pitching fermentations at 18°C. From successive re-pitching fermentations, pitching yeast has increasingly higher trehalose content possibly because of repeated cropping and handling processes. This leads to more sensitive membrane integrity because of continuous high osmotic, ethanol and nutrient starvation conditions (Jenkins *et al*, 2003). Guldfeldt and Arneborg (1997) determined trehalose concentrations of 14 mg/g dry weight at 14°C after 96h in stationary growth phase. These results closely correspond to the trehalose content in RFs 5 and 6, which were 12.5 and 10.2 mg/g dry weight respectively, at 96h.

Trehalose is an important vitality marker for the determination of the yeast's vitality. However, changes in trehalose concentration during and after handling processes, from cropping, storage and pitching could be a much more valuable marker (O'Connor Cox, 1998). This study revealed that trehalose concentrations varied considerably with each re-pitching fermentation although the concentration appeared

to increase with each re-pitching. Therefore, trehalose concentration as a marker for yeast vitality does not appear to be reliable enough.

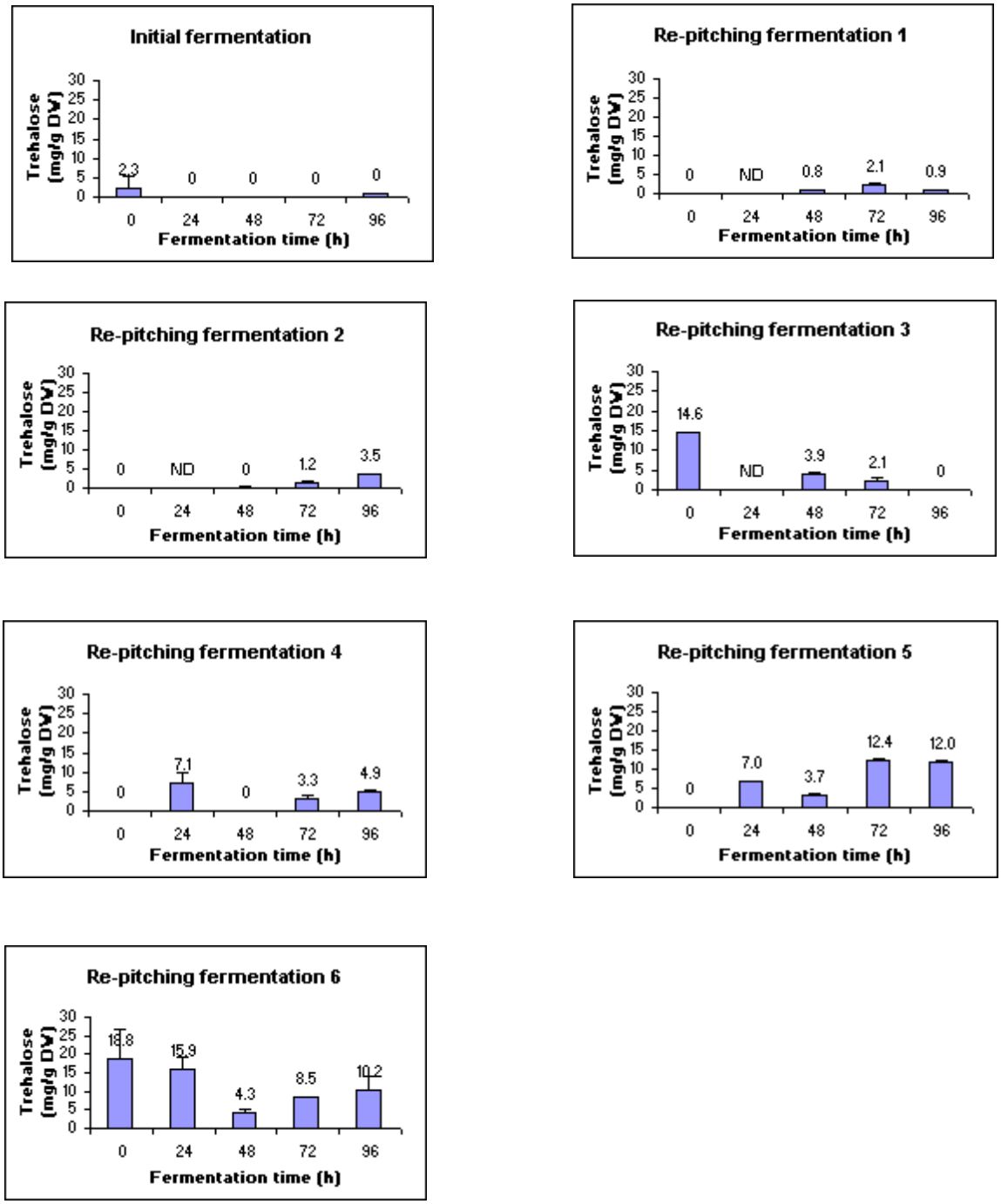
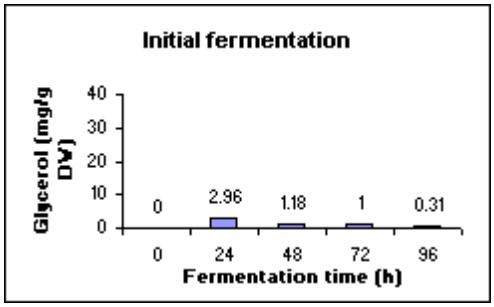
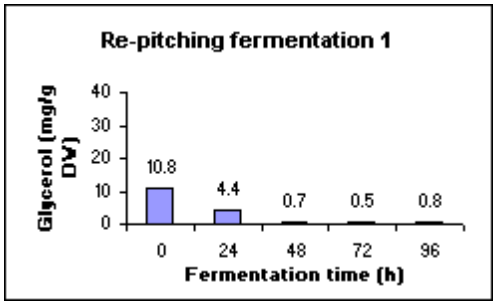


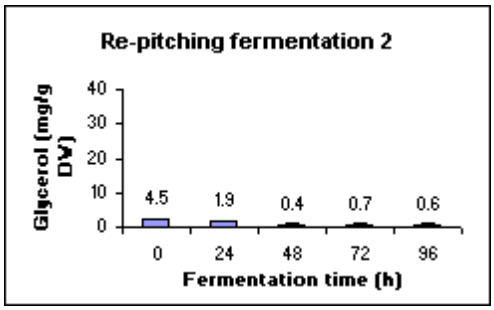
Fig. 4.11: Mean intracellular trehalose concentration over six successive re-pitchings at a fermentation temperature of 14°C. (Bars indicate the range or standard deviations of triplicate determinations. ND indicates not determined.)

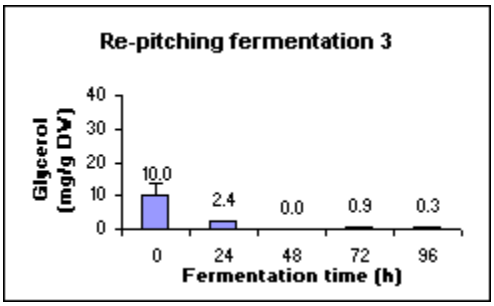
4.3.2.2 Glycerol

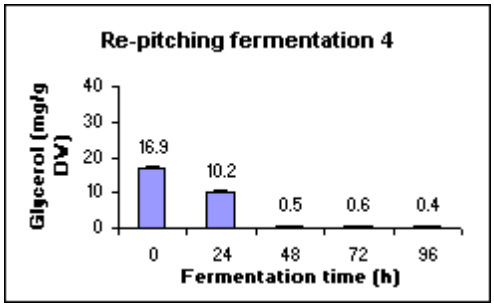
Intracellular glycerol concentrations by *S. cerevisiae* in six re-pitching fermentations cultivated at 18°C are presented in Fig. 4.12**.** During the switch from the respirative to the fermentative phase, the presence of high initial glucose concentrations at the start of fermentation directly inhibits the yeast growth rate and fermentation activity because of high osmotic pressure and low water activity (Amore, 1991). Panchal and Stewart (1979) made similar observations of total glycerol concentration values of around 5.8 mg/ml between 13h to 50h in the presence of 10 and 30% sucrose and sorbitol, which approximately correspond to the higher values of 3.0 and 4.4 mg/g dry weight from RFs 1 and 2 respectively at 48h (Fig 4.12). These high glucose concentrations of up to 25% induce a high glycolytic flux, using glucose for glycerol production, as a direct result of high water stress, and allow the cell to osmotically adapt to these stressful conditions (Nevoigt and Stahl, 1997). This rapid glycerol accumulation is enhanced through increased activity of the glycerol-3-phosphate dehydrogenase enzyme (GPDH) which catalyzes the synthesis of glycerol, and whose activity is greatly induced under osmotic stress (Hohmann and Prior, 1997). The initial glycerol accumulation is a direct measurement of applied external osmotic pressures (Reed *et al*, 1987). However, from RFs 2 to 6, glycerol concentrations decreased during fermentation as cells entered the stationary phase, in contrast to the increase in trehalose concentration. Glycerol offers protection against elevated osmotic conditions (Albertyn *et al*, 1994; Hohmann, 2002), whereas trehalose protects against elevated ethanol and nutrient depletion in the later course of fermentation. However, whether each molecule can interact and replace one another requires further study.

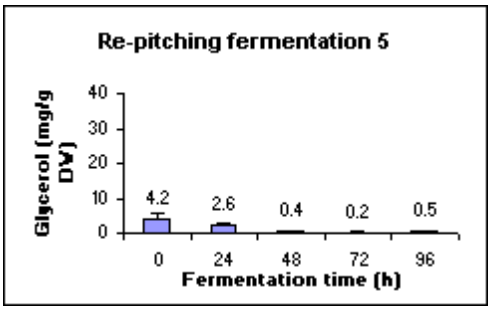












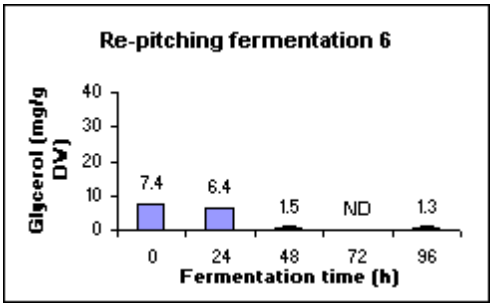
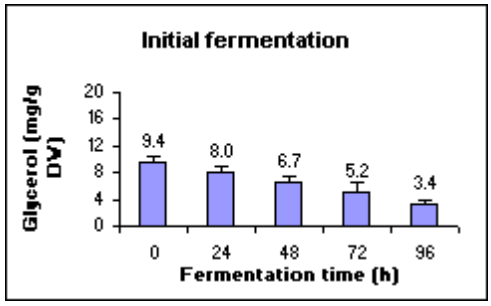
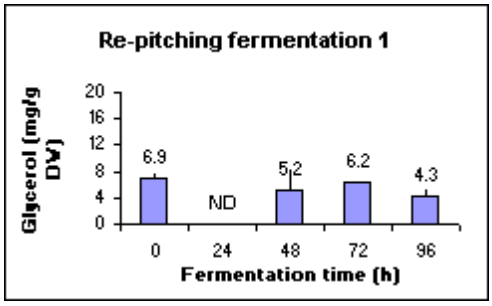
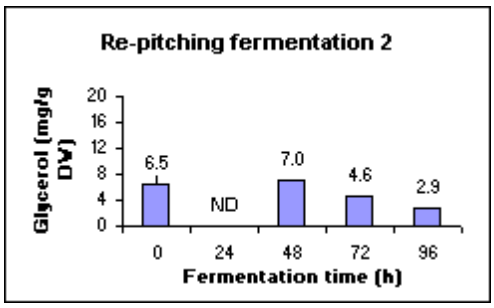


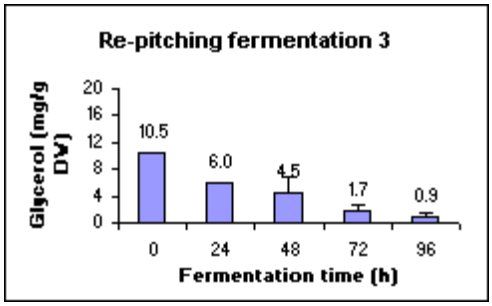
Fig. 4.12: Mean intracellular glycerol concentrations in *S. cerevisiae* re-pitched six times at a fermentation temperature of 18°C. (Bars represent the range of triplicate determinations. ND indicates not determined.)

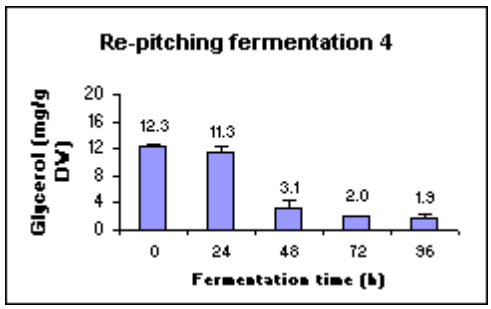
Glycerol production over six re-pitching fermentations at 14°C is presented in Fig 4.13. Generally, higher pitching intracellular glycerol concentrations were observed, followed by a gradual decrease in glycerol towards the end of fermentation at both temperatures. This same pattern applies for the yeast fermenting at 14°C with a slower growth rate at the beginning of fermentation producing higher glycerol concentrations. This implies an increased osmotic stress exposure from fermentable sugars on yeast cells due to slower metabolism (Panchal and Stewart, 1979). High initial glycerol production from RFs 3 to 6 appears to be mainly associated with high sugar concentrations in the original wort. Initial pitching and RF 1 maintain slightly higher glycerol concentrations during the entire fermentation because of lower ethanol production, which reduces the ethanol toxicity, so improving the membrane integrity towards increased glycerol diffusion (Nevoigt and Stahl, 1997). This is mainly true for the initial fermentation and RF 1 because the glucose concentration in both these fermentations were over 100 mM for up to 72h thereby possibly causing an extended osmotic stress (Fig 4.10). Furthermore, pitching and exponential yeast have weaker plasma membrane integrity because of poor sterols content and are more directly sensitive to high external solutes concentration present in wort (Cunningham and Stewart, 1998).

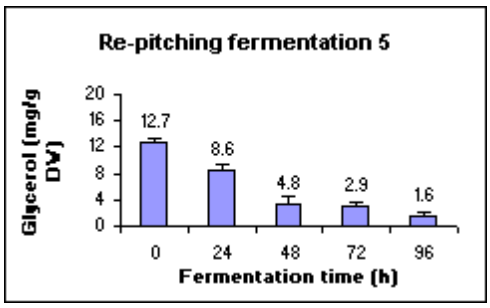












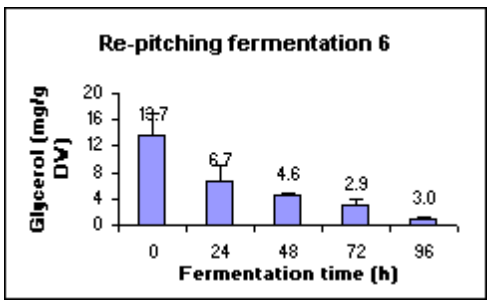


Fig. 4.13: Mean intracellular glycerol concentrations in *S. cerevisiae* re-pitched six times at a fermentation temperature of 14°C. (Bars represent the range of triplicate determinations. ND indicates not determined.)

4.3.2.3 Glycogen

The establishment of a rapid and simple method for the determination of glycogen in the brewing industry was deemed to be beneficial for the evaluation of pitching yeast's vitality (Quain, 1988). The main method used for glycogen determination is the iodine staining of yeast cells by using a rapid spectrophotometric assay (660nm) to measure the intensity of the brown colour (Appendix C) (Cahill *et al*, 2000).

No definite quantitative results were obtained because highly variable data were obtained from the re-pitching fermentations. This particular method might be more useful for qualitative rather than quantitative determination of yeast glycogen content since sufficient pitching glycogen can be visually examined from a dark brown (glycogen-rich cells) to a yellow colour (glycogen-poor cells) (Fig. 4.14) (Quain and Tubb, 1983).

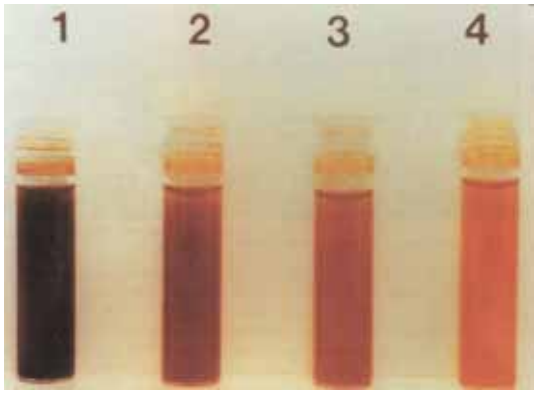


Fig. 4.14: Visual assessment of glycogen in *S. cerevisiae* determined by the intensity of iodine staining. Glycogen concentrations (in mg/ml) and their absorbancies (at 660 nm), followed in four respective tubes, were: (1) 1, 1.1; (2) 0.62, 0.98; (3) 0.43, 0.83; (4) 0.09, 0.29 (Quain and Tubb, 1983).

Another common method of determining glycogen content is the alkaline extraction of glycogen together with trehalose, by boiling washed yeast cells in sodium carbonate for 20 min. It is followed by enzymatic glycogen hydrolysis to glucose by the amyloglucosidase enzyme for 2h at 37°C. The released glucose was determined by HPLC. This method was rated as more time-consuming and labour intensive than the iodine-staining method (Schulze *et al*, 1995). No results are shown for this particular method because the mg of glucose equivalents/g dry weight measured by HPLC after enzymatic degradation for the majority of samples were highly variable and failed to yield any trend in glycogen concentration comparable to previously reported data for brewer's yeast. Schulze *et al* (1995) have obtained up to 90 mg/g dry weight of glucose equivalent concentrations after enzymatic treatment but these studies failed to yield comparable data.

4.3.2.4 Hsp12p protein expression

Heat shock protein Hsp12p is located in the cell wall and plasma membrane of *S. cerevisae*, and together with trehalose is thought to stabilize membrane integrity upon osmotic, ethanol, and starvation stresses (Mothshwene *et al*, 2004). The hydrophilic Hsp12p protein electrostatically interacts with membrane proteins by means of hydrogen bonding to reduce the charge repulsion of exposed phosphate groups (Sales *et al*, 2000). Hsp12p expression is shown in Fig. 4.15. This expression was found to increase from 48 to 96h, at the start of the stationary phase, as has been described by Sales *et al* (2000).

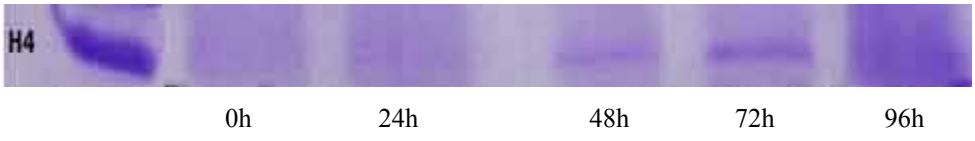


Fig. 4.15: Hsp12p expression, evaluated by 20% SDS/PAGE, of samples taken at 0, 24, 48, 72 and 96h during the initial fermentation at 14°C. (On the left hand side of the figure is the corresponding H4 standard histone marker.)

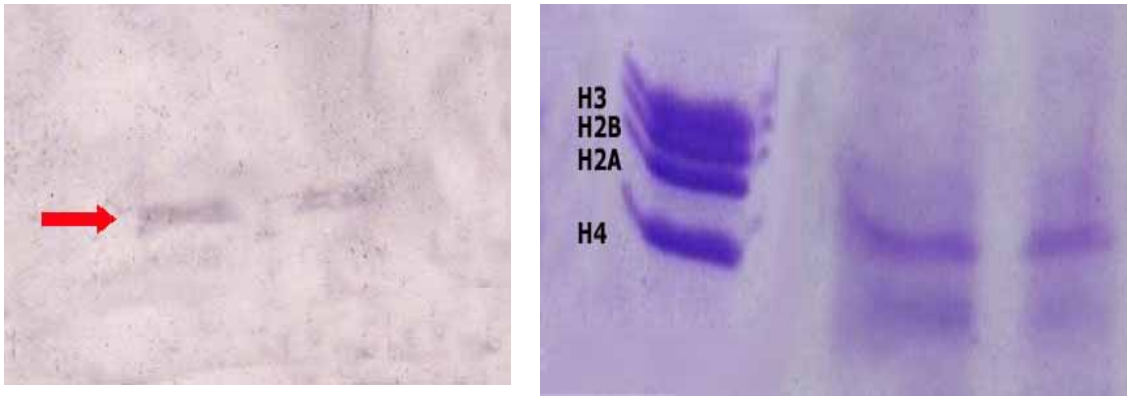
Based on this observation (Fig. 4.15), samples were taken at 48, 72 and 96h for each re-pitched fermentation. The heat shock protein was extracted and separated on a 20% SDS/PAGE gel in order to visualize Hsp12p expression. The average pixel value for selected samples (Section 3.3.5.2) is shown in Table 4.1. The percentage increase represents the increase in Hsp12p expression, as yeast undergoes consecutive fermentations. Compared to the initial intensity of Hsp12p expression, a low increase in expression was observed for most fermentation with the highest increases of 1.4-fold for RF 1 at 48h and RFs 2, 4 and 5 at 72h (Table 4.1). Trabalzini and others (2003) have identified a 2.5-fold increase of Hsp12p protein at 44h by *S. cerevisiae* K310 wine strain grown in YPD medium containing 100 g/l of glucose at 25°C, as analysed by 2D electrophoresis. During the course of fermentations, a lower Hsp12p protein induction was observed at lower temperatures exposed to a gradual ethanol increase during the stationary phase, reaching a maximum ethanol yield of 5% (w/v) (Fig. 4.4). These differences could be due to relatively mild stress conditions occurring in brewing fermentation and relatively greater stress tolerance of industrial yeast strains (James *et al,* 2003).

Table 4.1. Mean Hsp12p pixel values (± range of duplicate determinations) for each re-pitching

RE-PITCHING OF FERMENTATION	SAMPLING TIME (H)	% OF Hsp12p EXPRESSION RELATIVE TO 48H SAMPLE OF INITIAL FERMENTATION
Initial	48	100±6
	72	130.4±8
	96	124.8±5
1	48	143.3±5
	72	141.2±24
	96	139.7±37
2	48	141.9±13
	72	145.0±4
	96	ND
3	48	110.9±18
	72	ND
	96	101.4±3
4	48	139.1±1
	72	136.1±14
	96	126.2±8
5	48	132.8±12
	72	136.4±18
	96	126.4±20
6	48	118.5±6
	72	121.8±7
	96	119.6±7

ND: not determined

The Hsp12p expression was confirmed by means of western blot analysis (Fig. 4.16a), using rabbit antiserum, prepared as described in Appendix B. The immunoblot band is shown with its corresponding SDS/PAGE band (Fig. 4.16b). For the evaluation of the western blot technique, many technical steps were required that can lead to experimental errors and which can explain the poor resolution of the blot. For the evaluation of Hsp12p as a vitality marker, this quantitative method was found to be time-consuming, labour intensive, and expensive for the production of Hsp12p antibodies. Furthermore, very good operator skills are required.



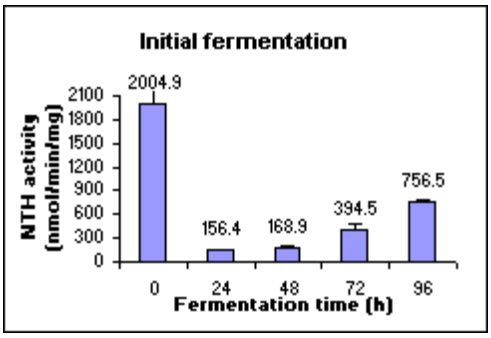
(a) (b)

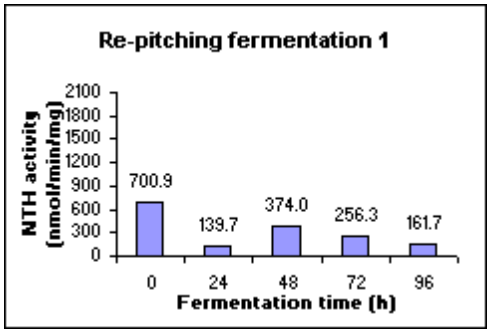
Fig. 4.16: (a) Western blot analysis of 3µg/µl Hsp12p protein (the black arrow indicates the position of the Hsp12p band), and (b) SDS/PAGE of duplicate Hsp12p samples. Standard on the left side of the total extract of chicken erythrocytes histones. The migration of molecular weight markers of appropriate molecular masses is denoted on the right-hand side of the gel; these are: H3, 15.3kDa; H2B, 13.7kDa; H2A, 14kDa and H4, 11.2kDa (Motshwene *et al*, 2003).

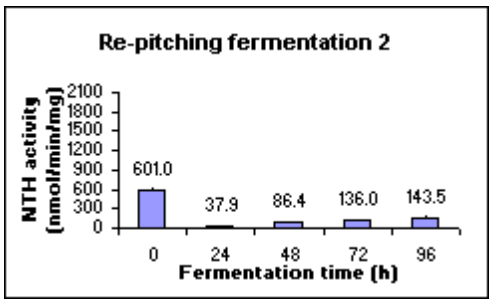
4.3.2.5 Neutral trehalase enzyme activity

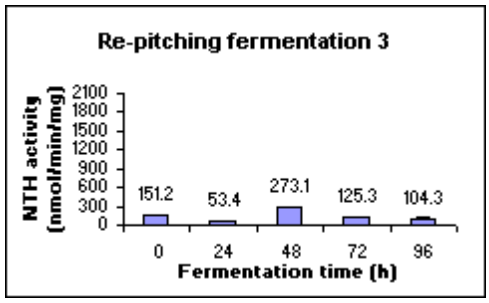
Yeast neutral trehalase activity from six re-pitching fermentations at 18°C and 14°C are represented in Figs. 4.17 and 4.18 respectively. The function of the enzyme neutral trehalase encoded by the gene *NTH1* is to catalyze trehalose hydrolysis. *NTH1* expression is activated by stress conditions such as nutrient depletion or heat shock (Zähringer *et al,* 1999). High neutral trehalase activities at the start of the fermentation inoculated with fresh yeast were observed. After 24h these had dropped considerably, followed by a steady increase in activity. These high initial neutral trehalase activities might be related to the mobilization of trehalose (Figs. 4.10 and 4.11) due to high glucose concentrations that induce neutral trehalase activity (Miguel and Argüelles, 1994). Neutral trehalase activities after 24h at both 18°C and 14°C were considerably lower possibly because of glucose repression on exponentially growing cells (De Virgilio *et al*, 1991). These activities gradually increase close to the end of fermentation during the stationary phase after completion of glucose consumption. High trehalose production in parallel with high neutral trehalase activities from nutrient limitation and high ethanol conditions both contribute to the stress response (Nwaka and Holzer, 1998). Neutral trehalase activities at 14°C (Fig. 4.18) were observed to be lower than at 18°C (Fig. 4.17). These lower activities might

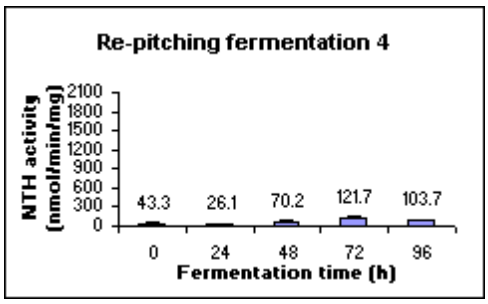
be associated with the large residual glucose concentrations present in the wort throughout fermentation, causing a glucose repression effect on both trehalose production and neutral trehalase activity (Zähringer *et al*, 1997). Neutral trehalase activity in RFs 1 and 2 at both temperatures followed a similar pattern over time, except that enzyme activities were in most instances considerably lower in fermentations conducted at 14ºC than at 18°C (Figs. 4.17 and 4.18). Neutral trehalase activity in RFs 3 to 6 at 18°C did not follow the same pattern as in previous fermentations. The RF 3 at 14°C had an exceptionally high enzyme activity level at inoculation compared to the other fermentations (Fig. 4.18). These lower neutral trehalase activities in fermentations at 14ºC and 18ºC might be related to progressive repression on enzyme activity by glucose after serial re-pitching, in particularly at 14°C (Fig. 4.18) (Nwaka and Holzer, 1998). Miguel and Argüelles (1994) reported a similar example of a neutral trehalase activities pattern in a culture incubated at 30°C in glucose. They demonstrated that neutral trehalase activity starts to increase once glucose in the medium has been consumed. Neutral trehalase activity levels appeared to decline with the number of re-pitchings. Further research is required before the assay for the enzyme could possibly be used as a useful marker of yeast viability.

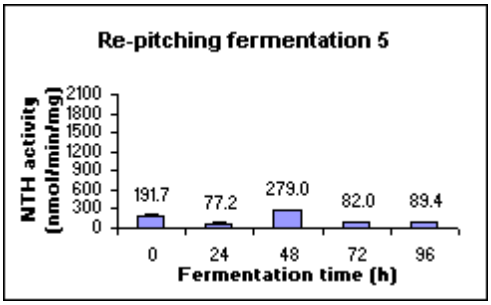












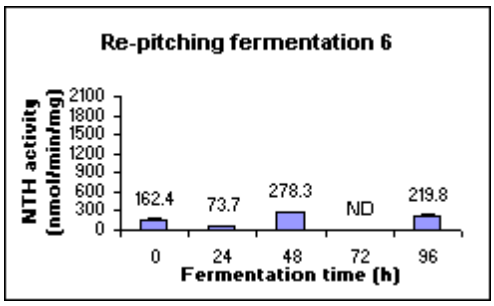
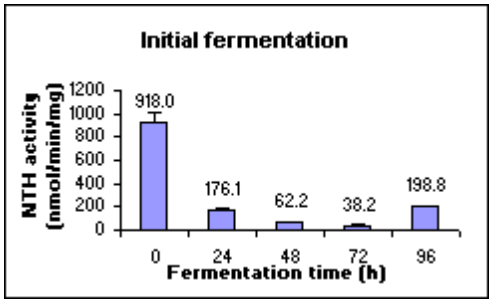
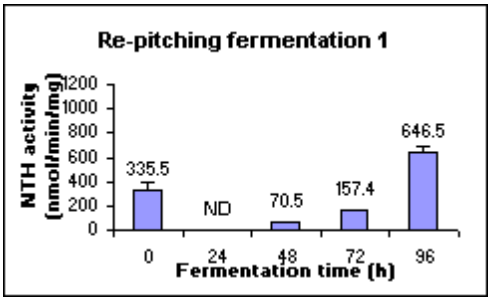
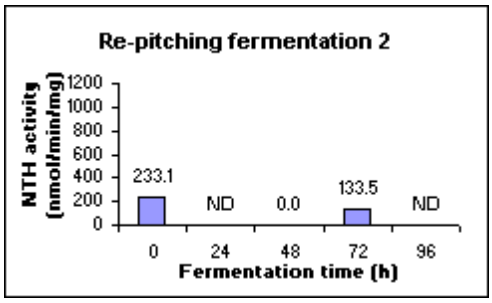
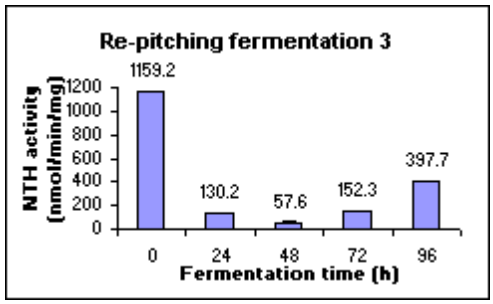


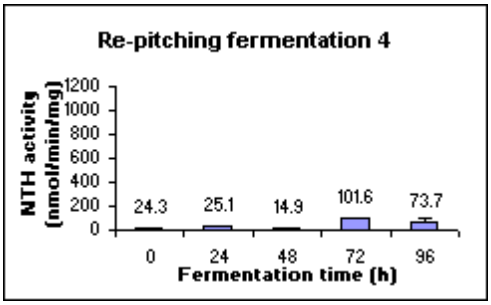
Fig. 4.17: Mean intracellular neutral trehalase enzyme activity at a fermentation temperature of 18°C. (bars represent the standards deviation of triplicate determinations. ND indicates not determined.)

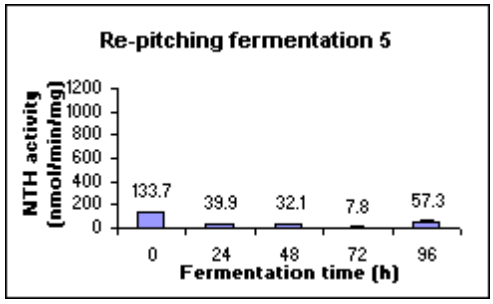












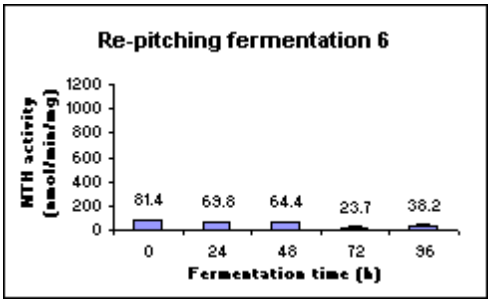


Fig. 4.18: Mean intracellular neutral trehalase enzyme activity at a fermentation temperature of 18°C. (bars represent the standards deviation of triplicate determinations. ND indicated not determined.)

4.3.2.6 Esterase enzyme activity

Yeast esterase activity from six re-pitching fermentations at 18°C and 14°C are represented in Figs. 4.19 and 4.20 respectively**.** High initial esterase activity in the initial fermentation and RFs 2 and 4 at 18°C (Fig. 4.19) and RFs 3, 4 and 6 at 14°C (Fig. 4.20) were observed. These high initial esterase activities at pitching might be related to the wort composition, in particular from addition of an adjunct containing a high nitrogen ratio in dextrose syrup that favours the production of esters (Peddie, 1989). This high ester synthesis is directly followed by a sharp decrease in esterase activity levels at 24h for most re-pitching fermentations, with the exception of RF 1 and 6 (Fig. 4.19) and RF 6 (Fig. 4.20). The precursor of esters, acetyl-coA, is involved in yeast growth mainly during the synthesis of sterols and unsaturated fatty acids synthesis in the presence of oxygen and nitrogen. This causes a drop in ester synthesis, related to lipid metabolism (Stewart *et al*, 1999). The majority of re-pitching fermentations at 72h show a slight decrease in esterase activity because of prolonged yeast growth, mainly at 14°C, where the yeast's growth rate is lower in comparison to growth at 18°C (see Figs. 4.1 and 4.2). Esterase activity maintained constant activities between 2 and 6 nmol/min/mg at both temperatures in the fermentations inoculated on re-pitched yeast. However, for the initial fermentations at both 14ºC and 18ºC the esterase activity remained generally higher in comparison to the rest of the re-pitching fermentations. Guldfeldt *et al* (1998) reported similar esterase over a four-day period under equivalent fermentation conditions in wort at 16°P at 14°C. High intracellular esterase activities in the first hours of fermentation were followed by a decrease to minimum values, after which there was an increase to constant values.

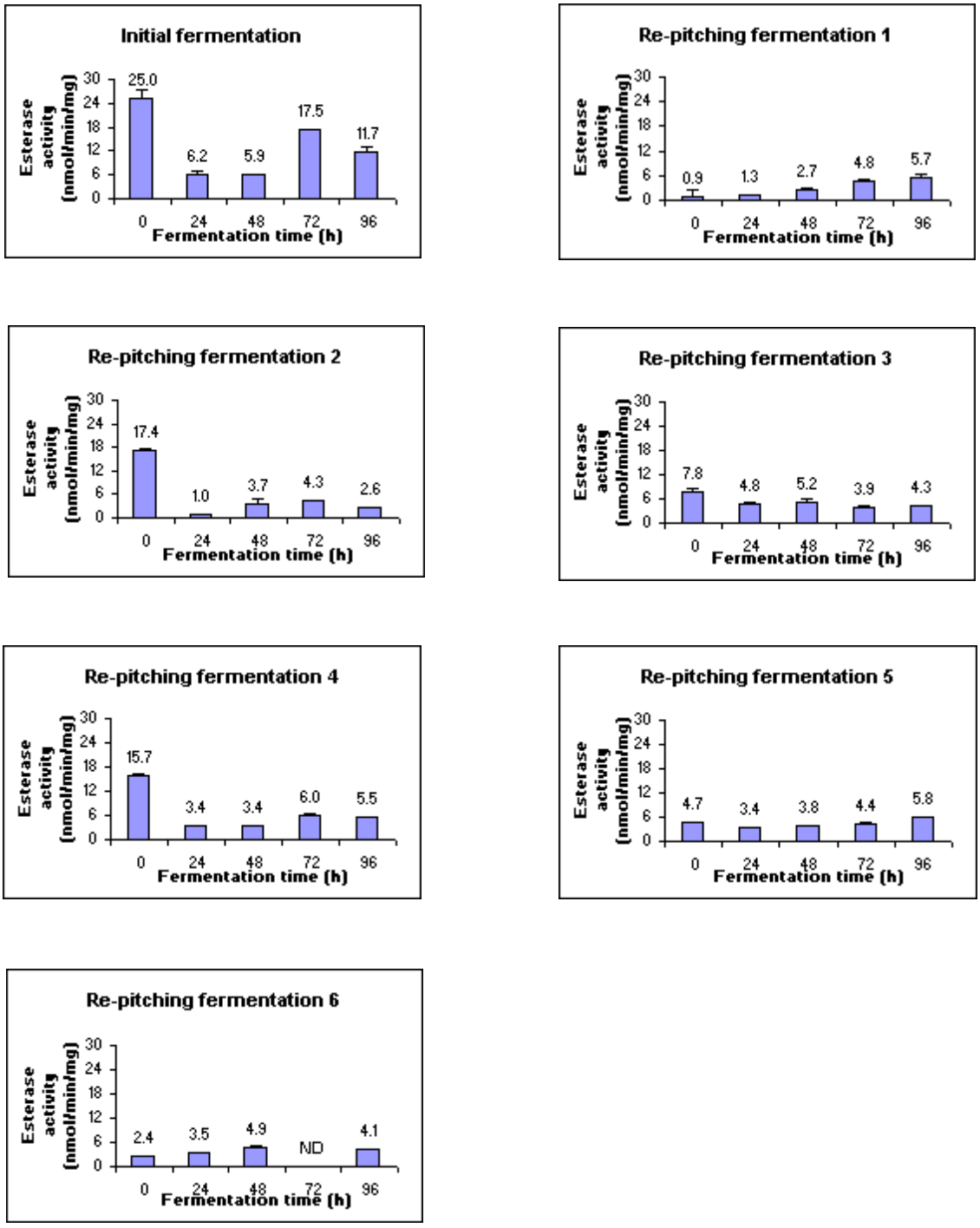


Fig. 4.19: Mean intracellular esterase enzyme activity at a fermentation temperature of 18°C. (bars represent the standards deviation of triplicate determinations. ND indicates not determined.)

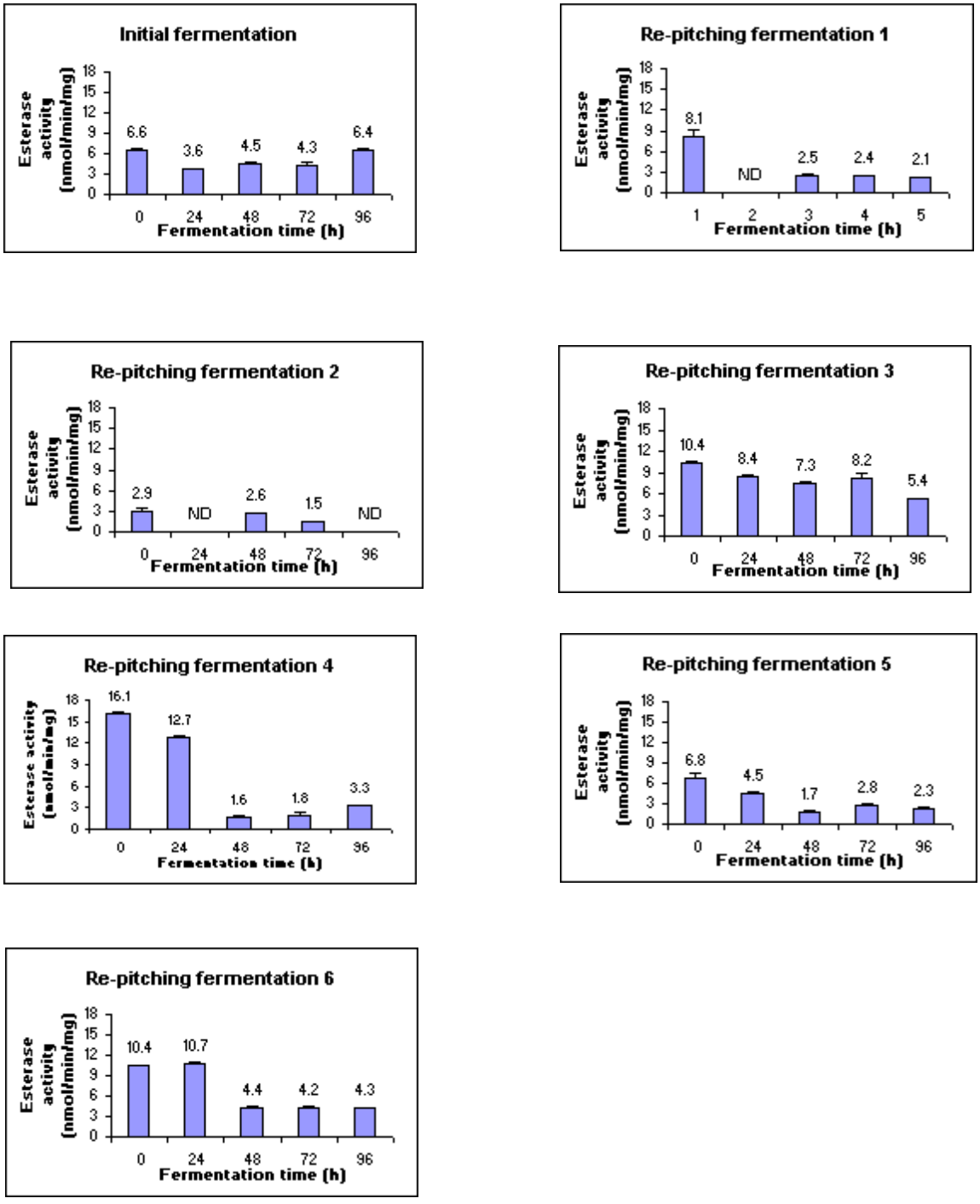


Fig. 4.20: Mean intracellular esterase enzyme activity at a fermentation temperature of 14°C. (bars represent the standards deviations of triplicate determinations. ND indicates not determined.)

4.4 Assessment of fermentation ability after exposure to stress

4.4.1 Introduction

The evaluation of yeast tolerance to common stress conditions in high-gravity fermentations is important to determine subsequent fermentation performance (O'Connor Cox, 1998). Distinctive stresses, including ethanol toxicity, high osmotic pressure and cold shock, were selected to assess the yeast's fermentation ability after stress exposure for a defined period. Powell *et al* (2000; 2003) and Deans *et al* (1997) have found that the cell age of yeast has a small impact on fermentation performance. The impact of yeast's cell age in regards to fermentation performance is based on specific differences between virgin, middle-aged and older cells. The following examples of this are given.

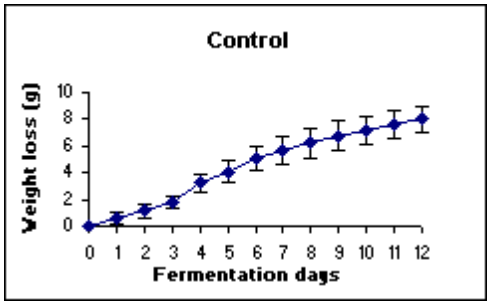
(a) Young, newly budded cells take longer to reach a critical biomass required for the first division. This result in an extended lag phase accompanied with a slight delay in onset of growth and utilization of sugars (Powell *et al*, 2000; 2003).
(b) Virgin cells will consequently start to flocculate, depending on their replicative age during later stages, which may have a biochemical effect on the beer quality.
(c) Older cells flocculate first at the bottom of the fermenter cone.
(d) Older cells, as described previously, have a larger number of budding scars limiting the surface area availability and reducing the number of divisions allowed (Deans *et al*, 1999).
(e) As yeast gets older they increase in size, shape alterations with surface appearance occur, and altered generation time and decline in metabolism all affect the fermentation performance in terms of viability and vitality (Powell *et al*, 2000; 2003).
(f) Young to middle-aged cells are the best-selected cells for active fermentation since they rapidly divide, with a fast biomass production (Powell *et al*, 2000).

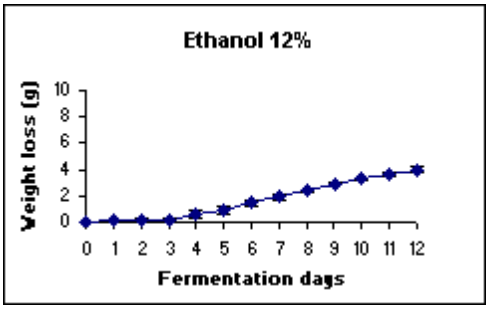
From all the above reasons, an unbalanced age selection process during cropping can have negative consequences on successive fermentation performances during serial re-pitching (Deans *et al.* 1999).

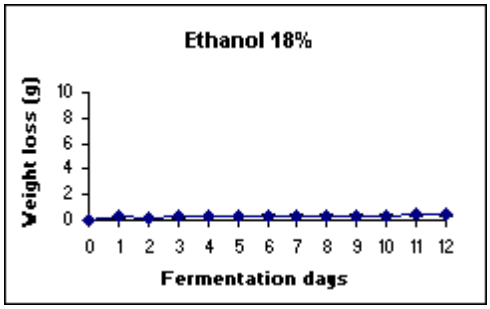
In this investigation, young to middle-aged yeast cells in suspension in beer were collected from the middle of a fermentation tube and flocculated older yeast within the slurry at the bottom of a fermentation tube were harvested. The two types were then compared with regard to their successive fermentation activity after stress exposure (Section 3.4).

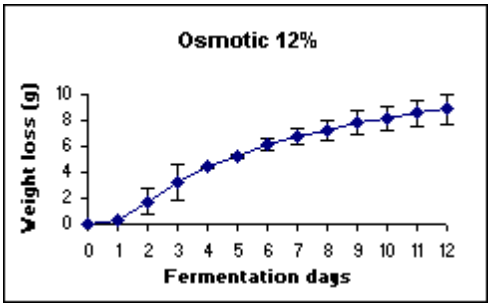
4.4.2 Fermentation rates

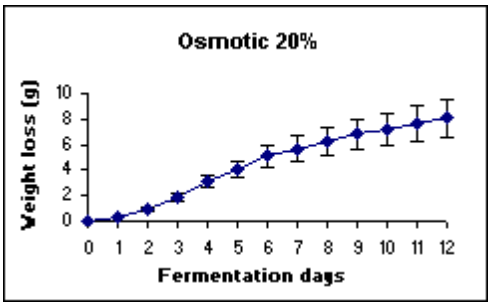
Typical fermentation profiles as determined from the weight loss / day (assumed to be loss of CO_2 by fermentation) for yeast obtained from the middle of a fermentation tube are shown for a control and cells exposed to stress prior to fermentation (Fig. 4.21). The maximum rates of CO_2 production were calculated from data obtained from slurry yeast and suspended yeast for the six re-pitching fermentations and are presented in Tables 4.2 and 4.3 respectively.

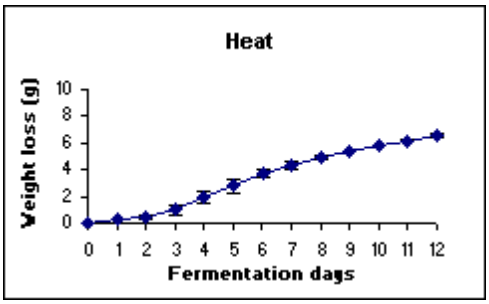












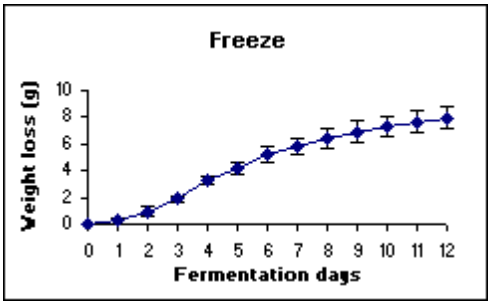


Fig. 4.21: Typical fermentation rates for the fermentation by slurry yeast exposed to various stresses prior to pitching (each data point represent the mean and the bars represent the range of the data of duplicate determinations.)

Table 4.2: Maximum fermentation rates in g CO_2 (weight loss)/day for *S. cerevisiae* harvested in suspension from E.B.C tubes and exposed to various stress

RE-PITCHING FERMENTATIONS	CONTROL	ETHANOL 12%	ETHANOL 18%	OSMOTIC 12%	OSMOTIC 20%	HEAT (40°C)	FREEZE (-20°C)
Initial	0.28	0.21	0.01	0.39	0.30	0.45	0.56
1	0.39	0.34	0.02	0.34	0.31	0.41	0.3
2	0.41	0.22	0.04	0.49	0.34	0.49	0.53
3	0.55	0.09	0.01	0.46	0.53	0.56	0.43
4	0.61	0.33	0.06	0.60	0.60	0.55	0.51
5	0.61	0.46	0.03	0.61	0.63	0.6	0.61
6	0.49	0.56	0.07	0.40	0.64	0.58	0.54

Table 4.3: Maximum fermentation rates in g CO_2 (weight loss) / day for *S. cerevisiae* harvested from the slurry at the bottom of E.B.C tubes and exposed to various stress

RE-PITCHING FERMENTATIONS	CONTROL	ETHANOL 12%	ETHANOL 18%	OSMOTIC 12%	OSMOTIC 20%	HEAT (40°C)	FREEZE (-20°C)
Initial	0.66	0.45	0.02	0.63	0.67	0.63	0.67
1	0.43	0.33	0.03	0.44	0.60	0.27	0.52
2	0.45	0.46	0.02	0.67	0.59	0.62	0.60
3	0.36	0.06	0.05	0.56	0.54	0.56	0.35
4	0.53	0.02	0.02	0.70	0.53	0.55	0.52
5	0.41	0.11	0.02	0.45	0.46	0.61	0.67
6	0.51	0.36	0.05	0.60	0.6	0.55	0.39

Control fermentations

Control cells without applied stress and cells exposed to various stresses were incubated for 4 h at 26°C for the stress to affect the yeast metabolism. Stressed and control cells were pitched into small-scale fermentation bottles and the weight loss per day was recorded (sections 3.5.1 and 3.5.2). The "middle" section yeast cells were observed to be the least active in the initial fermentation, with the lowest fermentation rate of 0.28 g CO_2/day (Table 4.2). This fermentation rate gradually increased by 1.1- to 1.4-fold with serial re-pitching, reaching the highest fermentation rate of 0.61 g CO_2/day by RF 5. Through serial re-pitching, the fermentation rate appeared to be more efficient after being re-pitched. However, this conclusion is difficult to confirm in these particular mini-fermentations. Older, middle-aged cells, present in the slurry at the bottom of the fermenter, were recognized to be more stress tolerant and demonstrated enhanced fermentation performance (Majara *et al*, 1996). Table 4.3

shows that the initial fermentation had the greatest fermentation rate of 0.66 g CO_2/day, which decreased by up to 1.8-fold in RF 3 and increases to 0.51 g CO_2/day by RF 6. The suggestion of Powell et al (2003) that older cells show enhanced fermentation performance compared to younger cells is not supported here as no particular trend was observed.

Ethanol stress

The ethanol tolerance to inhibitory concentrations with regard to subsequent fermentation performance was evaluated. Results are tabulated in Tables 4.2 and 4.3. Both tables demonstrate a net decrease in fermentation rates at both ethanol concentrations (12 and 18%). Ethanol concentrations of 12% in most re-pitching fermentations for suspended yeast reduced the fermentation rate between 1.1 to 1.8-fold, in comparison to the control, and only RF 3 showed a marked decrease by six-fold. The slurry yeast was more susceptible to these ethanol conditions as observed in RFs 3 to 5, with a decrease in fermentation rate. RFs 3 and 5 showed a decrease as high as 6-fold and 3.8-fold respectively. Furthermore an almost absence of fermentation was observed for RF 4. This unusual decrease in fermentation rates for the slurry yeast under such ethanol exposure is difficult to explain in terms of the yeast's ethanol tolerance, since other stress exposures do not show such a large fermentative inhibition. Previous re-pitching fermentations (initial to RF 2) showed a similar 1.4-fold decrease in comparison to the suspended yeast. Both suspended and slurry yeast appeared to loose resistance from RF 3, the fermentation rates stabilized (similar to the control) for the suspended yeast and decreased by 1.4-fold for the slurry yeast in RF 6. This general inhibition of fermentation rates by ethanol (12%) is most likely due to the sensitivity to exogenously added ethanol on yeast cells of any cell age, to which the extent of ethanol damage is dependent on ethanol concentration and duration of exposure (Hallsworth, 1998). Ethanol concentrations of 8% were determined as the maximum allowed ethanol concentration for growth of *S. cerevisiae*, whereas a 12% concentration created an inhibitory effect on the yeast fermentation ability (Dombek and Ingram, 1987). The 18% ethanol concentrations showed an overall inhibition of fermentation rates for both suspended and slurry yeast with the majority of re-pitching fermentation rates observed in the range between 0.01 and 0.07 g CO_2/day. The addition of such a large ethanol concentration to the medium

has been found to cause a complete metabolic activity arrest, resulting in irreversible cell lysis (Hallsworth, 1998). Amore (1992) showed that an 11% (w/v) ethanol concentration caused a 50% cell death in a lager strain after 20 min incubation at 30°C, with complete cell death at 15% (w/v) ethanol.

Osmotic stress

Stationary-phase cells were exposed to osmotic stress conditions with 12% and 20% sorbitol in the medium for a 4h period (Section 3.5.1). The fermentation rates of osmotic-stressed cells (Table 4.2) were similar to the control cells and no particular trend in osmotic tolerance was observed from the number of times the yeast was re-pitched. An increase in fermentation rate in comparison to the control was observed for the initial fermentation for both sorbitol concentrations for the suspended yeast, with the 12% concentration being slightly higher to the 20% (Table 4.2). As the suspended yeast was further re-pitched, a very small inhibition of fermentative activity could be observed throughout the rest of the re-pitching fermentations, with a 1.1- to 1.3-fold decrease of fermentation rate. The suspended yeast showed an enhanced osmotic tolerance to the 20% sorbitol concentration in comparison to the 12% concentration. This sudden change in water activity from 20% sorbitol exposure induces a parallel over-production of glycerol to restore the original cell volume and to maintain the normal cell's metabolic functions (Nevoigt and Stahl, 1997). The majority of re-pitching fermentations increase for both sorbitol concentrations by approximately between 1.2 to 1.5-fold (Table 4.3). Enhanced fermentation rates were observed in the initial fermentation and RF 1 under 20% to the 12% sorbitol concentration, after which the fermentation activity improved at 12% sorbitol between RF 2 to 4 and finally both stabilized by RF 6. It was considered that the compatible solute glycerol immediately accumulated at the start of osmotic stress exposure in order to counteract the water stress, and once production stabilized the water activity between cells and medium was restored (Nevoigt and Stahl, 1997).

Heat and freezing

Exposure to high temperatures such as sub-lethal heat shock of 40°C for 60 min causes cell death, mainly because of the disruption of the cell membrane permeability

(Heggart *et al*, 1999). The thermotolerance by suspended and slurry yeast are shown in Tables 4.2 and 4.3. The suspended yeast for the majority of re-pitching fermentations (initial to 3, including 6) demonstrated increased fermentation rates between 1.1- and 1.6-fold (Table 4.2). These cells appeared to survive moderate heat shock in comparison to control rates. Only RFs 4 and 5 showed a slight inhibition in fermentation rate by less than 1.1-fold. The slurry yeast showed a small inhibition of fermentation rates of between 1.05- to 1.6-fold, as well as in comparison to control rates in the initial and RFs 1, 4 to 6. RFs 2 and 3 demonstrated an enhanced fermentation rate of 1.4- and 1.6-fold respectively (Table 4.3). Such experimental thermotolerance was found to be directly related to the accumulation of intracellular trehalose and the synthesis of heat-shock proteins `for physical protection of membrane damage and to prevent denaturation of proteins functions to cell survival (Van Dijck *et al*, 1995). No particular trend was observed for the relationship in fermentation rates between re-pitching fermentations for both yeasts.

A small degree of freezing tolerance was also observed for both suspended and slurry yeast (Tables 4.2 and 4.3). The suspended yeast exhibited increased fermentation rates for the initial and RFs 2 and 6, by 2-, 1.3- and 1.1-fold respectively. The other re-pitching fermentations demonstrated an inhibition in fermentation rates of between 1.1- to 1.3-fold (Table 4.2). The slurry yeast similarly showed improved fermentation rates in the initial and RFs 1, 2 and 5, of between 1.02- to 1.6-fold (Table 4.3). Only RF 6 showed a 1.3-fold inhibition in fermentation rate compared to the control. No real trend in freezing tolerance in terms of inhibition level and re-pitching fermentation number were detected in both yeast types. The freezing tolerance of suspended and slurry yeast to low storage temperatures of -20°C might be related to accumulation of trehalose and heat-shock proteins. The latter may play a large role in desiccation resistance, important to the yeast at freezing temperatures (Lewis *et al*, 1995).

Chapter 5: Conclusions

Evaluation of the vitality of yeast in the brewing industry has become increasingly important in predicting successive fermentation performance because of severe stress conditions experienced during high-gravity brewing. The staining of yeast with methylene blue is a well-established method used in breweries to analyze yeast vitality, and an indicator of the state of the plasma membrane or physiological state of the cell at various growth stages and ages. This technique is considered simple and cost effective on the basis of a rapid incubation period of a few minutes and using an equal amount of staining solution. In this method the total yeast count is determined: the dead cells stained with a dark blue colour are counted, from which a viability percentage is calculated. Results with yeast viabilities less than 95% are apparently unreliable for the accurate determination of viability (Mochaba *et al*, 1998) and therefore this study attempted to investigate other methods as indicators of yeast viability. Similarly, Smart *et al.* (1999) have shown 100% viabilities by methylene blue staining in a lager yeast strain in exponential and stationary phases as well as under starved conditions, demonstrating a viability overestimation. In these studies, the yeast viability was usually greater than 95%.

Various types of indicators or markers of fermentation performance have been investigated in terms of reproducibility and ease of carrying out the techniques. Levels of intracellular stress protectant metabolites such as trehalose, glycerol, glycogen, and heat shock protein 12 were extracted and measured by HPLC and by SDS/PAGE. Trehalose levels were shown to increase mostly during the stationary phase at 18ºC for the majority of the re-pitching fermentations in the presence of higher ethanol concentrations. Lower trehalose levels were detected at 14ºC than at 18ºC, and appeared to be associated with lower ethanol yield. In general, later re-pitching fermentations had lower trehalose levels that those in the initial fermentation. Similar findings were reported by Majara *et al.* (1996); trehalose concentrations started to increase in a wort fermentation from 1% at 4 h to approximately 8% (w/w dry weight) at 120h. Higher glycerol concentrations were found at the time of pitching because of high osmotic pressures encountered by the yeast in high gravity wort, supplemented with high glucose concentrations, and the glycerol concentration

gradually decreased in parallel with the sugar levels. Lower glycerol levels were observed when the fermentation was conducted at 14ºC compared to 18ºC. These results suggest that temperature and osmotic pressure influence glycerol concentrations, as has been reported previously (Albers *et al*, 1996).

Higher glycerol concentrations are produced at higher temperatures (Singh and Norton, 1991; Panchal and Stewart, 1979). Glycogen is often described as a rapid, reliable marker of yeast vitality (Quain, 1981; 1988, Quain and Tubb, 1983), but has been found to be quite difficult to quantify and results are highly variable with techniques like enzymatic degradation or iodine staining. The use of the near infra-red (NIR) detection technique for the determination of glycogen as an indicator to assess yeast vitality has been reviewed by Moonsamy *et al.* (1995), Mochaba *et al.* (1994) and O'Connor Cox *et al.* (1996). This particular technique is more efficient and reliable and samples can be analysed in less than 1 min compared to other techniques to measure glycogen. The heat shock protein Hsp12p, on the other hand, is a marker whose level of expression was shown in this study to be influenced by growth conditions. The expression of the protein increased considerably under stress conditions, especially under ethanol stress and heat shock (Sales *et* al, 2000; de Groot *et al*, 2000). An increase in Hsp12p protein at the entry of stationary phase has been demonstrated by Sales *et al* (2000). However, in this present study the entire analysis procedure of protein extraction, quantification by SDS / PAGE and identification by western blotting, was found to be extremely sensitive to non-specific binding that could lead to poor band resolution. Therefore this current method is not recommended for use as a routine brewing technique. It could however possibly be successful after further developments of the technique.

The enzyme neutral trehalase was observed by Causton *et al.* (2001) to be induced under stress conditions as part of the environmental stress response. In the present study very little neutral trehalase activity was detected in most re-pitching fermentations; the highest activity was observed at the time of pitching the yeast. This enzyme may result in trehalose concentrations in re-pitched yeast being mobilized but further production of the enzyme is repressed by high glucose concentrations present in the wort (Majara *et al*, 1996). This high activity quickly decreases as trehalose if formed but increases slightly again towards the end of

fermentation, possibly due to the general stress response. San Miguel *et al.* (1994) also found this inverse reverse relationship between trehalose formation and neutral trehalase activity, with neutral trehalase activity decreasing from approximately 24 to 11 U/mg protein as trehalose concentrations increased from 5 to 22 mg/g dry weight. The activity of esterase, an enzyme that detoxifies unsaturated fatty acids (Peddie, 1989; Stewart *et al,* 1999), did not show any particular trend during the fermentations. Both these enzymes were therefore not particularly useful as potential vitality markers.

The fermentative activities after exposing the yeast to various environmental stresses were evaluated. It was found that the aged cells in the slurry appeared to ferment better, demonstrating a greater stress tolerance. The most damaging stress was observed to be ethanol at concentrations of 12% and above. On the other hand, high osmotic stress up to 20% sorbitol, did not impact on the subsequent fermentation ability indicating that this stress did not result in irreversible damage (Nevoigt and Stahl, 1997). Heat and freezing stresses also did not seem to disturb the yeast fermentative behaviour since, in most re-pitching fermentations, an increase in fermentation rate was observed. Majara *et al.* (1996) reported on trehalose, protein and glycogen contents in yeast when exposed to various stresses, and found that maximum trehalose is produced under cold shock, maximum glycogen under ethanol shock, and maximum protein synthesis under heat shock. In comparison to this study, trehalose and glycogen concentrations under these experimental fermentation conditions were difficult to confirm because trehalose was not measured under similar cold shock conditions used by Majara *et al.* (1996), and glycogen results were highly variable throughout the serial re-pitching fermentations.

Overall, this study failed to identify a new technique that could be satisfactorily used as an indicator of yeast viability in the brewery. Some methods, such as the measurement of Hsp12p expression, do however require further investigation to confirm whether they have possible applications. The introduction of rapid techniques, such as the use of the NIR for vitality assessment for glycogen and trehalose concentration determinations might further improve the detection reliability (Moonsamy *et al.,* 1995; Mochaba *et al,* 1994). It was difficult to obtain reproducible and reliable results when the studies were carried out in E.B.C. fermentation tubes.

It is recommended that future studies on markers as indicators of yeast viability be conducted under more defined laboratory conditions, were the ages of the yeast cells can be more accurately controlled.

Chapter 6: References

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Appendix A:

Black Label wort consists of malt black imported, Malt American SAM, Malt hemisphere SAM, calcium and zinc sulphate, lactic acid, dextrose syrup and yeast nutrient.

Dextrose syrup specification:
Description: High glucose syrup derived from maize starch
Physical appearance: Water white, viscous, sweet tasting syrup
Function: Refined source of fermentable sugars
Solids content: 71-72%
Residual maltodextrines (starch test): 0.199
Colour: <2.0 ° EBC
Taste and odour: Free from off-flavours/taints when 10% solution made up in carbonated water
Carbohydrates profile: 91% dextrose
Hydroxy methyl furfural: <2 mg/kg

Appendix B:

1. Hsp12p purification

Two millilitres of the supernatant fraction and 10 µl of the histone marker of a concentration of 1 mg/ml were electrophoresed over a 200 mm x 200 mm 20% SDS gel for 16h run. The sample and marker were prepared by a dilution factor 1 to 4.

The desired area of the Coomassie-stained gel was manually removed and cut into small pieces. The pieces were suspended in 0.5% (w/v) cetyltrimethylammonium bromide for 1h before being electroeluted at a constant current in 0.9% acetic acid for 5 to 6h.
The protein extract was subsequently removed at different time intervals into eppendorf tubes in a 100 µl volume each and stored at -80°C. The protein extracts were freeze-dried for 3 to 4h, dissolved in 500 µl double-distilled water, and the protein was precipitated by the addition of 55 µl of 50% trichloroacetic acid. The protein extracts were incubated at 4°C for 30 min and centrifuged at 3000 g for 10 min at 4°C. The precipitated proteins were washed with 1 ml of ice cold acetone / 20 mM HCl, followed by centrifugation. The precipitated protein were washed once again with 1 ml ice cold pure acetone, air-dried and re-dissolved in two volumes of 50 µl of double distilled water. The dissolved protein was run on an 80 mm x 100 mm 20% SDS gel to confirm Hsp12p purity.

2. Preparation of antiserum:

One mg of pure Hsp12p protein was injected in Freund's complete adjuvant into rabbits. After the initial inoculation, three booster injections at weekly intervals were given to the rabbits, followed by two further inoculations after a three-week interval. Titres and specificity were determined by ELISA to confirm antibodies, and which were used directly from the bleeds (Mtwisha *et al*, 1998).

Appendix C

1. Measurement of glycogen

The yeasts were grown as described previously in YPD media for 48h. The absorbance at 660nm was measured to determine the dry weight in mg/ml from the dry weight standard curve. A known volume of yeasts sufficient to give a final concentration of up to but not exceeding 4 mg/ml dry weight, was harvested by centrifugation (5000 g for 5 min at 4°C), and washed once with an equal volume of ice-cold double-distilled water. The cells were maintained on ice to prevent glycogen break down (Quain and Tubb, 1983).

Freshly prepared potassium iodide solution was added to the yeast cells to give a final yeast concentration of 20-25 mg/ml (wet weight). The cells were mixed carefully and incubated on ice for 10-15 min. The absorbance at 660 nm was measured and the glycogen concentration could be determined from the following formula: $X = (Y-0.26)/1.48$, where X equals the glycogen concentration in mg/ml and Y equals the absorbance at 660 nm. The calculated glycogen concentration can be thus converted to mg/g dry weight.

Appendix D

1. Measurement of aldo-keto reductase enzyme activity

The preparation of the reaction mixture to a final volume of 1ml, contained 800 µl of 50 mM potassium phosphate buffer at pH5.0 (850 µl for blank), 50 µl of 0.12 mM NADPH (Roche-10277839), 50 µl of 10 mM mercapto-ethanol and 10 mM of DL-glyceraldehyde (Sigma-Aldricht, G-5001) as the substrate added last. A water bath was connected to the spectrophotometer to keep the enzyme assay at a constant temperature of 25°C. Before the start of the assay, 50 µl of the enzyme extract was added to both blank and samples, and 50 µl of substrate to samples only. The decrease in absorbance at 340 nm was measured for a period of time of 5 min (Kuhn *et al*, 1995). The rate of the enzyme activity in U/min was determined for the most linear section of the graph by using the equation: $V/(v*d*\varepsilon)* \Delta A/\Delta t$ where $\Delta A/\Delta t$ is the change in absorbance over time, V is the total reaction volume in cuvette, v is the enzyme extract volume, ε is the extinction coefficient and d is the thickness of cuvette which equals to 1 cm.