SEPARATION OF A BREWING YEAST STRAIN OF *SACCHAROMYCES CEREVISIAE* BASED ON CELLULAR AGE

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment for the requirements for the degree of Master of Science.

August 2002

Your file Votre référence
ISBN: 0-612-88169-5
Our file Notre référence
ISBN: 0-612-88169-5

Abstract

SEPARATION OF *SACCHAROMYCES CEREVISIAE* BASED ON CELLULAR AGE

In yeast, aging appears to be marked by a progessive impairment in cellular mechanisms, resulting in irreversible changes in physiology and morphology. To date, very little has been reported about the biochemical changes that occur in yeast as a function of individual cell aging. To investigate this further, six generations of a brewing yeast strain of *Saccharomyces cerevisiae* (NCYC 1239) were separated according to cellular age using continuous phased culturing and biotin-streptavidin magnetic cell sorting.

To obtain cells with no bud scars (virgin cells), a concentrated yeast slurry was layered onto sucrose density gradients and centrifuged. The uppermost band from the gradients was collected and cells were biotinylated with biotinamidocaproate-*N*-hydroxysuccinimide ester, that covalently binds to lysine residues on the yeast cell wall. For continuous phased culturing, biotinylated cells were added to a carbon-limited nutrient medium and growth was synchronized using the doubling time of the cells. Harvested cells were incubated with streptavidin superparamagnetic beads and sorted with a strong permanent magnet. In total, approximately 75% of the biotinylated cells were recovered. Viability testing was conducted using vital staining and plate counts, with >98% viability reported with the vital stain and 37% viability with the agar plates.

In conclusion, continuous phased culture, together with magnetic cell sorting has the potential to become a powerful tool for the study of age-related biochemical changes in yeast. Further studies will focus on ensuring the reproducibility of the method and using the recovered cells to study biochemical changes occurring during yeasts' replicative lifespan.

Résumé

SÉPARATION DE *SACHAROMYCES CEREVISIAE* SELON L'ÂGE CELLULAIRE

Dans la levure, des changements irréversibles d'ordre physiologique et morphologique semblent être la résultante de la détérioration progressive des mécanismes cellulaires accompagnant le vieillissement. Jusqu'à présent, très peu d'information existait sur les changements biochimiques qui prennent place dans la levure en fonction du vieillisement cellulaire individuel. Dans le but de remédier à cette lacune, six générations provenant d'une souche de levure couvante de *Sacharomyces cerevisiae* (NYC 1239) ont été séparées selon leur âge cellulaire grâce à une culture de phase continue et le triage de cellules magnétiques biotine-streptavidine.

Pour obtenir des cellules exemptes de cicatrices laissées par le bourgeonnement (cellules vierges), de la levure concentrée à été étagée sur des gradients de densité de sacharose, puis centrifugée. La bande supérieure des gradients a ensuite été récoltée et les cellules, biotinylisées à l'aide d'ester Biotiamidocaproate-*N*-hydroxysuccinimide qui se lie de façon covalente aux résidus de lysine présents sur la paroi cellulaire de la levure. La culture continuelle de phase fut obtenue en ajoutant les cellules biotinylisées à un milieu nutritif limité en carbone et leur croissance, synchronisée, en se servant du temps nécessaire à la population pour doubler de taille. Les cellules ainsi récoltées furent finalement incubées avec des billes de streptavidine superparamagnétiques et triées avec un aimant permanent de grande force. Au total, approximativement 75% des cellules biotinylisées ont pu être récupérées. Des taux de viabilité supérieurs à 98% furent obtenus à l'aide de la technique de coloration vitale, et de 37% avec la méthode de comptage d'assiette de gélose.

En conclusion, la culture de phase continue de concert avec le triage de cellules magnétiques a le potentiel de devenir un outil puissant pour l'étude des changements biochimiques prenant place dans la levure, en relation avec l'âge cellulaire. Des études subséquentes focaliseront sur la reproductibilité de la méthode et sur l'usage des cellules récoltées pour étudier les changements biochimiques prenant place pendant la durée de vie réplicative des levures.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my co-supervisors, Dr. J.D. Sheppard and Dr. J.P. Smith, for their guidance, support, and knowledge.

I would also like to thank Ms. Ilsemarie Tarte and Mr. Wassim El-Khoury for their generous assistance and advice in carrying out the laboratory experiments.

Special thanks to Dr. Knowles for kindly assisting me with the fluorescent microscopy.

Grateful acknowledgments to Mr. Bernard Cayouette for translating the abstract into French.

Many thanks to everyone in my research group for their friendship and advice, including John Koukoutsis, Daphe Phillips Daifas, Sharon Rutherford, Sameer Al-Zenki, Barry van Bergen, Lucas Vann, Lamin Kassama, and Stephanie Briggs.

Special thanks to Dina Spigelski for her thoughtful encouragement, Carl White for his interest, and my family for their support.

Finally, I am most indebted to my husband, Giles Bishop, for his financial support, assistance, and encouragement throughout this effort.

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1.1. The brewing industry

1.1.1. The history of brewing

The brewing of beer is a multistage process involving the biological conversion of raw materials into a final product (Walker,1998a). Beer, as it is known, is a beverage made predominantly by fermenting an extract of malted barley and hops using a chosen strain of yeast. In modern brewing practices, some of the barley is replaced with starchy adjuncts, such as those derived from rice or corn (Reed and Nagodawithana,1991).

Scientific and archaelogical evidence suggests that beer was first produced in the late fourth millennium B.C. by the Sumerians in southern Babylonia. Brewing flourished in Egypt until the end of the eighth century A.D. when Muslim Arabs conquered the region and forbade the consumption of alcohol. However, by this time, the art of brewing had spread far beyond the confines of the Middle East, as traders, to and from the region, learned the essentials of beer-making. It is assumed that it was via traders that the production of beer reached the British Isles (Hornsey, 1999).

Although the brewing of beer has a history extending back several centuries, it is only within the past 150 years that the underlying science of the brewing process has been understood (Bamforth, 2000). In the 1870s, the French scientist, Louis Pasteur, demonstrated the essential requirement for live yeast in the fermentation process. He devised a new method of brewing which reduced contact between the beer and the atmosphere, thereby reducing the potential for contamination, a significant problem at that time. Subsequently,

Emil Hansen (1842-1909), who was employed by the Jacobsen's Carlsberg Laboratory in Copenhagen, concluded that not all contamination was caused by bacteria as Pasteur had surmised and showed that wild yeasts, other than *Saccharomyces cerevisiae*, were involved. He developed methods for isolating single yeast cells which eventually led to a pure culture system being used on a production scale in 1883, a method which is still used to this day (Hornsey, 1999).

1.1.2. The economic importance of the brewing industry in Canada and worldwide

The brewing of beer is an enormous enterprise and probably represents the largest biotechnology industry in the world. The amount of beer produced commercially each year is 10^{11} litres (Oliver, 1991). Canada is the 12^{th} largest producer of beer in the world (Vallee, 1996). In 1997, Canadians consumed nearly 20 million hectolitres of beer and exported another 3.5 million hectolitres. The industry employs over 17,000 workers directly and another 130,000 indirectly in Canada, and its contribution to economy was 11 billion dollars in 1997 (Brewers' Association of Canada, 2001).

1.1.3. The production of beer

1.1.3.1. The brewing process

Most beers are classified into two main types, ale and lager. This difference is based mainly on the strain of yeast used and the conditions during the fermentation process (Hornsey, 1999). The most popular type of beer in North American is lager (Munroe, 1995). A general scheme for a conventional lager type brewing process, which is similar to the production of ales, is outlined in Figure 1. The entire production process consists of four stages:

(1) Malting: The principal raw material used in the production of beer is barley. However, in its native form, barley cannot be used directly and must undergo a highly controlled process known as malting, in which the barley is germinated, kilned, and finally milled, at the brewery, to produce a coarse flour (Reed and Nagodawithana, 1991).

(2) Mashing: Since brewing strains of yeast cannot ferment starch and other polysaccharides in the malt, a process known as mashing has been developed for converting these polysaccharides into fermentable sugar. During this operation, the soluble materials are extracted from the ground malt that had been partially solubilized during malting, and the remaining insoluble components are converted to soluble assimilable products under controlled conditions with the aid of enzymes present in malt. Mashing involves two important enzymatic reactions. The first is the conversion of starch into fermentable sugar by α- and β-amylases found in malt and the second is the conversion of proteins and polypeptides into amino acids and smaller peptides. The fermentable sugars formed during the mashing process are used by yeast to produce ethanol and carbon dioxide, while some of the amino acids are required for yeast growth during early stages of fermentation (Reed and Nagodawithana, 1991).

(3) Fermentation: In most cases, this stage is divided into primary and secondary fermentation. During primary fermentation, most of the principal flavor and aroma compounds, critical for the final beer flavor, are formed. These include higher alcohols, esters, sulfur compounds, and organic acids that eventually characterize a specific beer. Secondary fermentation is a maturation process that renders the beer more palatable and stable. Yeast is harvested at the end of the primary fermentation process and is used to inoculate a second batch of wort (Reed and Nagodawithana, 1991).

(4) Down-stream processing: This includes operations such as filtering, pasteurization, carbonation, and bottling (Linko *et al.*, 1998).

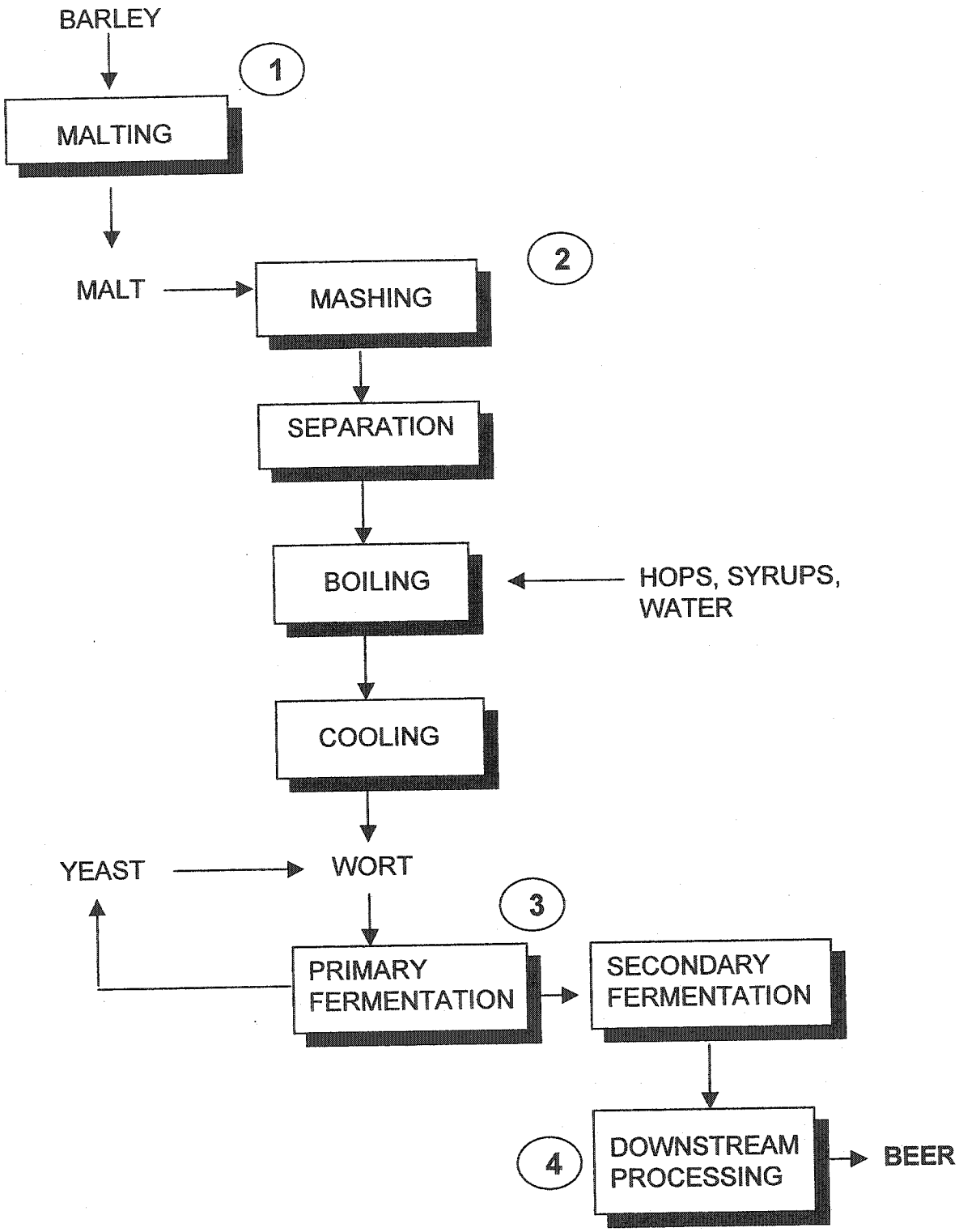

Figure 1. Simplified scheme for lager-type brewing (Adapted from Linko *et al.*, 1998).

1.1.3.2. Brewing yeasts

Two species of the genus *Saccharomyces*, *Saccharomyces cerevisiae* and *Saccharomyces uvarum (calsbergensis)*, are most commonly used in the brewing of beer (Reed and Nagodawithana, 1991). Ale brewing yeasts are comprised of strains of *Saccharomyces cerevisiae*. Lager yeasts are also strains of *S. cerevisiae*; however, for historical and practical, rather than taxonomic reasons, they are usually referred to as *S. carlsbergensis* (Walker, 1998a) or *S. uvarum* (Reed and Nagodawithana, 1991). Brewing strains are selected for the flavour characteristics they impart as well as other desirable characteristics, such as yeast flocculation, genetic stability, attenuation of wort carbohydrates, acceptable foam characteristics in the beer, and interaction with fining materials for effective filtration (Munroe, 1995).

While most laboratory yeast strains are haploid, most brewing strains are diploid, polyploid, or aneuploid. Haploid means the nucleus contains a single set of chromosomes; diploid means that the nucleus contains two homologous sets of chromosomes, while polyploid means the nucleus has three, four, or more integral sets of the haploid number of chromosomes (Russell, 1995).

Although a great deal of information describing the genetics and biochemistry is available for laboratory strains of *S. cerevisiae*, similar information is lacking for industrial strains. The haploid strains, typically used in the laboratory, are unsuitable for industrial purposes. Brewing yeasts have been selected, over time, for characteristics that prevent genetic manipulation in the laboratory. Besides being polyploid or aneuploid, brewing strains lack a mating type characteristic and sporulate poorly. Due to their polyloid nature, their multiple gene structure makes them genetically more stable and less susceptible to mutational changes and can thus be recycled with a higher degree of confidence than either haploid or diploid yeast strains (Stewart and Russell, 1986).

Brewers have traditionally relied upon natural selection of those yeasts that ferment well and impart desirable organoleptic properties to beer. Nonetheless, several genetic opportunities exist to improve fermentation processes and it appears that the most significant contributions to strain improvement of industrial yeasts involve recombinant DNA technology (Walker, 1998a). The world's first genetically modified brewing yeast was approved by government agencies for commercial exploitation in 1994. To date, it remains in the yeast collection and will likely remain until the public approves the use of modified organisms in the beer-making process, or until the brewing companies can prove the benefits of using such organisms (Bamforth, 2000).

Brewing yeast fermentation performance has been defined as "the ability of yeasts to consistently metabolize wort constituents into ethanol and other fermentation products in order to produce beer with a satisfactory quality and stability" (Stewart and Russell, 1995). Yeast performance is influenced and controlled by a number of factors including (Stewart and Russell, 1995):

(i) genetic characteristics, i.e. the choice of yeast strain used;
(ii) cell physiology, e.g. the stress tolerance of yeast cells;
(iii) viability and vitality of the cells;
(iv) inoculum cell density (or pitching rate);
(v) nutritional availability, e.g. the concentration and category of assimilable nitrogen, the spectrum of wort sugars and the concentration and availability of metal ions; and
(vi) physical environment, e.g. temperature, pH, dissolved oxygen, and wort gravity.

1.1.3.3. Yeast fermentation technology developments

In the production of ethanol during fermentation, the microorganisms used in the brewing process should meet certain criteria. These are (Stewart and Russell, 1986):

(i) a rapid and significant carbohydrate fermentation ability;
(ii) appropriate flocculation and sedimentation characteristics;
(iii) genetic stability;
(iv) osmotolerance (i.e. an ability to ferment high concentrations of carbohydrate);
(v) ethanol tolerance and the ability to produce high concentrations of ethanol;
(vi) high cell viability for repeated re-pitching; and
(vii) temperature tolerance.

Most of the current research with industrial yeast strains is aimed at improving the above listed characteristics (Stewart and Russell, 1986). Brewing improvements centered on the activities of yeast can be made not only through genetic modifications, but also through process-related modifications. These latter considerations relate to the design, operation, monitoring and control of yeast fermentations. Examples of process control developments include on-line measurement and modulation of parameters, such as yeast biomass, ethanol concentrations, specific wort gravities, and CO_2 evolution. Fermenter design developments include the use of immobilized yeast bioreactors for primary fermentations and beer maturation (Walker, 1998a).

1.1.4. Recent advances in the brewing industry

In the past decades, yeast research for the brewing industry has focused on trying to understand the mechanisms of yeast growth, fermentation, and flocculation. Early research focused on the biochemical and physiological aspects of yeast. Today, the trend is focused at the molecular and genetic levels in order to modify industrial yeast for specific purposes (Stewart and Russell, 1986).

According to Bamforth (2000), there are four forces driving technological change in the brewing industry: cost savings, quality enhancement, safety and wholesomeness, and enhanced sales opportunities. The contribution of raw materials to the overall cost is relatively minor (Figure 2). However, the cost of production, especially packaging, far outweighs that of raw materials. This has driven brewers to enhance profitability through increased automation, enhanced use of fermentation tanks, and exploration of new ways to reduce energy costs (Bamforth, 2000).

Perhaps the most important technical innovation in brewing operations during recent years has been the use of high gravity or very high gravity brewing. This means that the wort is produced and subsequently fermented at a considerably higher concentration than required for the final beer. After fermentation, the beer is adjusted to the desired concentration with oxygen-free water. Very high gravity worts, that is, over 20 °Plato, are achieved by adding additional carbohydrate, typically in syrup form, to the wort kettle (Linko *et al.*, 1998). The Plato unit is defined as the quantity of fermentable extract in percent by weight of the wort, beer, or other solution (Weissler, 1995). Therefore, a 20 °Plato wort would contain approximately 20% carbohydrate.

Among the many advantages cited for high-gravity brewing are (i) more consistent beers because of simple dilution adjustments made at later stages of processing, (ii) improved physical stability, better use of equipment, and (iii) lower energy costs. However, the disadvantages of high gravity brewing include (i) longer fermentation times, (ii) variable flavour characteristics, (iii) additional equipment for water preparation, (iv) greater yeast handling, and (v) potentially higher yeast mortality (Munroe, 1995). Adverse effects on the yeast may be due to increased wort osmotic pressure, elevated ethanol concentrations in the wort, and nutrient limitation. These factors can result in a decreased rate and extent of fermentation, decreased yeast viability and activity, and affect yeast flocculation characteristics (Casey *et al.*, 1993).

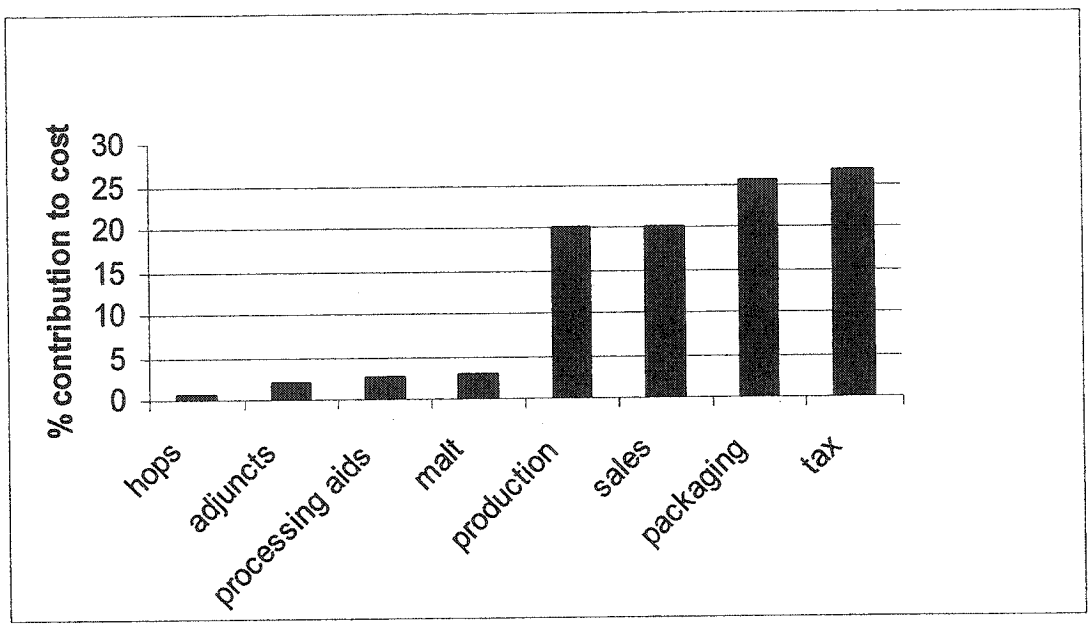

Figure 2. Cost components of beer production (U.S. estimates) (Adapted from Bamforth, 2000).

1.2. Brewer's yeast, Saccharomyces cerevisiae

1.2.1. Taxonomy

Generally, yeasts are recognized as being unicellular fungi that reproduce vegetatively by budding or fission (Walker, 1998a). The most widely used yeasts in the brewing industry are members of the fungal genus *Saccharomyces*, of which there are over 30 known species. They are eukaryotic organisms, possessing double unit membrane-bound organelles (such as nuclei and mitochondria). *Saccharomyces* spp. are found within the family *Saccharomycetaceae* and can be distinguished from each other based on morphological and physiological characteristics (Reed and Nagodawithana, 1991).

It is at the strain level that interest in brewing yeasts centres, and there are a least 1000 separate strains of *S. cerevisiae*. These strains encompass brewing, baking, wine, distilling, and laboratory cultures. Taxonomically, two species, *S. uvarum* (*carlsbergensis*) and *S. cerevisiae*, have been distinguished on the basis of their ability to ferment the disaccharide melibiose. Strains of the former produce the extracellular enzyme melibiase and are able to use melibiose, whereas strains of *S. cerevisiae* lack this enzyme (Russell, 1995).

1.2.2. Ecology

Although not as ubiquitous as bacteria, yeast are widespread in the natural environment. Most yeasts grow well in warm, moist, carbohydrate-rich, acidic, and aerobic environments. Since they are non-motile, yeasts rely on aerosols, animal vectors and human activity for their natural dispersal (Walker, 1998a).

Yeasts can be isolated from air, water and soil. Preferred habitats are plant tissues but some species are found in parasitic relationships with animals. Some yeasts may be regarded as extremophiles, especially certain osmophilic yeasts which are able to thrive in solute-rich environments. Several of these yeast species are encountered as food spoilage organisms (Walker, 1998a). The term 'wild yeast', has generally been ascribed to any yeast that contaminates the pure culture employed in processes such as brewing, wine making, or baking. Wild yeasts encountered in brewing operations are often strains of *S. cerevisiae*, *S. uvarum*, *S. bayanus*, and *S. diataticus*. Common defects caused by these 'wild yeasts' include the production of off-flavors and turbidity (Reed and Nagodawithana, 1991).

1.2.3. Yeast Nutrition and Metabolism

1.2.3.1. Yeast nutritional requirements and basic metabolism

The overall growth requirements of yeast are a carbon energy source, a nitrogen source, vitamins, organic ions, oxygen, and water (Hornsey, 1999). In addition, yeast growth depends on both temperature and pH. Most laboratory and industrial strains grow best between 20-30°C but, as with all microorganisms, yeasts exhibit characteristic minimum, optimum and maximum growth temperatures. For example, for *S. cerevisiae* strains, maximum temperature values range from 35-43°C. Most yeasts grow well between pH 4.5 and 6.5, but nearly all species are able to grow, to a lesser extent, in more acidic or alkaline environments, around pH 3 or pH 8, respectively (Walker, 1998a).

1.2.3.1.1. Sources of carbon and basic metabolism

Yeasts are strictly chemoorganotrophic, meaning that they require fixed, organic forms of carbon for growth. These are provided in part, by the

carbohydrates, or sugars, in the wort. (Walker, 1998a). The major carbohydrates in an all-malt wort are shown in Table 1.

Both glucose and fructose are passively taken up by the yeast cell. Sucrose is first enzymatically hydrolysed outside the cell and then absorbed, whereas maltose and maltotriose are actively transported across the yeast cell membrane and hydrolysed in the cytosol of the cell. Dextrins, such as maltotetraose and larger starch breakdown products, are not metabolised and small amounts of pentose sugars are also left unfermented (Hornsey, 1999). The general order of uptake of fermentable sugars from wort during fermentation by brewing yeast strains is sucrose-glucose-fructose-maltose-maltotriose (Hornsey, 1999) although some degree of overlap does occur (Russell, 1995).

During the brewing process, the rate and degree of wort sugar uptake is controlled by several factors. These include (i) the yeast strains employed, (ii) the concentration and type of assimilable nitrogen, (iii) the concentration of ions, (iv) the fermentation temperature, (v) the pitching rate, (vi) the tolerance of yeast cells to ethanol, (vii) the wort gravity, (viii) the wort oxygen level at yeast pitching, and (ix) the wort sugar spectrum (Russell, 1995).

1.2.3.1.2. Sources of nitrogen and basic metabolism

Brewer's yeast cannot assimilate elemental nitrogen or nitrate ions. Some strains can use ammonium ions but the majority of the nitrogen required for the synthesis of essential cell constituents is derived from the amino acids and the di- and tri-peptides in the wort (Hornsey, 1999).

As a result of interactions between the various amino acid transport systems in yeast, amino acids are removed from wort in an orderly manner. Four groups of amino acids, based on relative uptake rates, have been identified in ale fermentations and are described in Table 2 (Hammond, 1987). In lager fermentations, the uptake order is similar although arginine, aspartate and glutamate tend to be taken up more slowly (Hammond, 1987).

Once amino acids and peptides have been taken up they are not assimilated intact but pass through a transaminase system which removes the amino group, leaving the carbon skeleton to be metabolised. This is of great value to yeast since the released amino groups can be donated to other carbon skeletons formed by anabolic pathways. In this way, the cell is not reliant on a sufficient supply of amino acids but can synthesise those it needs from sugars and sources of amino nitrogen present in wort. These reactions are also of great importance to the brewer since keto acids produced, by the action of transaminase, are precursors of aldehydes, higher alcohols, and vicinal diketones which play an important part in beer flavour (Hammond, 1987).

Table 1. Profile of the major carbohydrates in an all-malt wort (Hornsey, 1999).

Carbohydrate	Percent composition (%)
Maltose	50-60
Dextrins	20-30
Maltotriose	15-20
Glucose	10-15
Fructose	1-2
Sucrose	1-2

Table 2. Relative rates of amino acid absorption by yeast (Hammond, 1987).

Rate of absorption	Amino acid
Immediate	Arginine, Lysine, Aspartate, Asparginine, Glutamate, Glutamine, Serine, Threonine
Gradual	Leucine, Isoleucine, Valine, Methionine, Histidine
Slow	Alanine, Glycine, Phenylalanine, Tryptophan, Tyrosine
Negligible	Proline

1.2.3.1.3. Growth factors, vitamins, inorganic ions and minerals

Growth factors are organic compounds required in very low amounts for specific catalytic or structural roles, but are not used to supply energy to the cell. These include vitamins, purines and pyrimidines, nucleosides and nucleotides, amino acids, fatty acids, and sterols (Walker, 1998a). Wort generally provides a rich source of vitamins for yeast which generally require biotin, thiamin (B1), nicotinic acid, riboflavin, calcium pantothenate, inositol and pyridoxine, pyridoxal and pyridoxamine (Hornsey, 1999).

A number of inorganic ions are also required by yeast during fermentation, some having a structural role, others being required for enzymatic purposes. Yeasts require sulfur mainly for the biosynthesis of sulphur-containing amino acids and sources include sulphate, sulphite, thiosulphate, methionine, and glutathione (Walker, 1998a). Phosphorous is present in nucleic acids and in phospholipids and is therefore an essential nutrient for all yeasts. Much of the negative charge of the yeast cytoplasm is due to the presence of inorganic phosphates and the phosphate groups in organic compounds. Potassium and magnesium are regarded as the principal macroelements and are required in substantial quantities (2-4 mM optimally) to establish the main metallic cationic environment in the yeast cell. Trace elements, which are generally required in the micromolar or nanomolar range include manganese, calcium, iron, zinc, copper, nickel, cobalt, and molybdenum. Heavy metals, including silver, arsenic, mercury, and selenium are considered toxic to yeast in concentrations above 100 μM (Walker, 1998a).

During fermentation, the concentrations of magnesium, zinc, potassium, and cobalt are most important where the optimum concentration of one is partly dependent on the concentration of other ions. This relationship has been found to occur between zinc and manganese and between calcium and magnesium. Most cationic elements are present in wort in several forms but not all are bioavailable, due to wort pH, anion availability, and presence of chelating agents

(Hornsey, 1999). As well, a poor quality barley crop can lead to worts that are deficient in these metals, which can lead to inconsistencies in the process and product, including lagging fermentations and poor yeast quality (Bromberg *et al.*, 1997).

1.2.3.1.4. Oxygen and water

Yeasts require oxygen, not only as the terminal electron acceptor in aerobic metabolism, but also as an essential growth factor for membrane fatty acid and sterol biosynthesis (Walker, 1998a). The influence of oxygen and sugar availability on yeast carbohydrate metabolism has been categorized under various regulatory phenomena, such as the Pasteur, Crabtree, Custers and Kluyver effects. *S. cerevisiae* is facutatively fermentative and Crabtree-positive since it can ferment high sugar-containing media in the presence of oxygen (Walker, 1998a).

Finally, yeasts, like all organisms, need water in high concentrations for growth and metabolism. Substrates and enzymes are all in aqueous solution or colloidal suspension and no enzyme activity can occur in the absence of water (Walker, 1998a).

1.2.3.2. Yeast cultivation media

Due to their relatively simple nutritional requirements, it is easy to grow yeasts. For general laboratory cultivation, several synthetic and complex media are commercially available and formulations for standard media can be found in many microbiology handbooks. For vigorous and extensive yeast growth, malt extract broth (or beer wort) is traditionally used as a growth medium. Another common growth medium uses yeast extract, obtained by autolysing yeast cells, and supplements it with malt extract, peptone, and glucose (Walker, 1998a).

Examples of synthetic media include Yeast Nitrogen Base, that was originally formulated by Wickerham, for carbon assimilation and fermentation tests. It contains ammonium sulfate and asparagine as nitrogen sources, the principal minerals, a range of vitamins, and a selection of amino acids. A carbon source of choice is added. Yeast Carbon Base is also available and requires the addition of a nitrogen source (Walker, 1998a).

1.2.4. Yeast Growth

1.2.4.1. Vegetative Reproduction in Saccharomyces spp.

Saccharomyces species reproduce by a mechanism known as multilateral budding, which is initiated by lytic enzymes that weaken the cell wall in a localised area. Vesicles from the endoplasmic reticulum congregate in this area and internal pressure forces the cytoplasm through the weakened wall. This "bulge" immediately becomes surrounded by newly synthesized cell wall material. The nucleus migrates to the bud-forming site and when cell organelles have passed into the embryonic bud, a septum of cell wall material separates the bud from the so-called mother cell, constricting and pinching off a portion of the nucleus (Hornsey, 1999).

As a cell reproduces by budding, a bud scar is formed on the mother cell but not on the daughter cell (Figure 3). By counting the number of bud scars on a cell it is possible to estimate a cell's age. Although individual cells age, the total collection of cells does not since, assuming a healthy population, within most generations, 50% of yeast cells have no bud scars, 25% of cells have one bud scar, 12.5% of cells have two bud scars, etc. In theory, a well-maintained yeast culture can be used indefinitely (Russell, 1995).

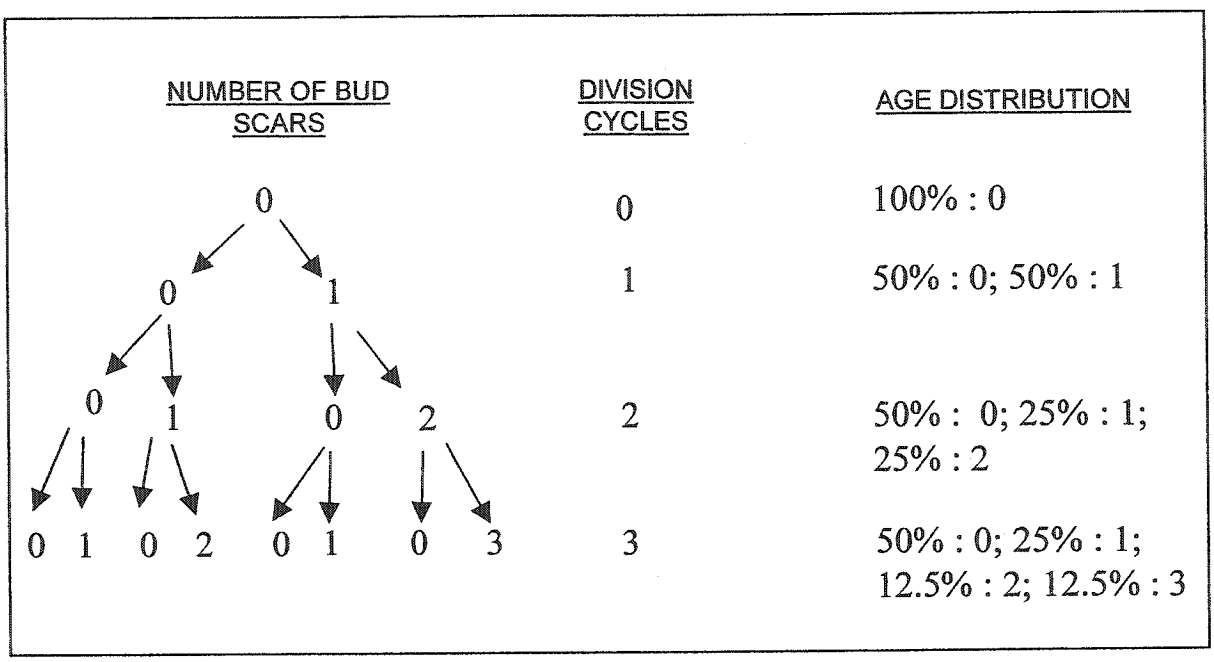

Figure 3. Development of age heterogeneity during yeast growth (Adapted from Jones, 1997).

1.2.4.2. The yeast cell cycle

The normal yeast cell cycle involves the progression through a series of events that allows the cell to complete a round of cellular reproduction known as division. For the brewing industry, this process is critical for the formation of sufficient yeast biomass to generate the final product, and sufficient biomass for subsequent repitching (Smart, 1999).

The yeast cell cycle involves both continuous events, such as cell growth, and periodic events, e.g., DNA synthesis and mitosis. The culmination of these events is cell division (Walker, 1998a). Periodic events can be divided into four phases: G1, S, G2, and M. After cell division has occurred, there is a time gap (G1 phase) before the new cell begins to replicate its genetic material (DNA) during the S phase. Once DNA replication is complete, there is a second time gap (G2 phase) before mitosis (M phase) occurs and the cycle is repeated (Smart, 1999).

One of the most important events during the cell cycle is known as 'Start', which resides in the G1 phase (Figure 4). Failure to progress through Start, due to the absence of nutrients, results in cell division arrest and entry into the cell cycle stationary phase, G0. However, after Start has occurred, environmental factors, such as external stress and poor nutrients, are no longer able to prevent division. Budding is the obvious visible sign that a cell has passed Start and in general, the larger the bud, the closer the cell is to division from the mother cell (Smart, 1999).

Cell division rate is influenced by the rate of growth of an individual cell and the rate of protein synthesis (Johnston *et al.*, 1977). Cells can pass through Start only when they have reached a critical cell size. Thus, slow growing cells have a relatively long division time, and this additional time is largely spent in the G1 phase. Also, daughter cells are smaller than mother cells at cell division and thereby have longer G1 phases, since they must grow more than mother cells to reach a critical size, in order to pass Start. This differential is known as mother-

daughter asymmetry, where the mother and daughter cells may be distinguished by both cell size and division rate (Walker, 1998a).

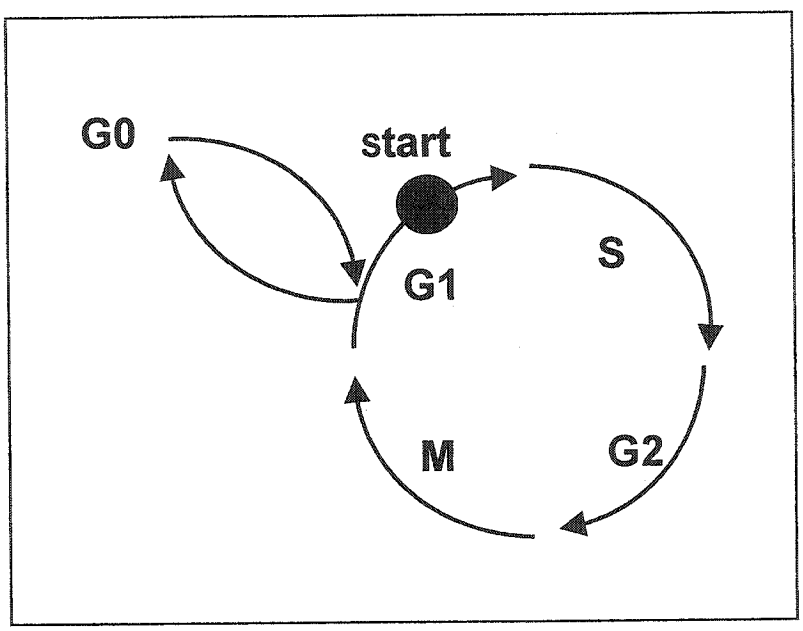

Figure 4. The yeast cell cycle (Adapted from Smart, 1999)

1.2.4.3. Yeast population growth

1.2.4.3.1. Growth kinetics of microorganisms

The required conditions for growth of biomass in a culture are a viable inoculum, an energy source, nutrients to provide the essential materials from which the biomass is synthesized, the absence of inhibitors that prevent growth, and suitable physio-chemical conditions (Pirt, 1975).

If all the requirements for growth are satisfied, then during an infinitely small time interval (*dt*), it is anticipated that the increase in biomass (*dx*) will be proportional to the amount of biomass present (x) and to the time interval such that,

$$dx/dt = \mu x \qquad (1.0)$$

The differential coefficient (*dx/dt*) expresses the population growth rate. The parameter μ, which represents the rate of growth per unit amount of biomass $(1/x)(dx/dt)$, is termed the specific growth rate and has the dimension of reciprocal time ($1/t$) (Pirt, 1975). The value of μ will vary between 0, for no growth, and some maxima, denoted μ_{max} (Walker, 1998a). When μ_{max} is constant, integration of the above equation gives:

$$\ln x = \ln x_o + \mu_{max} t \qquad (2.0)$$

By putting this equation in the form

$$\ln (x / x_o) = \mu_{max} t \qquad (3.0)$$

it follows that

$$x = x_o\, e^{(\mu max\, t)} \qquad (4.0)$$

Growth which follows this is called constant exponential or logarithmic growth (Pirt, 1975).

It is often useful to calculate the doubling time (t_d) of a culture from knowledge of μ. The relationship between μ and the doubling time (t_d) of the biomass is obtained from equation 3.0, by putting $x = 2x_o$ and $t = t_d$, which then becomes

$$t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} \qquad (5.0)$$

When yeast growth is limited by the concentration of one substrate, *S*, the relationship between μ and *S* can be defined by the Monod equation:

$$\mu = \mu [\,[S\,]/\,([S] + K_s)] \qquad (6.0)$$

where K_s (the saturation constant) is the value of *S* which limits the growth rate to ½ μ (Walker, 1998a).

1.2.4.3.2. Batch growth of yeasts

When yeast cells are inoculated in a suitable liquid nutrient medium and incubated under optimal physical growth conditions, a typical batch growth curve will result when the viable yeast cell population is plotted against time. The yeast growth curve, obtained from a batch culture, is composed of three main phases: lag, exponential, and stationary phases.

During the lag phase, cells do not grow and therefore the specific growth rate, μ, is equal to zero. The length of the lag phase depends on growth conditions, inoculum density, and the manner in which the cells were grown and

handled prior to inoculation. The lag phase reflects the time required for the yeast to adapt to their new physical and chemical environment. Ribosomes and enzymes, needed to establish growth at a higher rate, are synthesized during this phase. Once cells exit the lag phase and begin active cell division, they enter an exponential growth phase. (Walker, 1999).

In batch culture, the exponential phase is fairly short-lived due to depletion of essential nutrients, accumulation of inhibitory metabolites, or excessive cell flocculation. One way to prolong the exponential phase is to use a fed-batch culture, in which nutrients are fed incrementally in step with yeast growth. Fed-batch culturing is commonly employed in the industrial propagation of baker's yeast (Walker, 1998a).

As growth becomes limited due to nutrient depletion, cells enter the phase of zero growth, known as the stationary phase. Cells in this phase are known to accumulate glycogen and trehalose, become thermotolerant, and develop thick cell walls (Werner-Washburne *et al.*, 1996). In addition to nutrient limitation, other physiological causes may promote entry of yeast cells into stationary phase. These include toxic metabolites (e.g. ethanol), low pH, high CO_2 concentrations, variable oxygen levels, and high temperature (Walker, 1998a).

1.2.4.3.3. Continuous cultivation of yeasts

Yeast cells can be propagated in continuous culture for prolonged periods of exponential growth without lag or stationary phases. The change in yeast biomass per unit time in continuous culture vessels is described as the difference between the rate of growth and the rate of removal of cells;

$$dx/dt = \mu x - Dx \qquad (7.0)$$

where D represents the dilution rate, or flow rate of nutrient per unit volume. Once continuous growth is established, the yeast growth rate remains constant and a steady-state cell concentration is maintained such that $dx/dt = 0$.

Therefore, μ = D, and yeast growth rate will be governed by the dilution rate. In practical terms, this is accomplished by altering the rate at which the feed pump delivers nutrients into the continuous culture vessel (Walker, 1998a).

Continuous cultures, based on the controlled feeding of a sole growth-limiting substrate, are called chemostats. In yeast physiology, chemostats have proved valuable in studying the growth and metabolic effects of a single variable nutrient because all other factors are held constant. Yeast chemostats can be established based on limitation of sources of carbon (e.g. glucose), nitrogen (e.g. ammonium ions), sulphur, phosphorus, potassium, and magnesium (Dawson, 1972; Fiechter *et al.*, 1987).

1.2.4.3.4. Synchronous Yeast Growth

In conventional batch and continuous yeast cultivation systems, cell populations are randomized with respect to individual cell cycles. Non-random, or synchronous, yeast cultures are characterized by cells in the population dividing more or less in unison. The synchronization of yeast cell populations is an important experimental tool which can greatly assist the biochemical analysis of cell cycle events (Walker, 1999).

Two approaches are available for synchronizing populations: (i) induction synchrony and (ii) selection synchrony. In induction synchrony, the objective is to forcibly induce the entire yeast population to divide simultaneously (Walker, 1999). For example, randomly dividing cells are treated with a chemical or physical agent and cells gradually accumulate at the same point in the cycle. The agent is withdrawn and the cells recommence the cycle in phase. Occasionally, this treatment has to be repeated to fully effect synchrony (Wheals, 1987).

Dawson (1972,1985) developed a system for continuously synchronizing yeasts known as phased culture, which functions based on the theory of oscillator entrainment. In this technique, instead of supplying fresh medium as a steady stream of nutrient rations to randomly dividing cells in a chemostat, a single total addition of nutrients is made at doubling time intervals. Cells adjust

their growth rate to the nutrient dosing interval and become synchronized to it. By changing the time interval, the doubling time can also be changed. This system delivers a key growth-limiting nutrient at a concentration sufficient to complete one cell cycle. Over the next cell cycle time, the population will have doubled. This phasing procedure is repeated continually to maintain synchrony. However, it may be argued that in such nutrient-phased systems yeast cells are periodically starved (Walker, 1998a). Examples of induction synchrony methods for yeast cell populations are shown in Table 3.

The aim of selection synchrony is to take an exponentially growing population and, by some means, sort the cells with respect to cell age and size. Once sorted, one of the fractions is used to initiate a new culture, or the fractions are analyzed as being representative of cells of different ages (Wheals, 1987).

In batch synchronous cultures of yeast, synchrony of cell division can only be maintained for two or three cycles and cultures are unbalanced with respect to continual changes in nutrient status and cell growth rate. A more balanced yeast growth could be achieved under steady-state conditions, e.g., a chemostat (Walker, 1998a). Although yeasts propagated in traditional chemostats are generally assumed to be asynchronous, several workers have established continuous-synchronous yeast cell populations in modified chemostats (Munch *et al.*, 1992; Abel *et al.*, 1994).

An overview of various selection synchronization methods for yeast cell populations is shown in Table 4. None of the induction and selection methods results in perfect synchrony, due to imperfections in the methods, momentary variations in the size and density of cells of identical cell age, and the natural variability in the duration of mitotic cycles of individual cells (Wheals, 1987; Walker, 1999).

With all the selection methods, the majority of cells in the original population are discarded and as a result, the yield is smaller compared with induction methods (Walker, 1999). Regardless of the method, however, even the most gentle of procedures can subject cells to mild heat shock, oxygen deprivation and nutrient depletion, which is sufficient to disturb the normal

metabolic flux in the cell and produce artifacts (Wheals, 1987). There are many methods for synchronizing yeast cell populations and the one chosen depends on several factors including laboratory resources and degree and yield of synchrony required (Walker, 1999).

Yeast synchrony can be monitored either by counting the percentage of dividing cells (Dawson, 1972), by phase contrast microscopy, or by cell counting using either a microscope counting chamber (e.g. Neubauer) or Coulter counter (Walker, 1999).

Table 3. Examples of induction synchronization for yeast cell populations

Method	Description	References
Feeding and starving	Stationary phase cells are given short periods of nutrient feeding and starvation. After storage at 4°C in a resting condition, cells are inoculated into fresh medium to induce synchrony.	Mitchison and Vincent (1965)
Block and release	The DNA-division cycle is blocked (e.g. by inhibition of DNA synthesis) then cells are released from the block to initiate synchrony.	Mitchison and Creanor (1971)
α-Factor block	α-Factor mating pheromone will block haploid *S. cerevisiae MATa* cells of the opposite mating type in G1 phase of the cell cycle. Inoculation into α-factor free media allows cells to proceed synchronously.	Creanor and Toyne (1993)
Continuous synchrony	Dilution rate changes in chemostat cultures results in 'spontaneous' synchronization of cell division.	Duboc *et al.* (1996)
Continuous phasing	A single addition of nutrients is made at doubling time intervals. Cells adjust their growth rate to the nutrient dosing interval and become synchronized to it.	Dawson (1972, 1985)

Adapted from Walker (1999)

Table 4. Selection synchronization methods for yeast cell populations

Method	Description	References
Velocity separation	A concentrated cell suspension is layered on a gradient (e.g. sucrose) in a tube and centrifuged at low speed (e.g. 500xg) separating cells by size. The top layer of small cells is removed and resuspended in growth medium to initiate the synchronous culture.	Egilmez *et al.* (1990); Grzelak *et al.* (2001)
Zonal centrifugation	Similar to velocity separation, but scaled up by the use of a zonal rotor.	Mitchison (1988); Creanor and Toyne (1993)
Density separation	Cells with different buoyant density during their cell cycle may be separated by equilibrium high-speed (up to 45,000 x *g*) centrifugation in a tube gradient (e.g. dextrin).	Hartwell (1970)
Continuous-flow centrifugation	Asynchronous growing cells are introduced into a continuous-flow centrifuge rotor at a speed which retains about 90% of the cells. 10% of small cells are carried away with the effluent to initiate synchronous culture.	Lloyd *et al.* (1975)
Centrifugal elutriation	Successive layers of different sized yeast cells kept suspended in an elutriator rotor can be collected to initiate a synchronous culture by increasing the medium counter flow or decreasing the rotor speed.	Mitichison (1988); Creanor and Toyne (1993)

Adapted from Walker (1999)

1.2.4.4. Assessment of yeast viability and vitality

1.2.4.4.1. Definitions of yeast viability and vitality

Beer quality is influenced by the biochemical performance of yeast during the fermentation (Smart *et al.*, 1999). If yeast is to be repitched, then its physiological and genetic stability of the yeast is critical. Assessment of this stability generally involves the examination of yeast viability and vitality (Lentini, 1993). Most brewers generally agree that viability refers to whether a yeast cell population is 'alive' or 'dead', and that vitality is a measure of yeast cell activity (Heggart *et al.*, 1999).

Live cells are those that can grow and proliferate. This characteristic is usually measured based on the ability of cells to produce macrocolonies on agar plates or microcolonies on media covered microscope slides (Heggart *et al.*, 1999). While some cells, incapable of division, may still be capable of active metabolism, in dead cells all metabolic activity has ceased (Walker, 1998a).

However, there appears to be no single definition to describe the concept of vitality, since cells may be affected in many ways. In terms of brewing, vitality has been described as (Heggart *et al.*, 1999):

(i) the capacity of yeast cells to initiate metabolism rapidly following transfer from a nutrient-poor to a nutrient-rich environment;
(ii) a measure of yeast activity or fermentation performance;
(iii) the ability to endure stress and still perform; and
(iv) fitness for use.

1.2.4.4.2. Determining yeast viability and vitality

Several methods can be used to assess yeast viability and vitality (Tables 5 and 6). These techniques generally show varying degrees of correlation with fermentative performance and no single technique exists that accurately predicts the physiological activity of a yeast sample (Jones, 1997; Walker, 1998a).

Yeast viability can be determined directly by measuring loss of cell reproduction (e.g. plate count) and indirectly by assessing cellular damage (e.g. vital stains) or a loss of metabolic activity (e.g. ATP, NADH). The most accurate measures still remain the plate and slide culture methods (Russell and Stewart, 1995; Walker, 1998a) but these methods are considered too slow for routine use in a brewery laboratory (Mochaba *et al.*, 1997). Staining methods are more accurate but less precise than plate or slide culture methods, with the exception of certain fluorochromes (Walker, 1998a). The disadvantage of fluorochromes is the high cost of fluorescent microscopes and their impracticality for routine use (Mochaba *et al.*, 1997).

Of the vital stains, brightfield stains are still the most rapid and relatively inexpensive to use (Lentini, 1993). The only requirements are a light microscope with a hemocytometer or similar counting chamber (e.g. Neubauer, Coulter Counter) or normal slide. When using a hemocytometer (Figure 5), a coverslip is placed over the counting surface and the cell suspension is introduced into one of the V-shaped wells with a pipet. The area beneath the coverslip is filled by capillary action (Lech and Brent, 1988). The main divisions on the hemocytometer (Figure 6), separate the grid into nine large squares. Each square has a surface area of 1 mm^2, and the depth of the chamber is 0.1 mm. Therefore, each square of the hemocytometer represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 mL, the cell concentration per mL can be determined.

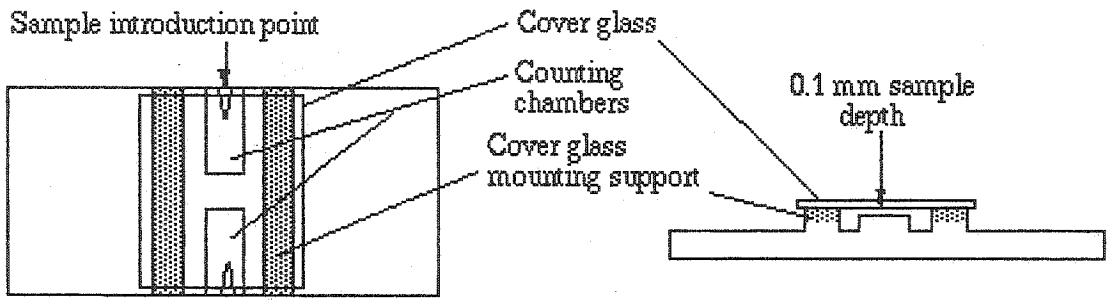

Figure 5. *Cell counting chamber (Adapted from Lech and Brent, 1988)*

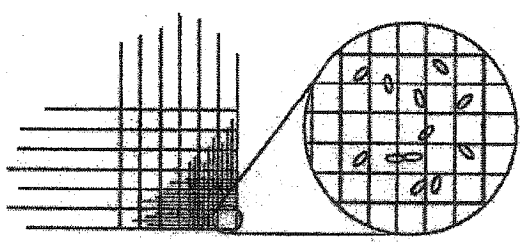

Figure 6. Hemocytometer cell counting grid (Adapted from Lech and Brent, 1988)

Methylene blue is the most common brightfield stain used (Maca *et al.*, 1994; Fukui *et al.*, 1996; Smart and Whisker, 1996; Cunningham and Stewart, 1998; Walker,1998b) and is recommended by the European Brewing Commission (EBC), the American Society of Brewing Chemists (ASBC), and the International Organization of Brewers (IOB), as the standard method for measuring yeast viability (Lentini, 1993). Generally, methylene blue stain results are accurate at viabilities above 90% (Lentini, 1993) but some researchers consider it useful only at viabilities greater than 95% (O'Connor-Cox *et al.*, 1997). When compared to plate counts, methylene blue consistently overestimates viability by 10-15%. When methylene blue viability decreases to between 90 and 95%, cell viability by the plate count method can be < 50%. It is also difficult to distinguish partially stained cells from unstained ones (Lentini, 1993). However, Russell (1995) claims that for an experienced microbiologist, methylene blue can quickly identify a problem, if the viability of a yeast strain is known prior to its use. Recently, it was found that the compound, Methylene Violet provided a good indication of viability (Smart *et al.*, 1999), since it exhibited greater resistance to oxidative demethylation, compared to methylene blue, resulting in reduced variations of color intensity, thereby increasing the accuracy of the method.

However, vitality, rather than viability, is thought to be a more reliable indicator of yeast quality and a better predictor of subsequent fermentation performance (Axcell and O'Connor-Cox, 1996; Mochaba *et al.*, 1997). Yeast vitality can be determined indirectly by measuring metabolic or fermentation activity, cellular storage molecules, intracellular and extracellular pH, and gaseous exchange coefficients, such as respiratory quotient (RQ) (Walker, 1998a). Mochaba *et al.* (1997) reported above-average fermentation performance when yeast cells released magnesium, potassium, and phosphate ions immediately after inoculation, and below-average performance when yeast absorbed these ions. They used a commercially available test kit to measure magnesium in wort prior to, and immediately following, yeast inoculation. The dye, Methylene Violet, has also been suggested as a marker of yeast vitality, as

dye, Methylene Violet, has also been suggested as a marker of yeast vitality, as it was found to give a good correlation with the Acidification Power Test (Smart *et al.*, 1999).

Table 5. Methods for assessing yeast viability

Method	Description	References
Plate count	Growth on nutrient agar plates; growth reported as the number of colony forming units (CFU)/ mL or g.	American Society of Brewing Chemists (1980)
Slide count	Microcolonies counted after 18 hours on a film of nutrient agar on a microscope slide.	Pierce (1970)
Vital stains	Based on membrane permeability and intracellular modification of two types of stains. Viable cells render stain colorless when absorbed; non-viable cells do not. (i) Brightfield stains e.g. Methelyne Blue; Methylene Violet (ii) Fluorochrome stains e.g. Carboxyfluorescein Diacetate; Mg-ANS	McCaig (1990); Breeuwer *et al.*, (1994); Smart and Whisker (1996); Smart *et al.* (1999);
Metabolic activity	ATP bioluminescence: Levels of ATP in cell with correlated with cell activity.	Jones (1987)
Cellular compounds	Glycogen and trehalose cell content: Stored energy source for the cell; depletion associated with stress or starvation.	Mochaba *et al.* (1994); Moonsamy *et al.* (1995)
Slurry pH	Above normal pH in yeast slurry indicative of autolysis.	Mochaba *et al.* (1997)
Protease Release Test	Release of free amino nitrogen (FAN) of stored yeast indicates autolysis.	Mochaba *et al.* (1993)
Dielectric permittivity	Capacitance probe: an electromagnetic wave is applied to a yeast suspension, allowing a dielectric charge to accumulate across the cell membrane; Resulting radio frequency depends on intact cells and their total area; potential in-line use.	Austin *et al.* (1994); Maca *et al.* (1994); Markx and Kell (1995)

Table 6. Methods for assessing yeast vitality

Method	Description	References
Cellular components	Cellular membrane sterol concentration at start of fermentation indicates yeast's ability to grow and ferment the wort.	Quain (1988)
Magnesium ion release	Quantified release or absorption of the magnesium ion is used to predict fermentation performance.	Mochaba *et al.* (1997)
Fermentation capacity	Carbon dioxide evolution is monitored as part of a normal fermentation.	Manson and Slaughter (1986)
Acidification Power Test	Measures yeast's ability to acidify the surrounding medium before and after suspension in a sugar solution. Spontaneous proton efflux measure of internal energy reserves.	Opekarova and Sigler (1982); Cunningham and Stewart (1998)
Membrane potential	Fluctuations in intracellular pH correlated with variations in cellular ATP levels.	Imai *et al.* (1994)

1.2.5. Effects of Environmental Stress on Yeast Viability and Vitality

Several factors are known to affect the viability and vitality of yeast. These factors, referred to as stresses, can be physical, chemical, and/or biological, as outlined in Table 7 (Walker, 1998a). Some of these factors, as they relate to the brewing and fermentation processes, will be briefly reviewed.

1.2.5.1. Physical stresses affecting yeasts

1.2.5.1.1. Temperature stress

Thermal damage to yeast cells results from disruption of hydrogen bonding and hydrophilic interaction leading to general denaturation of proteins and nucleic acids. Since yeasts have no means to regulate their internal temperature, the higher the temperature, the greater the cellular damage. While yeast, subjected to low temperature stress will share a similar fate, the exact mechanisms as to how it occurs remains unclear (Walker, 1998a).

Fargher and Smith (1995) have shown that cold shocking brewing yeast strains at 4°C inhibited budding. Cold storage of seed yeast for later pitching is a widespread practice in breweries, but can adversely affect subsequent fermentation performance (Walker, 1998a). Optimally, yeast should be collected and stored at temperatures between 0 and 2°C, with a minimum storage time (O'Connor-Cox, 1998). As storage time increases, especially at higher temperatures, the yeast assimilate glycogen reserves to produce the energy required to maintain metabolic activity, which can be detrimental to yeast performance upon repitching (Russell, 1995; Heggart *et al.*, 1999).

Table 7. Stress factors affecting yeasts

Stress	Effect
Physical	Temperature shock Osmotic shock Dessication/ dehydration High hydrostatic and gaseous pressures Shear stress
Chemical	Ethanol and other metabolite toxicity Nutrient limitation or starvation Oxidative stress pH shock Metal ion stress (toxicity and limitation)
Biological	Cellular ageing Competition from other organisms

Adapted from Walker (1998a)

Large cylindroconical fermentation vessels can also lead to problems with yeast cooling during collection since the tank's small surface area in relation to its volume, can make cooling potentially very slow, 0.5 °C h^{-1}. Conversely, fast cooling with jackets may lead to ice forming close to the jackets on the inside of the vessel while the average wort temperature is 10 °C. Furthermore, fermentation tanks are often situated outside in tank farms and can be subjected to extreme temperature variations, ranging from tropical heat to arctic cold (Hough, 1985).

1.2.5.1.2. Osmotic stress

When yeasts are exposed to beer or to wort, they are subjected to an osmotic pressure. Very high osmotic pressures, which can be encountered during high gravity brewing, may distort yeast metabolism or decrease yeast viability (Heggart *et al.*, 1999). The extent of osmotic pressure will depend upon the concentration of solutes surrounding the yeast cell. Due to the yeast cell's need for turgor pressure to initiate vegetative budding, yeasts subjected to high osmotic pressure may suffer dehydration, compromising their reproductive capacity (Walker, 1998a; Heggart *et al.*, 1999).

1.2.5.1.3. High hydrostatic and gaseous pressure

With regard to pressure, yeast cells grown in large-capacity bioreactors may face quite severe stress due to both hydrostatic and gaseous pressure. The former would occur in tall cylindroconical fermentation vessels, whereas the latter would result from endogenous CO_2 produced by yeast during fermentation in pressurized vessels (Walker, 1998a). Inhibition of yeast metabolism, particularly cell division, has been recognized at elevated levels of CO_2. The extent of the

inhibition appears to be a function of both temperature and pressure. At lower temperatures and higher pressure, CO_2 solubility increases, leading to an increase in cellular inhibition (Heggart *et al.*, 1999). Yeasts are not considered to be 'barotolerant', and fail to grow at pressures above 10 MPa (Walker, 1998a).

1.2.5.1.4. Mechanical stress

Brewing yeast experience mechanical stress during propagation, cropping, and pitching, or when agitated during storage and acid washing following fermentation (Munroe, 1995). Depending on their flocculation characteristics, yeast may be removed following fermentation using centrifugation, sedimentation, or skimming. Under centrifugation conditions normally used in the brewery, adverse stress placed on the cells is not considered shear-related, but rather is due to increased temperature and unintentional oxygen pickup (Munroe, 1995).

The mechanical stress encountered during routine yeast handling does not significantly contribute to a reduction in yeast viability and vitality (Heggart *et al.*, 1999). Due to their thick cell walls, yeast are very resistant to shear stresses caused by agitators in stirred-tank bioreactors (Walker, 1998a). However, it has been reported that yeast agitation resulted in the release of cell wall components and enzymes. This was attributed to cell wall shearing, rather than cell wall breakage (Heggart *et al.*, 1999).

1.2.5.2. Chemical stress factors affecting yeast

1.2.5.2.1. Ethanol stress

The effect of ethanol on yeast physiology is strain dependent. Brewing strains of *S. cerevisiae* are moderately tolerant to ethanol, while distillery and

wine yeasts show a greater tolerance. In general, ethanol inhibits brewing yeast growth above concentrations of 10% (v/v), while fermentation capacity is inhibited at 20% (v/v) ethanol. The extent of inhibition is related to other factors, such as high sugar concentrations and high temperatures (Heggart *et al.*, 1999).

According to O'Connor-Cox (1998), yeast should be held for less time in the handling vessel in high-gravity brewing compared to normal gravity brewing, since yeasts will have worked harder to ferment more sugar and will be suspended in beer containing higher levels of ethanol. Both stresses will result in yeast deterioration which will occur at a rate determined by both the quality of the yeast and the level of the applied stress. Yeast fermenting 20°P wort will probably deteriorate more rapidly during storage than yeast fermenting 14°P wort (O'Connor-Cox, 1998).

1.2.5.2.2. Acid washing

Some breweries incorporate a yeast-washing step into their process, to eliminate bacterial contamination and remove any proteinaceous or hop residues that may adhere to the yeast cell wall. Three yeast washing procedures are commonly used including, sterile water, acid, and acid-ammonium persulfate. Yeast slurries are normally acidified with dilute acid to pH 2.0 with continuous agitation then allowed to stand for a maximum of two hours (Heggart *et al.*, 1999). Acid washing can influence yeast fermentation performance by reducing yeast viability and vitality, reducing the rate or extent of fermentation, and changing yeast quality parameters, such as flocculation, fining, size of yeast crop and excretion of cell components (Heggart *et al.*, 1999). However, acid washing usually has no deleterious effects on yeast physiology and fermentation performance if properly performed. Successful washing depends on temperature control, pH, and duration of contact with the acid (Russell, 1995).

1.2.5.3. Biological stresses affecting yeasts

1.2.5.3.1. Yeast cell aging

For the brewer, an aged yeast usually refers to a crop which has been stored for extended periods or used for several successive fermentations. However, this form of population chronological aging does not truly relate to the individual age of the yeast cell (Smart, 1999). The age, or more strictly the 'lifespan' of a yeast cell, is defined as "the number of times it undergoes division", and its maximum division number is termed the Hayflick limit (Hayflick, 1965). Both genetic and environmental factors govern a yeast's maximum reproductive capacity, but a Hayflick limit of 25 appears to be typical for *S. cerevisiae* (Walker, 1998a).

As individual yeast cells age, they go through a series of biochemical changes as their cellular functions break down exponentially with age, leading to senescence (Sinclair, 1999). Numerous unsubstantiated models for yeast aging have been proposed, including bud scar excesses, membrane defects, cell wall changes, surface to volume limits, and increases in membrane fragility (Sinclair *et al.*, 1998). Yeasts, especially *S. cerevisiae* cells, have been widely studied at the molecular genetic level in an effort to understand the aging process in mammalian cells (Kennedy *et al.*, 1995; Austriaco, 1996; Jazwinski, 2001). However, this approach has recently been criticised by some researchers (Sinclair, 1999; Gershon and Gershon, 2000), who find it inherently problematic to compare aging in an unicellular organism (yeast) to that in a multicellular one (human).

To date, at least 20 age-related genes have been identified in laboratory yeast strains. These longevity genes specify a variety of biochemical functions that are age-related including, metabolic control, stress resistance, gene dysregulation, and genetic instability (Jazwinski, 2001). The exact functions of many of the genes involved in yeast aging remain undetermined (Sinclair *et al.*, 1998).

Austriaco (1996) has discussed mutations in *S. cerevisiae* which affect longevity. For example, *UTH* (youth) genes have been shown to increase both stress resistance and longevity in this yeast. Sinclair (1998) and his colleagues, found that the gene causing premature aging in humans, has a similar counterpart in yeast, the SGS1 gene. Mutant SGS1 cells accumulate extrachromosomal rDNA circles (ERCs) at a higher rate, resulting in nucleolar fragmentation in old cells. When a single ERC is released into a young cell, its lifespan is reduced by about 40% and cells subsequently become prematurely large, granular and sterile.

However, this observation was not supported by Jazwinski (2001), who found that as ERCs increased, lifespan was extended. Instead, Jazwinski (2001) has suggested that at least two metabolic mechanisms affect aging in yeast. One mechanism is the retrograde response, which signals mitochondrial dysfunction to the nucleus, leading to significant adjustments in metabolism. The retrograde response partly compensates for mitochondrial damage which increases with age. The second mechanism involves restricting caloric intake of yeast cells, which seems to prevent or postpone certain aging characteristics (Jazwinski, 2001).

Genetic research on the yeast aging process is still in its infancy and almost no information has been collected for brewing yeasts. Most of the current information on aging of brewing yeast has been derived from observable morphological changes (Heggart *et al.*, 1999). Brewing and laboratory strains of *S. cerevisiae* display similar morphological and physiological changes during aging and senescence (Barker and Smart, 1996). Morphological observations include the accumulation of bud scars (Johnston, 1966; Jazwinski, 1990), increased mean cell volume of both mother and daughter cells (Mortimer and Johnston, 1959; Kennedy *et al.*, 1994), old cells arresting in the G1 phase (Johnston, 1966), cell surface wrinkling (Barker and Smart, 1996), and extended generation times (Mortimer and Johnston, 1959; Egilmez *et al.*, 1990; Barker and Smart, 1996).

As for physiological changes, Grzelak *et al.* (2001) observed that in *S. cerevisiae*, its antioxidant defense mechanism decreased during replicative aging, while Nestelbacher *et al.* (2000) found that yeast, depleted of catalase, had a reduced lifespan in the presence of oxygen. In aged brewing yeast, changes in flocculation characteristics have also been observed (Soares and Mota, 1996). Deans *et al.* (1997) suggested that fermentation performance could be influenced by cell age variations which occur when yeast is allowed to sediment, following fermentation or prior to pitching.

1.2.5.3.2. Competition from other organisms

In general, the level of contamination during the fermentation process is relatively small compared to the large number of culture yeasts in a fermentation. During the active stages of fermentation, pure culture yeasts, that are well adapted to wort as a nutrient, typically outgrow any contaminating microorganisms. Hence, the number of bacterial types that could survive in a brewing process is relatively small. Bacterial growth may be inhibited by a combination of factors including, hop resins, high ethanol concentration, low pH, lack of oxygen during much of the process, limited sugar during fermentation, early heating processes, and low temperature maintained during fermentation and processing (Reed and Nagodawithana, 1991).

Nevertheless, breweries employ a rigorous cleaning program, known as cleaning in place, or CIP, to prevent potential spoilage problems. CIP, in combination with good manufacturing practices (GMP), cleans vessels, lines, and fillers in the brewery, without introducing microbial contaminants. A range of detergents and sanitizers is available, and the latest ones, the peracids, incorporate octanoic acid and low foaming wetting agents, which have proven effective against resistant bacteria and wild yeasts (Duca, 1999).

1.2.5.4. Adaptive responses to stress exhibited by yeast

1.2.5.4.1. Heat shock response

All organisms respond to a wide range of environmental stresses by inducing the synthesis of a small set of proteins, the so-called stress proteins. When subjected to mild temperature shock, ranging from a few degrees to over 20°C above the conventional growth temperature, microorganisms respond by synthesizing proteins, collectively termed the heat-shock proteins, or HSPs. The HSPs, or closely related proteins, are also induced when organisms are exposed to diverse environmental stresses other than heat, including ethanol, heavy metals, oxidative agents, and anaerobiosis. Although not well understood, the function of these stress proteins appears to be to protect the cells from further, and potentially lethal, stress challenges (Watson, 1990).

The heat shock response causes changes in gene expression and most proteins, made prior to the heat shock, are synthesized at a greatly reduced level (Piper, 1993). Since other kinds of stress have similar effects, this process can be considered as a general cellular response to metabolic disturbances (Mager and Moradas Ferreira, 1993). Heat shock proteins have been implicated in all major growth-related processes, such as cell division, DNA synthesis, transcription, translation, protein folding and transport, and membrane function. In *S. cerevisiae*, a sudden temperature change generates considerable, but temporary, alterations in the pattern of protein biosynthesis (Mager and Moradas Ferreira, 1993).

Cells, however, are able to adapt to heat shock. Intrinsic thermotolerance in yeasts is observed following sudden heat shock, while induced thermotolerance occurs when cells are preconditioned by exposure to a mild heat shock prior to a more severe heat shock. Thermotolerance in yeast can be influenced by heat, certain chemicals, osmotic dehydration, low external pH, nutritional status, growth phase (Piper, 1993), and alterations in intracellular pH

(Coote *et al.*, 1991). The onset of thermotolerance in *S. cerevisiae* appears to occur between 34 and 37 °C (Coote *et al.*, 1991).

1.2.5.4.2. Osmostress response

Since yeasts have no way to actively transport water, they transport, synthesize, or prevent the loss of certain solutes, in order to maintain low cytosolic water activity when the external solute concentration is high. These solutes, known as osmolytes, can effectively replace cellular water, restore cell volume, and enable enzyme activity to continue. Osmolytes also stabilize membrane proteins and maintain the liquid crystal state of membrane phospholipids during dehydration. In yeast, the most effective osmolytes are the polyols, specifically, glycerol (Walker, 1998a).

The disaccharide trehalose, has also been found to be an effective stabilizer of biological membranes against osmotic shock (Crowe *et al.*, 1984). Trehalose binds to the polar head groups of lipids, where it replaces water and acts to preserve the properties of a hydrated membrane during dessication (Piper, 1993). In high gravity brewing, yeast trehalose levels were observed to increase more than those of glycerol early in fermentation, suggesting that trehalose was an important osmoprotectant and stress indicator (Majara *et al.*, 1996).

Mager and Varela (1993) hypothesized that a sudden increase in external osmolarity was sensed mainly in the plasma membrane. As a consequence of the disturbance of the ion gradients (e.g. Na^+, K^+, H^+), and the resulting loss of turgor pressure, a series of molecular events may occur resulting in modification of enzyme activity and gene expression. Part of the induced gene expression may lead to protection and recovery processes such as trehalose and heat shock protein synthesis.

1.2.5.4.3. Other stress-related responses

In response to ethanol stress, *S. cerevisiae* cells display several physiological changes, including an increase in membrane monounsaturated fatty acids, a decrease in saturated fatty acids, higher levels of trehalose, HSP synthesis, and an increase in antioxidant enzymes. As well, tolerance to ethanol may be affected by osmotic pressure, metabolic by-product formation, and media composition. Prior exposure to lower levels of ethanol may induce tolerance to higher levels of ethanol, i.e. a similar mechanism to thermotolerance (Heggart *et al.*, 1999).

Metal ions, in particular, Mg^{2+} appear to protect yeast from ethanol stress (Walker, 1998b). Thomas *et al.* (1994) found that certain amino acids and particulate matter may also act as osmoprotectants during high-gravity brewing. Tiligada *et al.* (1999) found that *S. cerevisiae* cells acquired thermotolerance after incubation with sodium molybdate. This inhibited the dephosphorylation of cellular components but the exact mechanism is still unknown.

1.2.5.5. Periodic yeast renewal in the brewery

The most common observation by brewers of behavioural changes in yeast concerns the phenomenon referred to as 'weakness' or 'aging', which can be defined as "the deterioration in yeast performance with successive fermentations" (Jones, 1997; Smart, 1999).

Current fermentation practices generally produce two to four times the amount of yeast needed for repitching (Jones, 1997). However, reusing cropped yeast cannot be continued indefinitely due to an increased risk of infection, spontaneous yeast mutation (Jones, 1997; Cahill *et al.*, 2000), and changes in gene expression in response to environmental conditions (Jones, 1997).

To date, little is known about the effect of extended serial repitching on subsequent fermentation performance, cropped yeast condition, and flocculation

(Smart, 1999). Studies that have tried to link serial repitching and fermentation performance have focused on the area of flocculation (Smart and Whisker, 1996). It was found that extended serial repitching significantly affected cropped yeast flocculation and activity, and a progressive deterioration in flocculation and viability was observed. Furthermore, cropped yeast obtained from serially repitched laboratory fermentations, using both wort and standard medium, exhibited a similar response, indicating that batch wort variation was not responsible for the deterioration in yeast condition (Smart and Whisker, 1996). Smart (1999) hypothesized that extended serial repitching could constitute a form of 'repetitive stress injury', in which the yeast population is repeatedly transferred from the cell cycle to stationary phase to stress conditions and back to the cell cycle. This could result in the progressive deterioration of the yeast biomass, and require the periodic renewal of the yeast culture.

Brewers typically renew their yeast by propagation in a plant located within the brewery (Jones, 1997). Jones (1997) reported that Emil Hansen's original protocol of scale-up propagation was no longer used in most breweries, as it was considered too time consuming. Instead, yeasts are propagated in large vessels, often aerobically and at higher temperatures, in high-gravity worts, and yeast is pitched into the wort during its exponential growth phase. However, brewery yeast propagation is not just a matter of production of maximal quantities of yeast to pitch fermentations. The yeast must be in an appropriate physiological state and be capable of producing standard beer from its first generation (Cahill *et al.*, 2000). It is widely recognized, but not well understood, that the first full-scale fermentation, following propagation, is often atypical in terms of attenuation rate, yeast crop, and finished product (Jones, 1997).

As high-gravity brewing continues to become widespread in the fermentation industry (O'Connor-Cox, 1998), it is likely that high-gravity propagation will become a regular feature of brewhouse operations (Cahill *et al.*, 2000). However, studies have shown that high-gravity propagation alters cell volume and has a negative impact on the quality of first-generation cropped yeast (Cahill *et al.*, 2000).

1.2.6. Cell separation techniques

1.2.6.1. Introduction

One of the main obstacles to the biochemical study of yeast aging has been the difficulty in isolating large quantities of aging cells. The mathematics of exponential growth are such that to obtain a single old cell after 25 generations, it must be separated away from 2^{25} daughter cells. Thus, obtaining a pure population of 10^8 old cells becomes a challenge (Sinclair *et al.*, 1998). Various techniques, such as elutriation (Egilmez and Jazwinski, 1989) and biotin-streptavidin magnetic sorting (Smeal *et al.*, 1996), have now been developed to permit the isolation of relatively pure populations of aged yeast cells.

The history of cell separation dates back to the 1960s. Physical characteristics, for example, cell density, or biochemical characteristics, such as the availability of selectable enzymes, were used to develop cell separation methods. In the late 1960s, a paper was published on Ficoll-density gradients for the isolation of lymphocytes from blood and shortly after, several methods based on antigen-antibody reactions were developed for the separation of various cell types (Esser, 1998).

Designing the optimal strategy for any specific cell separation depends on (Radbruch *et al.*, 1994):

(i) the availability of suitable cell-surface markers;
(ii) the composition of the original cell mixture;
(iii) the frequency of selected cells and the characteristics of the other cells; and
(iv) specific requirements regarding purity and recovery of sorted cells.

In many cell-separation protocols, the target cells are initially enriched from a complex cell mixture by density gradient centrifugation. This makes further purification by techniques using specific cell binding agents, such as

antibodies, in combination with magnetic beads (MACS), solid phase adsorption, or fluorescence-activated cell separation (FACS), easier and more cost effective (Van Vlasselaer *et al.*, 1998). Density gradient centrifugation, FACS, and biotin-streptavidin magnetic cell sorting will be considered below.

1.2.6.2. Density gradient centrifugation

Cells can differ in density, depending on the size of cell. This knowledge can be used to separate cells on density gradients by sedimentation in a centrifuge (Esser, 1998). Cells behave in suspension as small spherical particles that sediment at a rate proportional to their size and the difference between their density and that of the surrounding medium. The sedimentation of spherical particles in solution occurs according to Stoke's law, whether at unit gravity or during centrifugation (Van Vlasselaer *et al.*, 1998).

Depending upon the magnitude of the centrifugal force and the density of the medium, particles will separate on the basis of either their density, their size, or a combination of both. The separation of particles during centrifugation, at high gravitational force over an increasingly dense medium, occurs on the basis of density and is called isopycnic separation. Velocity or rate-zonal sedimentation, occurs at low gravitational force, and refers to the separation of particles based on size (Van Vlasselaer *et al.*, 1998); it can also be used to obtain cells for synchronizing cultures (see Table 4, section ***1.2.4.3.4.***).

There are two stages of separation during centrifugation. The first stage takes advantage of the difference in the terminal velocities of different particles as determined by Stoke's law:

$$V_t = 2R^2 (p_s - p)\, a/ (9\mu)$$

where 'V_t' is the terminal velocity of the particle, 'R' is the radius of the particle, 'a' is the centrifugal acceleration of the centrifuge, 'μ' is the viscosity of the medium, 'p_s' is the density of the particle, and 'p' is the density of the medium. Therefore,

the terminal velocity is a function of the particle radius and density. On average, larger and heavier particles will travel through the medium more quickly and settle at the bottom of the centrifuge tube in a shorter time (Howland, 1973). However, since the size, morphology, and density of cells vary, and are determined by the prior history of the cells or the stage in the cell cycle, Stoke's law only partially predicts the extent of cell separation on a density gradient (Van Vlasselaer *et al.*, 1998).

1.2.6.2.1. Gradient forming materials

The function of any gradient material is to provide a dense medium on which the initial cell suspension can be layered and to stabilize the contents of the tube against stirring when the centrifuge is accelerated and decelerated (Mitchison and Carter, 1975). Characteristics of an ideal density gradient material are (Van Vlasselaer *et al.*, 1998):

(i) it allows the formulation of solutions within a range of densities;
(ii) it has a low ionic strength, so as to not affect the osmolality or the pH of the final solution;
(iii) it is isotonic at its final density;
(iv) it is iso-osmotic throughout the gradient; and
(v) it has a low viscosity.

Gradient forming materials can be broadly categorized into two groups: simple sugars, or low molecular weight solutes, and complex polymers, or high molecular weight solutes.

1.2.6.2.1.1. Simple sugars

Sucrose is a widely used solute for density-gradient separations. Solutions are prepared on a weight-for-weight (w/w) basis, by weighing both

water and sucrose. The concentration of solutions can be measured using an Abbe refractometer with a sugar scale or a hydrometer. RNAse-free sucrose is marketed by several companies specifically for density gradient work, but is comparatively expensive. In some cases, analytical reagent grade sucrose, mineral water grade and table sugar may all be of acceptable purity for gradient work, but can contain significant amounts of heavy metals and ribonuclease activity (Ridge, 1978).

The presence of carbon bonds linked to hydroxyl groups make sugar both stable and hydrophilic. However, the glucosidic links of disaccharides, especially sucrose, are liable to acid hydrolysis at pH values less than 3 and at elevated temperatures (Rickwood, 1978). Although the solubility of sucrose in aqueous media is extremely high, concentrated solutions become extremely viscous, especially at 5°C and can form a "glass". The high viscosity means that it takes particles longer to reach their equilibrium, or isopycnic, positions within the centrifuge tube (Rickwood, 1978).

The most commonly used density range for sucrose solutions is 1.00-1.20 g/cm^3. This is sufficient to band cells, membranes, membrane-associated organelles and viruses (Rickwood, 1978). Although cells may be sensitive to osmotic changes (Rickwood, 1978 ; Esser, 1998), sucrose has been used in several studies involving the separation of yeast cells, without any negative effects being reported (Egilmez and Jazwinski, 1989; Barker and Smart, 1996; Grzelak *et al.*, 2001).

Lactose, glucose and glycerol are also suitable media for gradient preparation (Mitchison and Carter, 1975). On a weight-per-weight basis, glycerol solutions are less dense and less viscous than sucrose solutions. However, glycerol solutions are more viscous than sucrose solutions of similar density (Ridge, 1978).

1.2.6.2.1.2. Complex polymers

One of the main disadvantages of using monosaccharides and disaccharides as density-gradient media is the high osmolarity of the concentrated solutions. Complex polymers, or high molecular weight solutes, protect osmotically sensitive particles, such as mitochondria and cells (Rickwood, 1978). Examples of such solutes include dextrans, dextrins, protein (e.g. serum albumin), Percoll, and Ficoll (Mitchison and Carter, 1975).

High molecular weight gradient solutes have several advantages, including low osmotic pressure, exclusion from intact membrane-bound structures, and slow diffusion, which minimizes the possibility of instability developing during loading of the sample (Ridge, 1978).

For cell separation, the most widely used polymers are albumin, colloidal silica, colloidal silica coated with polyvinylpyrrolidone (Percoll), and Ficoll. Many of these media have specific disadvantages. For example, Ficoll is more viscous than most gradient media and requires very high centrifugal forces for isopycnic centrifugation. Albumin and Percoll are probably the best and most often used media for the isopycnic sedimentation of cells (Pretlow and Pretlow, 1991).

Although some solutes can be prepared from biological sources, Ficoll is prepared by the random chemical co-polymerization of epichlorohydrin and sucrose. It has a bead form, making it very stable, but its molecular structure is still not well-defined. It is relatively inert osmotically, but its viscosity becomes very high at concentrations greater than 20% (w/v), which increases the time required for particles to reach their isopycnic positions (Rickwood, 1978).

Proteins, while sharing many of the same advantages and disadvantages of other solutes, have an additional advantage in that they can protect biological material from denaturation. Overall, polymers can show charge interactions with the suspended sample particles, leading to aggregation, and making the choice of gradient material often one based on trial and error (Ridge, 1978).

1.2.6.2.2. Gradient formation

A density gradient may be established naturally by simply placing sucrose crystals at the bottom of a test tube. The sugar dissolves in the solution and diffuses towards the top. However, the time required to establish a sugar gradient in this way in unacceptably long, and the process is not well controlled. A more practical approach is to place layer after layer of sucrose solutions of different concentrations in a test tube, with the heaviest layer on the bottom and the lightest on the top. This forms a discontinuous gradient with sharp boundaries between the layers, which will diffuse into a continuous gradient, usually over 18-24 h (Howland, 1973). Several techniques exist for forming these layers, including overlayering, in which successively less dense liquids are layered on top of each other, and underlayering, in which the lightest layer is pipetted first, followed by increasingly dense layers, which displace the lighter layers towards the top of the centrifuge tube (Ridge, 1978).

Continuous gradients can also be formed automatically by using a two-chamber gradient maker, which consists of two identical chambers, connected near their bases by a tapped channel. Dense liquid in one chamber is mixed continuously with a less dense liquid in the other chamber and the flow is controlled by a peristaltic pump which delivers the increasingly dense liquid to the bottom of the centrifuge tube (Ridge, 1978).

1.2.6.3. FACS and MACS

The extension of affinity techniques for the isolation of molecules, to that of whole cells, involves many parameters. Cell membranes are complex and have a heterogeneous chemical nature. Moreover, cells are much larger than molecules and there is an increased risk, due to complications arising from cell death, and of spillage of complex cell components into the system (Liberti and

Feeley, 1991). To overcome these problems, fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) allow highly accurate sorting of cells from heterogeneous cell mixtures. An important factor influencing the success of these techniques is the variety and quality of antibodies available as cell surface markers (Graepler *et al.*, 1998).

FACS is an extension of the technique known as flow cytometry. Flow cytometry is a means of measuring the physical and chemical characteristics of cells or particles as they travel in suspension, one by one, past a sensing point. With this instrument, it is possible to measure physical characteristics, such as cell size and shape. Any cell component or function that can be detected by a fluorescent compound, can also be examined. In addition to cell analysis, many flow cytometers have the ability to "sort", which is defined as "the physical separation of a cell or particle from a heterogeneous population" (Waggoner, 1990). In MACS, separation is achieved using magnetic particles and an external magnetic field (Polysciences, 2000).

In FACS, cells are analyzed individually and sorted sequentially. As a serial sorting device, its capacity is limited by the frequency of analysis and sorting, which is about 5000 cells per second, or 10^8 cells in 6 h. The capacity of MACS is limited by the sorting device and all cells are sorted simultaneously; typically, about 10^{10} cells can be sorted in 30 minutes (Radbruch *et al.*, 1994). However, successive "sorts" may be necessary to increase the purity of the labelled cells (Polysciences, 2000).

Since FACS requires experienced operating personnel and expensive equipment, techniques involving magnetic beads and antigen-antibody labelling have been developed over the past 15 years (Seidl *et al.*, 1999). Most early developments involved fairly large permanently magnetized beads of at least a few micrometers in diameter, which only required somewhat weak forces to produce an effective magnetism. Today, the use of colloidal magnetic labels which are much smaller than the cells to be separated, are more common. These smaller particles are less magnetic, and therefore require stronger magnetic fields for the separation process (Owen and Liberti, 1987).

Several companies manufacture their own magnetic cell sorting systems. Manufacturers include Polysciences, Dynal, Miltenyi Biotech, Cortex Biotech, StemCell, and Bangs Laboratories (Vettese-Dadey, 1999). Two of these commercial products are described below.

Polysciences manufacture a product called BioMag, a 1-μm non-uniform solid support consisting of an iron oxide core and a silane (SiH_4) coating. The particle surface is functionalized with amine or carboxyl groups for the covalent attachment of proteins, glycoproteins, and other ligands. The magnetic beads are superparamagnetic, that is, they respond well to magnetic fields but do not become magnetized, permitting repeated magnetic extraction without aggregation. Targeted cells are incubated with between 20 and 100 beads per cell and are isolated by applying a rare earth magnet against the side of the tube. Unlabelled cells are removed in the supernatant and labelled cells are collected. Magnetic beads can be detached from the cell by culturing or by using specific proteases (Polysciences, 2000).

Miltenyi Biotech manufactures superparamagnetic MicroBeads, which are made of iron oxide and a polysaccharide and average 50 nm in diameter. Cells are incubated with the magnetic beads and are loaded onto a column packed with ferromagnetic spheres coated with a biopolymer. The column is placed within a magnetic field and the flow-through contains unlabelled cells. Removal of the separator and flushing of the column elutes the labelled cells. An advantage of these beads is that they are biodegradable. (Vettesse-Dadey, 1999).

1.2.6.3.1. Biotin-streptavidin magnetic cell sorting

Skutelsky and Bayer (1979) were the first to suggest the use of the biotin-avidin complex for the separation of parent and daughter cell populations. Nowadays, this complex, and similar biotin-streptavidin complexes, are commonly employed in separation studies of many cell types, including parietal cells and blood neutrophils (Jareo *et al.*, 1997; Graepler *et al.*, 1998) and yeast cells (Smeal *et al.*, 1996).

Avidin, a protein found in egg white, and streptavidin, a similar protein found in *Streptomyces avidinii*, have the ability to bind, with high affinity, the vitamin biotin. The biotin-avidin interaction is thought to represent a natural defense mechanism since the binding with avidin or streptavidin of biotinylated enzymes that participate in CO_2 transfer inactivates enzymes and inhibits the growth of bacteria that depend on biotinylated enzymes (Diamandis and Christopoulos, 1991).

The biotin-avidin, or biotin-streptavidin, interaction has some unique properties (Diamandis and Christopoulos, 1991):

(i) the interaction of avidin or streptavidin with biotin is characterized by an affinity constant of 10^{15} L/mol. This high affinity ensures that once formed, the complex is not disturbed by changes in pH or physical manipulation;

(ii) avidin or streptavidin binding to biotin is specific enough to ensure that the binding is directed only at the target of interest;

(iii) both streptavidin and avidin have four binding sites per molecule, making it possible to use multiple biotinylated moieties;

(iv) biotin, being a small molecule (244 Da), generally does not affect the biological activity of the target molecule; and

(v) both avidin and streptavidin are very stable molecules and their biotin-binding activity can survive harsh reaction conditions and extensive derivatization.

Avidin is a 67 kDa glycoprotein, consisting of four identical subunits of 128 amino acids each. It contains both mannose and *N*-acetylglucosamine and has an isoelectric point (pI) of approximately 10. The major constraint in using avidin in some applications is the high non-specific binding, which is attributed to both the presence of the sugars and the high pI. Streptavidin also consists of four subunits, but contains no carbohydrate, and has a pI of 5 - 6. It is widely used in place of avidin because of its low non-specific binding. Biotin's binding with

avidin or streptavidin occurs between the bicyclic ring of biotin and a pocket within each of the four subunits of the proteins (Figure 7) (Korpela, 1984).

Biotin derivatives may be prepared that contain reactive portions able to couple with particular functional groups in proteins and other molecules. The biotin molecule may be attached directly to a protein via its valeric acid side chain or derivatized with other organic components to create spacer arms and various reactive groups. In most cases, proteins are biotinylated via the ε-amino groups of lysines by using an *N*-hydroxysuccinimide ester (NHS-ester) of a biotin analog. NHS ester-containing reagents react with nucleophiles with the release of the NHS to form an acylated product (Figure 8) (Hermanson, 1996).

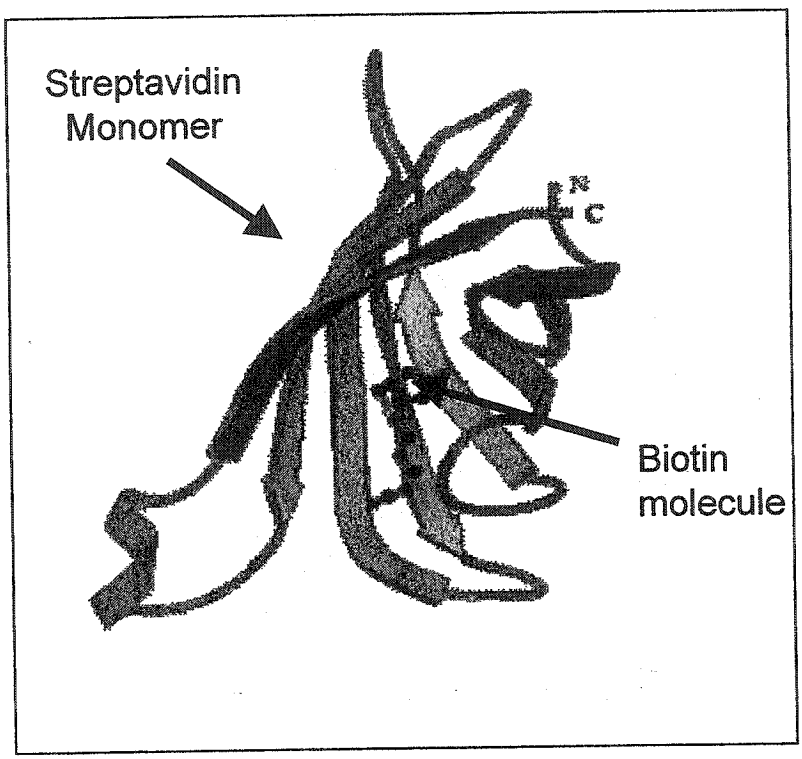

Figure 7. Streptavidin-biotin complex

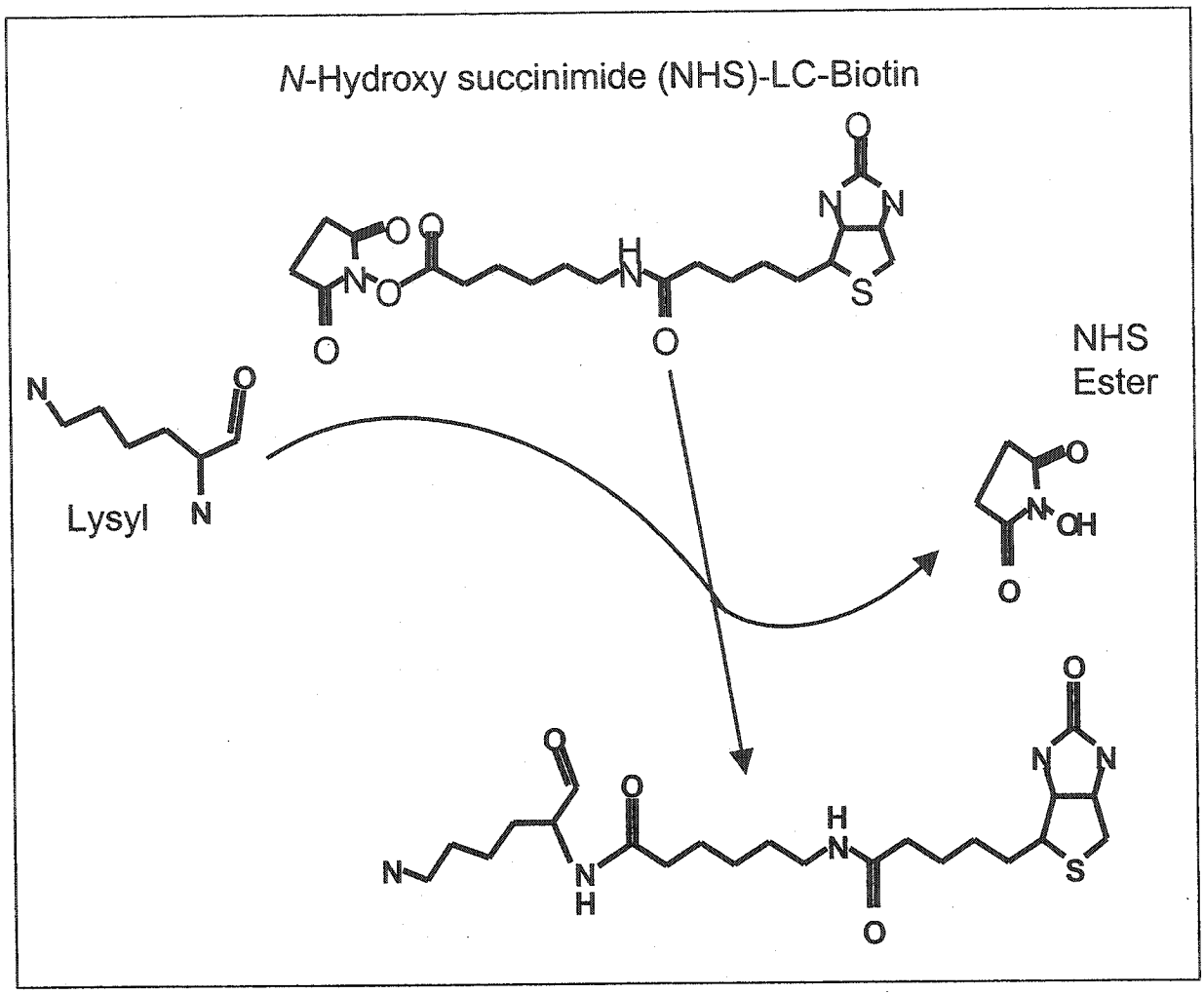

Figure 8. The NHS ester end of NHS-LC-Biotin reacts with the amine containing lysyl on the yeast cell wall, forming a stable amide linkage (Adapted from Hermanson, 1996).

1.3. Objectives of research

In the brewing industry, in order to ensure a high quality finished product it is necessary that yeast cells be able to effectively assimilate required nutrients from the wort, tolerate their environment, and produce the desired flavour profile within a relatively consistent time period. Achieving this requires a thorough understanding of cellular responses to changes in environment. In yeast, aging appears to be marked by a progessive impairment in cellular mechanisms, resulting in irreversible changes in physiology and morphology. To date, very little has been reported about the biochemical changes which occur as a function of individual cell aging. Thus, the objectives of this research were:

1. To isolate unbudded, or virgin, yeast cells from a mixed-aged population of a lager strain of *Saccharomyces cerevisiae*.
2. To label the unbudded yeast cell surfaces with a biotin ester derivative.
3. To grow biotin-labelled cells using a phased culture system for several consecutive generations.
4. To isolate biotin-labelled cells using streptavidin magnetic beads and verify the effectiveness of the separation procedure, based on yield and subsequent cell viability.

Chapter 2

Isolation of Virgin *Saccharomyces cerevisiae* Cells by Density-Gradient Centrifugation

2.1. Introduction

Effective control of yeast performance in brewing requires an awareness of the many factors that influence yeast physiology (Heggart *et al.*, 1999). To date, very little has been reported regarding the biochemical changes that occur in yeast as cells age and their environment changes (Smart, 1999). In order to study age-related changes at the molecular level, the availability of aging cells is a prerequisite (Egilmez *et al.*, 1990). Egilmez *et al.* (1990) and Barker and Smart (1996) have shown that bulk-quantities of yeast cells that have not yet commenced their reproductive cycles (i.e. virgin cells) can be obtained using density-gradient centrifugation.

2.1.1. Objectives

The objectives of this initial study were to obtain virgin yeast cells from a mixed-age yeast population using density-gradient centrifugation for subsequent use in biotin-streptavidin magnetic cell sorting.

2.2. Materials and methods

2.2.1. Microorganism and inoculum preparation

A lager brewing yeast strain of *Saccharomyces cerevisiae* (NCYC 1239), obtained from the National Collection of Yeast Cultures (NCYC, Norwich, England), was used in this study. The lyophilized yeast was rehydrated according to the NCYC's instructions by resuspending it in 0.5 mL of Malt Yeast Peptone Dextrose (MYPD) broth and then transferring it to a flask containing 10 mL of this broth. The MYPD broth was prepared from 0.3% (w/v) malt extract, 0.3% (w/v) yeast extract (both Difco, MD, USA), 0.5% (w/v) bacteriological peptone (Difco, Detroit, MI, USA), and 1.0% (w/v) dextrose (Fisher Scientific, NJ, USA) dissolved in distilled water. The broth was autoclaved at 121°C and 15 pound per square inch (psi) for 15 min immediately after preparation. The final pH of the broth was 6.1.

Following inoculation, the broth was incubated at room temperature for 24 h on a platform shaker set at ~250 rpm (New Brunswick Scientific, New Brunswick, NJ). Aliquots of the culture were subsequently transferred to MYPD agar plates (1.5% agar) and stored at 4°C, or combined with sterile glycerol (ICN Biomedicals, Aurora, OH, USA) in a 1:1 ratio and stored at –20°C. Cultures grown on MYPD agar plates were sub-cultured every month to ensure cell viability. All cultures were transferred aseptically in a laminar flow cabinet to minimize contamination from other microorganisms.

2.2.2. Growth curve

The inoculum for the growth curve was prepared by transferring a loopful of pure culture from the MYPD plates into 100 mL of MYPD broth, followed by incubation for 24 h at 28°C in a water-bath shaker (250 rpm) (New Brunswick Scientific, Edison, NJ, USA). Cells from the stock solution were enumerated using an Improved Neubauer hemacytometer and appropriate dilutions were made using sterile peptone water (0.1% w/v) (Difco, Detroit, MI, USA) to give a

working suspension of 10^4 cells/mL in 100 mL of MYPD broth. The inoculated flask was then incubated as previously described and monitored for growth every hour for ~28 hours by visible cell count and surface plating on agar plates. The visible cell number, as well as the viable and non-viable cell numbers, were determined by staining the cells with citrate methylene violet solution, placing the cells in an Improved Neubauer hemacytometer, and examining the cells in a microscope (Cambridge Instruments, Buffalo, NY). Plate counts were made by preparing serial dilutions, using sterile peptone water as described, followed by surface plating onto Plate Count Agar plates (PCA) (Difco, Detroit, MI, USA). Plates were incubated at 30 °C for approximately 48 h and colonies were enumerated using a Darkfield Quebec Colony Counter (AO Scientific Instruments, QC, CAN).

Counts were expressed as the number of viable cells per mL (cells/mL) for hemacytometer counts, and as Colony Forming Units per mL (CFU/mL) for plate counts. The doubling time and specific growth rate were calculated as outlined by Walker (1998a), and both these values related to the behaviour of cells during the exponential phase of growth when the cell population was doubling at regular intervals. To calculate the specific growth rate, denoted by μ,, the following equation was used:

$$\mu = \frac{\log (N_t - N_o)}{t_2 - t_1} \qquad (8.0)$$

where, N_t and N_o represented initial and final cell numbers, respectively, and t_2 and t_1 the time in hours at N_t and N_o, respectively. To calculate the time required for the cells to double in number (t_D), the following formula was used:

$$t_D = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} \qquad (5.0)$$

2.2.3. *Batch culture growth*

2.2.3.1. *Bioreactor apparatus and inoculation*

In order to obtain sufficient quantities of virgin yeast cells, a 4-L laboratory-scale bioreactor was used to propagate the yeast. The bioreactor was equipped with an internal cooling coil and its temperature was maintained at 28 °C during cell cultivation using a Haake D1 water circulator (Haake, Karlsruke, Germany). In-going air was filtered using a Whatman Hepa Cap 36 air filter with a 0.2μm pore size (Arbor Technologies, Ann Arbor, Michigan, USA) and air flow was controlled with a flowmeter (Cole Parmer, Chicago, IL, USA). The aeration rate was 786 mL min^{-1}, as per the flowmeter calibration data sheet for the model of flowmeter used (Aalborg, Orangeburg, NY, USA). Air was supplied to the yeast culture via an aerator placed inside the bioreactor (Saint-Gobain Ceramics and Plastics, Northboro, MA, USA).

The interior walls of the bioreactor were equipped with baffles to prevent vortexing. Mixing was achieved with a Corning magnetic stirrer, model PC-520, operating at ~300 rpm (Corning, Corning, NY, USA). The dissolved oxygen concentration in the broth was measured continuously with an Ingold polarographic oxygen sensor connected to an Ingold Model 170 oxygen amplifier (Ingold, Wilmington, MA, USA). The output from the amplifier was monitored using a Goerz SE 120 chart recorder (ABB Goerz, Austria).

The MYPD medium provided sufficient buffering capacity for yeast growth, which precluded the need for addition of acid or base salts to regulate the pH. The initial pH of the medium was 6.1 and, following yeast growth, it was ~pH 5.4, as measured with an Accumet Model 25 pH/ Ion Meter (Fisher Scientific, Fair Lawn, NJ, USA).

Prior to use, the bioreactor was sterilized in an autoclave for 20 min at 121°C and 15 psi. Three litres of MYPD broth were sterilized in a similar manner and then added aseptically to the bioreactor. Once the broth had cooled to room

room temperature, the antibiotic Tetracycline Free Base Trihydrate (ICN Biomedicals, Aurora, OH, USA) was added at a concentration of 50 μg/mL to prevent bacterial contamination. The prepared broth was then inoculated with 10 mL of yeast suspension from a 48 h culture grown in MYPD broth as described previously. The yeast were grown in the bioreactor until the cells ceased reproducing and the stationary phase was reached, after ~ 90 h, as indicated by the output from the dissolved oxygen sensor. Cells were harvested by asceptically siphoning the yeast suspension into sterile centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min (IEC, Needham Heights, MA, USA), the supernatant was removed and phosphate buffer saline (PBS, pH 7) was added to the tubes, which were then stored at 4 °C until further use. The PBS was prepared using 13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4 \cdot 7H_2O$, and 0.14 mM KH_2PO_4 dissolved in 1 L distilled water. The pH was adjusted to 7 using 1M NaOH and the solution was sterilized by autoclaving.

2.2.3.2. Dry weight measurement

The biomass concentration of *S. cerevisiae* was monitored by dry weight analysis. Two 10 mL samples were removed asceptically from the bioreactor and centrifuged at 3400 rpm at room temperature for 5 min (IEC, Needham Heights, MA, USA). The supernatants were removed and the yeast pellets were suspended in 10 mL sterile distilled water and the samples were re-centrifuged. Following centrifugation, the supernatants were removed and approximately 3 mL of sterile distilled water was added to each yeast pellet to form a yeast slurry. The slurries were then poured into pre-weighed aluminium dishes and placed in an isotemp oven (Fisher Scientific, Fair Lawn, NJ, USA) at 85 °C for 2 hours. The dishes were then re-weighed and the quantity of yeast, as dry matter, in the original sample determined by subtracting the final weight of the dish from the initial weight.

2.2.3.3. Viability testing

To test for yeast cell viability, cells were stained with Methylene Violet 3 RAX dye (Aldrich Chemical, Milwakee, WI, USA). The methylene violet solution was prepared according to the method of Smart *et al.* (1999). A sodium citrate solution was prepared using sterile distilled water (2% w/v) (Anachemia, Montreal, QC, CAN) and the dye was dissolved in the sodium citrate solution to give a final concentration of 0.01% (w/v). The solution was then filter-sterilized through a 0.2 μm nylon filter (Nalge Co., Rochester, NY, USA) to remove any impurities. Yeast suspensions were mixed with equal parts of this citrate methylene violet solution and examined microscopically after a five-minute incubation period at room temperature.

The stained cells were transferred to an Improved Neubauer Hemacytometer counting chamber (American Optical, Buffalo, NY, USA) and were examined using a Reichert-Jung Diastar microscope (Cambridge Instruments, Buffalo, NY, USA). Cells were counted in 5 groups of 16 small squares at the centre and four corners of the central square millimeter (Figures 5 & 6). To avoid counting a cell twice, those on a line were counted only when on the top and left lines, or on the bottom and right lines. Cells touching two of the boundary lines were disregarded. In order for the count to be significant, a minimum of 200 cells was counted for each sample. Unstained cells were assumed to be viable and stained cells to be non-viable, since the latter were unable to metabolically reduce or exclude the cytological stain (Lentini, 1993).

To determine the cell number, expressed as the number of cells/mL, the following formula was used:

$$\frac{\#\text{ of cells counted}}{\#\text{ of squares counted}} \times \frac{4000\text{ squares}}{\text{mm}} \times \frac{1000\text{mm}^3}{\text{mL}} \times \text{Dilution Factor} \quad (9.0)$$

2.2.4. Density-gradient centrifugation

In order to separate populations of brewing yeast according to chronological age, a modified version of the protocols published by Egilmez *et al.* (1990) and Barker and Smart (1996) were used.

2.2.4.1. Gradient preparation

Sucrose solutions were prepared using sucrose dissolved in distilled water. Prior to the addition of sucrose, the water was filtered through a Whatman 1 qualitative filter (Arbor Technologies, Ann Arbor, Michigan, USA) to remove particles that could potentially disrupt the gradients. Two concentrations of sucrose solutions were prepared, 5% (w/w) and 20% (w/w). These solutions were sterilized in an autoclave and stored at 4 °C.

Sucrose gradients were prepared in Fisherbrand sterile graduated 50 mL centrifuge tubes (Fisher Scientific, Pittsburgh, PA, USA) in a UV-sterilized hood (Labconco, Kansas City, Missouri, USA) to minimize microbial contamination. Gradients were prepared by placing 15 mL of the 5% solution in a centrifuge tube, and then carefully pipetting 30 mL of the 20% solution from the bottom of the tube, so as to displace the 5% solution towards the top. The tubes were weighed to facilitate centrifugation and stored at 4 °C for at least 24 h to permit gradient formation (Figure 9).

2.2.4.2. Density-gradient centrifugation

Yeast cells, obtained from the bioreactor, were counted in an Improved Neubauer hemacytometer and resuspended in sterile distilled water to give a suspension of 1×10^9 cells/mL. This suspension was carefully layered onto the prepared sucrose gradients at a rate of 1 mL/ gradient, and the gradients were centrifuged in a fixed-angle rotor at 100 x *g* for 6 min using an IEC 21000R refrigerated centrifuge (IEC, Needham Heights, MA, USA). During this

procedure, two distinct bands of cells were obtained. The upper cell band was collected in sterile PBS solution (pH 7) and stored on ice to prevent further yeast growth. The upper cell band fractions were pooled and centrifuged at 500 x *g* for 10 min at 4 °C. The yeast cell pellet was then resuspended in ~5 mL of sterile distilled water, stained with methylene violet dye, and counted as described previously. Appropriate dilutions were made using sterile distilled water and aliquots containing ~ 7.5 x 10^8 cells/mL were layered onto a second set of gradients and centrifuged at 100 x *g* for 6 min. The upper two-thirds of each band was collected, pooled, and centrifuged as previously described. Cells collected from each band were washed twice by centrifugation at 750 x *g* and 4°C for 10 min (IEC, Needham Heights, MA, USA) in sterile distilled deionized water to remove excess sucrose, and stored in sterile PBS (pH 7) at 4 °C.

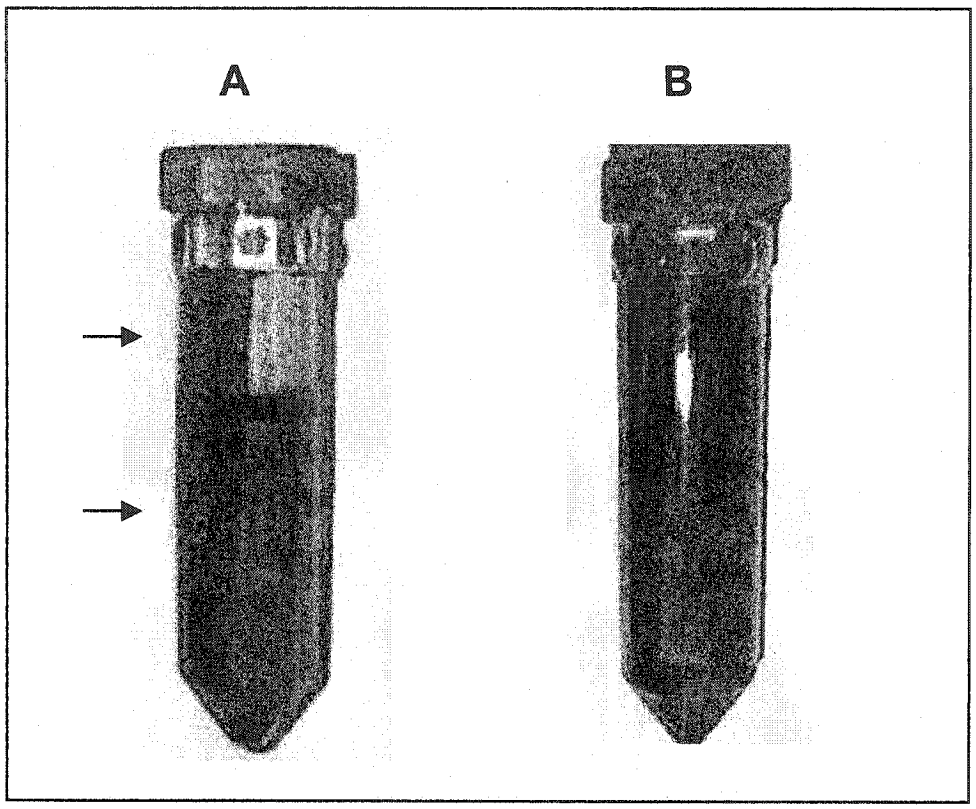

Figure 9. **(A)** Preparation of sucrose density gradients using 5% sucrose solution (top arrow) and 20% sucrose solution (bottom arrow). **(B)** After 24 h at 4°C, sucrose solutions diffuse to form the density gradient.

2.2.4.3. Fluorescent microscopy

The yeast cells collected from the sucrose density gradients were verified for the presence of bud scars using a Leitz Ortholux II fluorescence microscope (Leitz, Wetzlar, Germany) fitted with a Wild Photoautomat MPS 45 camera and a Wild photometer attachment (Wild, Heerbrugg, Switzerland). Cells were stained with Fluorescent Brightener 28 (ICN Biomedicals, Aurora, OH, USA), which is equivalent to Calcofluor White MZR, at a concentration of 1 mg/mL.

2.3 Results and discussion

2.3.1. Growth curve

The growth curves of *S. cerevisiae* are shown in Figures 10a and 10b. The maximum cell number (~10^8 CFU/mL) was reached after 24 h at 28 °C. The specific growth rate (μ) was 0.373 h^{-1} and the cell doubling time (t_d) was 1.86 h. Both these values were obtained from the mean of two replicate samples.

The relationship between visible cell count and plate count was determined using linear regression analysis and is shown graphically in Figure 10c. There was a high correlation between the two counting methods, indicating that either method could be used as a reliable indicator of *S.cerevisiae* NCYC 1239 growth in MYPD broth.

When cells were grown in the 4-L bioreactor, the yeast biomass averaged a dry weight of 4.4 g/L at the end of growth.

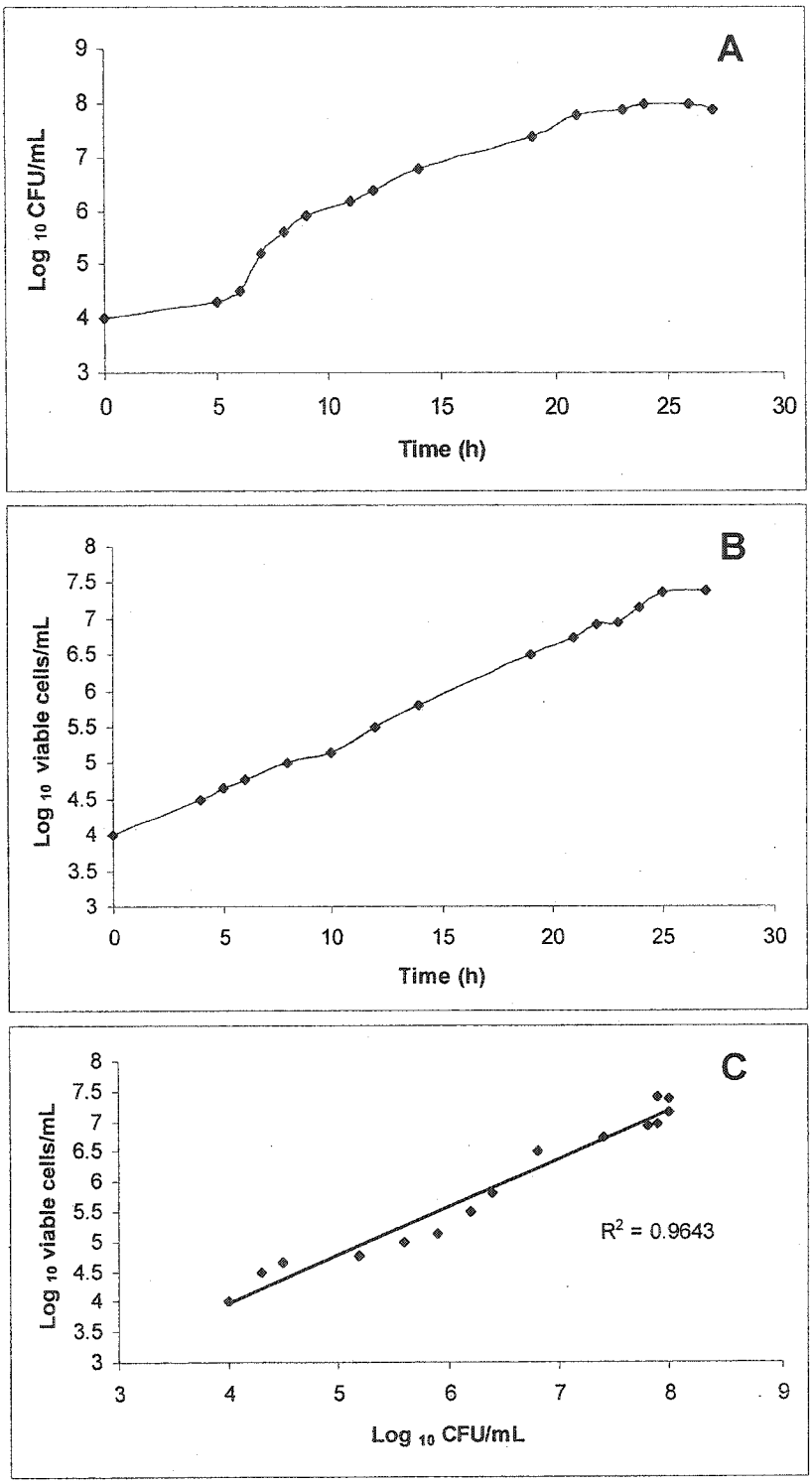

Figure 10. Growth curves of *S.cerevisiae* (NCYC 1239). (A) by plate count; (B) by visible cell count; and (C) by plate count vs visible cell count.

2.3.2. *Virgin cells*

Two bands were visible after centrifugation (Figure 11). The lower band was usually 2 to 3 times broader than the upper band. The upper band contained small virgin cells, while the lower band contained larger cells of various ages.

Sucrose gradients were chosen since they had been used successfully to fractionate yeast cells according to age (Mitchison and Carter, 1975; Egilmez *et al.*, 1990; Barker and Smart, 1996). Sucrose was also chosen for its convenience, availability, and low cost. Cell separations were first attempted with 10-30% linear gradients, as employed by Egilmez *et al.* (1990) and Barker and Smart (1996). The method developed for this study was initially optimized using these gradient concentrations. However, since only cells from the top band were required for our study, compared to previous studies which required cells from other parts of the gradient, it was determined that a gradient containing only two bands (5% and 20% sucrose) would be as effective and easier to prepare than a 10-30% gradient. Furthermore, this modification gave highly favourable results.

Parameters investigated in this study included centrifuge speed, temperature, time, and cell concentration. A centrifugation temperature of 4 °C was found to give better results than gradients centrifuged at room temperature. One reason for the improved separation of yeast cells at lower temperatures may be that the higher viscosity of the cold sucrose solutions, combined with cold interior of the centrifuge, had a stabilizing effect on the yeast cells. Streaming, in which cells sediment to the sides of the tube during centrifugation, is a common problem with fixed-angle rotors, due to the radial centrifugal field. However, this is not a problem with swinging-bucket rotors (Ridge, 1978). Although Egilmez *et al.* (1990) reported streaming, despite using a swinging-bucket rotor, it was acknowledged to be a compromise between cell concentration and the number of gradients required, i.e., lower cell concentrations produced less streaming; however, it required more gradients. Generally, it was found in this study that

higher centrifugation speeds, i.e. 150 x *g* for 4 min and 250 x *g* for 3 min, resulted in streaming. At lower speeds, cells were better resolved but the time taken for the cells to move through the gradient increased considerably taking ~10 min at 50 x *g* and ~8 min at 75 x *g*, respectively. Since yeasts are able to metabolize sucrose quite rapidly (Russell, 1995), minimizing its contact with yeast cells is important.

The maximum number of cells that could be loaded onto the 45 mL gradients was ~$1x10^9$ cells/mL. This was in agreement with the results of Barker and Smart (1996) and Egilmez *et al.* (1990) who found the optimum amount to be < 5×10^8 cells/mL and 1×10^9 cells/mL, respectively. Centrifuging cells a second time, through other gradients, and recovering only the upper two-thirds of the band was done to obtain a more homogeneous distribution of cells (Barker and Smart, 1996).

Finally, it was not necessary to sonicate the yeast cells, as outlined in previous methods, in order to disrupt cell clumps. Instead, cells were allowed to reach the stationary phase, at which time the cells were generally well separated from one another, when examined microscopically. Cell clumping, as noted by Egilmez *et al.* (1990), was not a problem with the yeast strain used in this study, perhaps due to its non-flocculent nature.

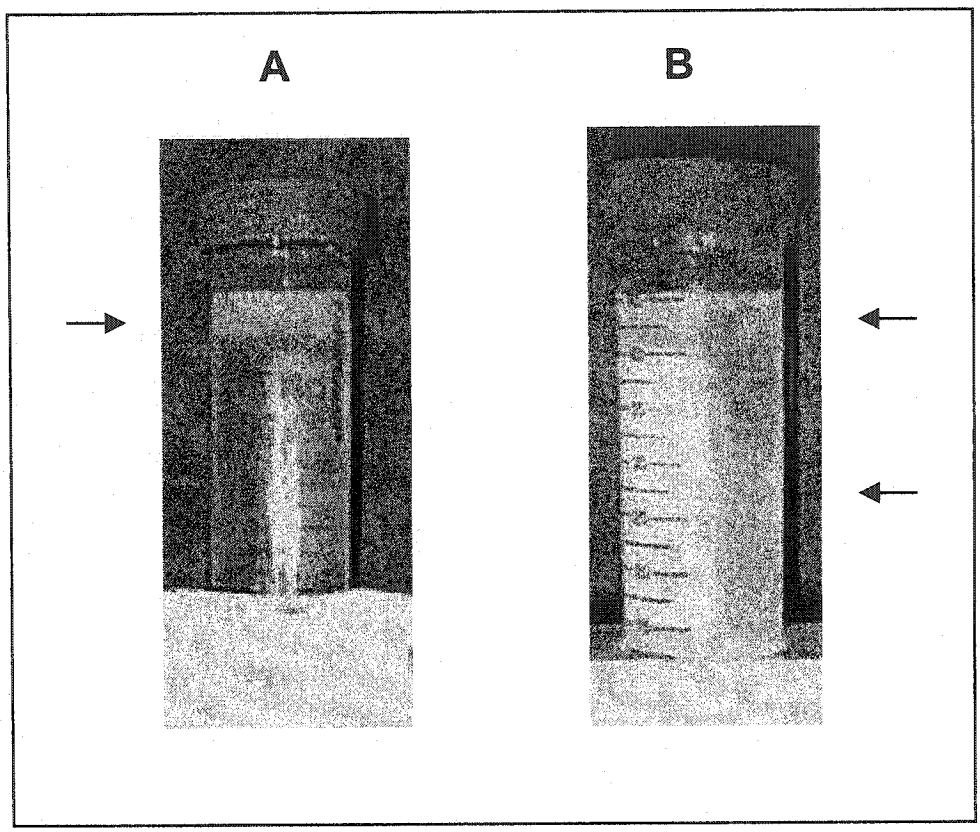

Figure 11. Separation of yeast cells on sucrose gradients. **(A)** Prior to centrifugation, yeast slurry is layered onto the gradient. **(B)** Following centrifugation, two bands of cells are visible; top band contains mostly virgin cells, bottom band contains cells of mixed-ages.

2.3.3. Fluorescent microscopy

Cells were sampled from both the upper and lower bands of the gradients to verify the effectiveness of the separation procedure. Cells, from both bands, were stained with Fluorescent Brightener 28 dye, which binds to the chitin in the bud scar, and then viewed under the microscope. No bud scars were visible on the cells from the upper band (Figure 12a), while some yeast cells from the lower band had 2-4 bud scars (Figure 12b). Furthermore, cells in Figure 12a were smaller compared to those in Figure 12b, suggesting that these were the smaller daughter and, therefore, virgin cells.

However, fluorescent microscopy is only an indication but not a guarantee of purity, unless the entire cell can be observed. A normal fluorescent microscope, as used in this study, only enables 60% of the yeast cell's surface to be observed. In contrast, a confocal microscope, which allows the entire surface of the cell to be visualized, could result in a more definitive yeast cell age (Smart, 1999). Other researchers (Egilmez *et al.*, 1990) have used an arbitrary fluorescent index, in combination with fluorescent microscopy, to assess the level of bud scars in a cell population, while Deans *et al.* (1997) have used fluorescence spectrophotometry to correlate fluorescence intensity with number of bud scars.

One problem observed in this study and previously unreported, was the problem of dissolving the Fluorescent Brightener 28 dye. Various techniques were tried, including heat, organic solvent, sonication, and filtering. Since none of these techniques proved satisfactory, the dye was partially dissolved in room temperature sterile distilled water and added to the cells. Small crystals of the dye were still visible under the microscope, making viewing and photographing the cells more difficult. Centrifugation, following an incubation period of the dye with the cells, was not tried due to the limited sample available and concern that too much of the sample would be lost to the process. However, this may be a possible solution to overcome the problem with this dye.

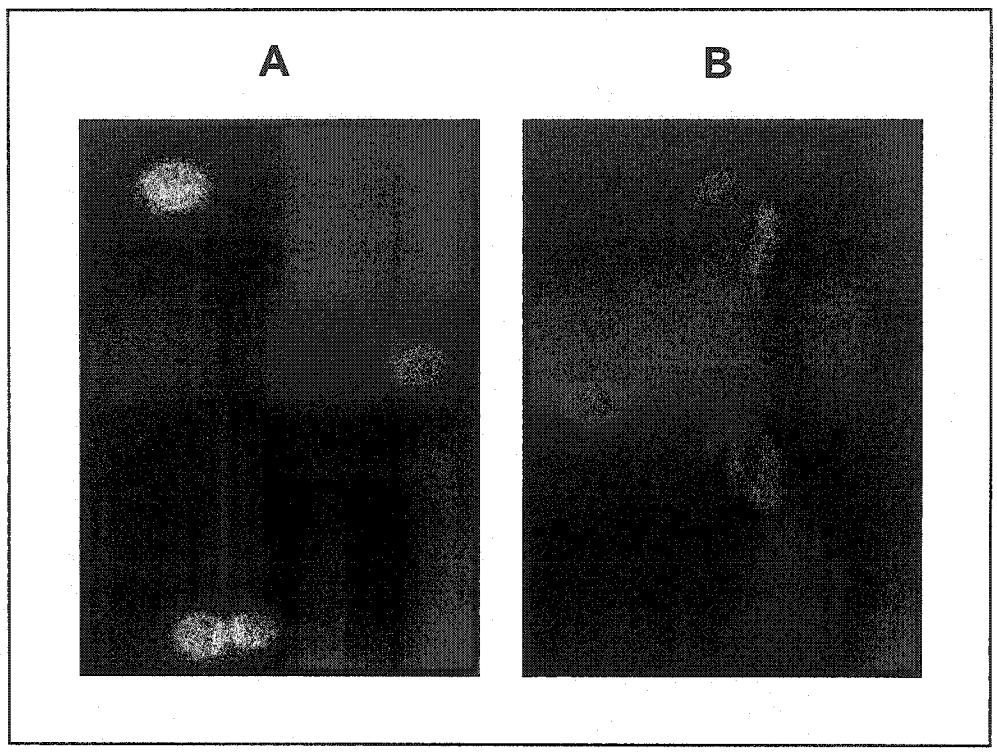

Figure 12. Yeast cells obtained from upper and lower bands of sucrose density gradients and stained with fluorescent dye. **(A)** Yeast cells from upper band show no signs of bud scars. **(B)** Bud scars are visible as small rings on the surface of yeast cells.

2.4. Conclusion

In conclusion, yeast cells with no or few bud scars (Figure 12A) could be obtained from non-linear sucrose density gradients (5-20% w/w). Yeast was grown aerobically in batch culture, concentrated to $1x10^9$ cells/mL, layered onto gradients, and centrifuged in a fixed-angle rotor at 100 x *g* and 4 °C. Fluorescence microscopy was used to verify the effectiveness of the separation, but due to some of its inherent limitations, other techniques, such as scanning electron microscopy (SEM), may prove more useful.

Chapter 3

Biotin-Streptavidin Magnetic Cell Sorting of *Saccharomyces cerevisiae*

3.1. Introduction

Selective separation of yeast cells requires a method to identify the target cells and a means to collect these targeted cells. In magnetic cell separations, identification is achieved using labelling agents, such as antibodies, proteins, or other compounds that are directed specifically against a cell-surface component that is not present in other cells in the mixture. Once the target cell has been identified, it must be linked to a solid phase that will be used to separate it physically from the mixture (Figure 8). This solid phase can consist of a superparamagnetic microsphere or bead that has been coated with a compound that will react and form a bond with the labelling agent. The cells can then be separated by exposing the cell mixture to a magnetic field. The non-target cells remain in suspension and can be collected by aspiration or decanting. The target cells which are attracted to the magnet and are retained against the separation vessel can then be recovered by removal of the magnetic field (Figure 13) (Gee, 1998).

A number of paramagnetic and superparamagnetic solid phases have been used for cell separation. These vary widely in their size, shape, and magnetic properties, with the major difference being the methods used to collect them. Larger particles (1-5 μm) can be collected in a magnetic field generated by a rare earth magnet, such as neodymium-iron-boron, whereas nanoparticles require entrapment in metallic wire within a magnetic field (Gee, 1998). One advantage of superparamagnetic particles is that they respond well to magnetic

fields but do not themselves become magnetized. This inability to become permanently magnetized allows repeated magnetic extractions without magnetically induced aggregation (Polysciences, 2000).

In *S. cerevisiae*, the cell surface of the daughter cells is due to *de novo* synthesis of the cell wall at the budding site on the mother cell. Therefore, if the mother cells were labelled with a agent that would be retained on the cell surface, the label would stay associated with these cells through multiple rounds of cell division. Subsequent recovery of labelled cells would give a pure population of cells that would have all divided a determinable number of times (Smeal *et al.*, 1996).

One of the most popular biological targeting systems makes use of the natural affinity of avidin to the vitamin D-biotin (Hermanson, 1996). D-biotin is present in minute quantities in all living cells, usually acting as a coenzyme in carboxylation reactions (Korpela, 1984). Avidin, a protein found in egg white, and streptavidin, a similar protein found in *Streptomyces avidinii*, can bind to D-biotin with an affinity of 10^{15} L/mol (Diamandis and Christopoulos, 1991).

D-biotin can also be modified to allow the covalent attachment of other molecules, with the *N*-hydroxysuccinimide (NHS) ester being the most common agent. The NHS ester reacts via a nucleophilic attack of an amine on a carbonyl group, releasing the NHS group and forming a stable amide linkage. In most cases, cells are biotinylated via the ε-amino groups of lysine on the cell surface (Figure 8) (Hermanson, 1996). This biotin-avidin interaction has been used successfully to separate *S. cerevisiae* cells. Furthermore, it has been shown that the biotinylation agent remained stationary on the parent cell surface during subsequent growth and division and did not interfere with these processes (Skutelsky and Bayer, 1979; Smeal *et al.*, 1996; Graepler *et al.*, 1998).

3.1.1. Objectives

The objectives of this study were to biotinylate *S. cerevisiae* cells and recover the cells using streptavidin superparamagnetic beads.

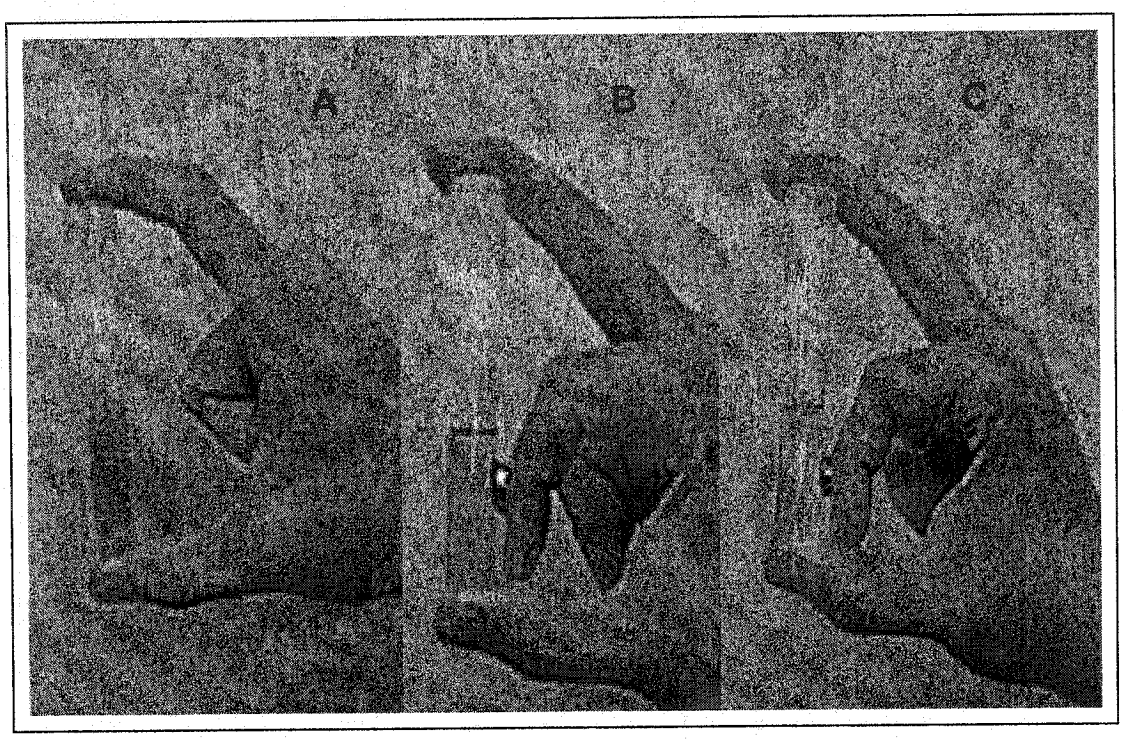

Figure 13. Collection of BioMag strepavidin superparamagnetic beads in a magnetic field. (A) Beads in suspension appear as a cloudy liquid. (B) A small permanent magnet held to the side of the tube starts to attract the beads within a few seconds. (C) Within a few minutes, all of the beads are attracted to the magnet and held against the side of the tube.

3.2. Materials and Methods

3.2.1. Yeast propagation

Stock suspensions of *S. cerevisiae* (NCYC 1239) were prepared as described in section **2.2.2.** Appropriate dilutions of the stock solutions were made using sterile distilled water to obtain working suspensions of the desired cell concentrations.

3.2.2. Initial cell labelling and recovery experiments

In the initial experiments involving the biotinylation of *S. cerevisiae* cells and recovery of these cells with streptavidin superparamagnetic beads, the methodology of Smeal *et al.* (1996) was followed. A flowchart of the unit operations in this method is shown in Figure 14.

Mixed-age yeast cells (~10^7 cells/mL), prepared as described previously, were centrifuged at 2500 rpm (IEC, Needham Heights, MA) for 3 min at ambient temperature. The cells were then washed with sterile distilled water and the yeast pellet resuspended in cold (4°C) phosphate buffered saline (PBS, pH 7.2) in a 1.5 mL capacity microcentrifuge tube (Fisher Scientific, Pittsburgh, PA). The cells were again centrifuged, the supernatant removed, and the pellet resuspended in 0.33 mL of concentrated PBS (10 x PBS, pH 7.2).

Separately, 3.5 mg of *N*-hydroxysuccinimide ester biotin (NHS-Biotin) (ICN, Aurora, OH) was dissolved in 0.67 ml of 10 x PBS at ambient temperature and immediately added to the cells.

The cells were mixed by vortexing at ambient temperature for 15 min then centrifuged and washed eight times in 1 mL of cold PBS. The pH was adjusted to 8.0 with 1 M KOH (Fisher Scientific, Fair Lawn, NJ) in PBS and the cells were added to 200 mL of sterile Yeast Nitrogen Base (Difco, Sparks, MD), supplemented with 10 g/L dextrose (Fisher Scientific, Fair Lawn, NJ) (YNBD),

centrifuged, washed with sterile distilled water, recentrifuged and the pellet was resuspended in a test tube containing 10 mL of cold 1 x PBS. The cells were then placed in a Branson 8210 Sonicator for 30 s (Branson Ultrasonics, Danbury, CT).

BioMag streptavidin superparamagnetic beads (Sigma, St-Louis, MO, USA) were added to the yeast suspension at a rate of 50 beads per biotinylated cell based on the following calculation:

1×10^7 cells x 50 beads/cell = 5×10^8 magnetic beads required

Since 1mg/ml BioMag streptavidin superparamagnetic beads contained 5×10^8 beads per mg, i.e. equivalent to 5×10^8 magnetic beads per ml, the volume of washed beads required was 1 ml.

Prior to the addition of beads to the yeast suspension, they were washed to remove the suspending medium containing sodium azide which is toxic to yeast. This was accomplished by washing the beads 3 times in an equal volume (1 ml) of sterile 1 x PBS and using a strong permanent magnet (Forcefield, USA) to pull the magnetic beads to the side of the tube. The supernatant was then decanted, 1 mL of buffer (PBS) was added and the beads were resuspended in 1 ml of sterile PBS.

The beads were then added to the prepared cell suspension, placed on ice and swirled every 15 min for 2 hours. A set of strong permanent magnets (Forcefied, USA) was applied to the side of the test tube, the supernatant was carefully aspirated and 10 ml of cold YNBD added to the tube and the solution mixed by inversion. This procedure was repeated seven times, after which the cells were resuspended in 10 ml YNBD and counted in an Improved Neubauer hemacytometer.

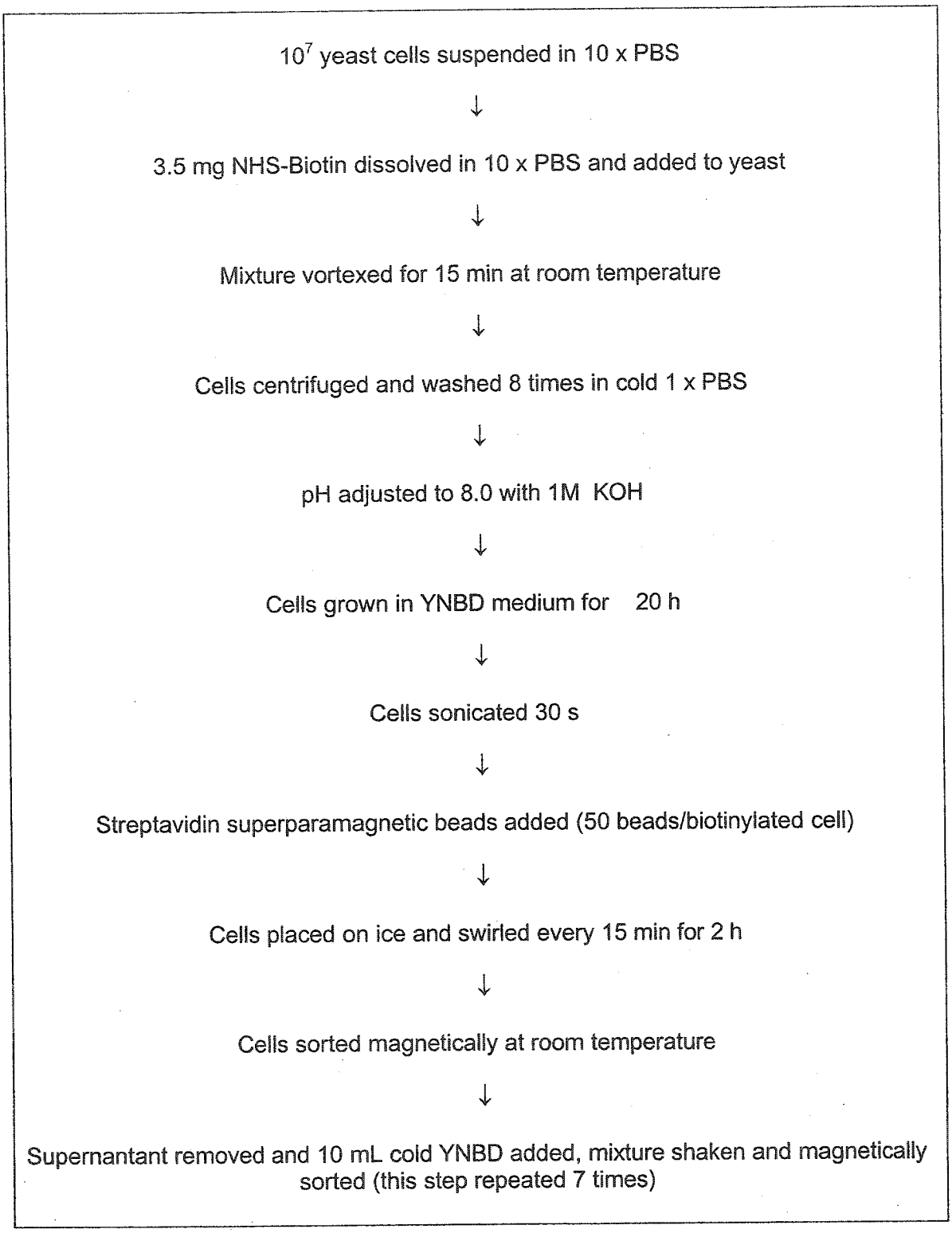

Figure 14. Experimental procedure for biotinylating yeast cell walls and recovering the cells with streptavidin magnetic beads (Adapted from Smeal *et al.*, 1996).

3.2.3. Optimization of the binding and separation process

3.2.3.1. Experimental Design

To help determine the optimal conditions for biotinylating and magnetically sorting *S. cerevisiae*, a 2^{k-p} fractional factorial design was used. Fractional factorial designs are two-level designs which include only a fraction of the total trials that comprise a full factorial (Box *et al.*, 1978). The utility of these designs is based on the premise that main effects and two-factor interactions are initially the most important effects for modeling. Since higher-order interactions, involving three or more factors, are relatively rare, their effects are assumed to be negligible, allowing the critical main effects and two-factor interactions to be estimated in a smaller number of trials (Strobel and Sullivan, 1999).

A full factorial study of two levels of five factors required 32 (2^5) trial runs. In this experiment, a ¼ fraction of the full factorial design was selected (i.e. 8 runs), in which case main effects were aliased with two-factor interactions. This design was chosen since it could be used to determine how significant main effects and interactions were on cell recovery.

Five variables were tested, each at a low (-1) and a high (+1) level and consisted of: (i) biotin concentration, (ii) biotin/yeast mixing conditions, (iii) removal of unreacted biotin, (iv) magnetic bead to cell ratio, and (v) magnetic bead/cell incubation conditions. These variables and their levels were chosen based on the results of preliminary experiments (section **3.3.1.**), and other published protocols for labelling biological cell walls or proteins with biotin (Bayer and Wilchek, 1990; Diamandis and Christopoulos, 1991; Hermanson, 1996; Current Protocols in Protein Science, 1997), and recovery of biotinylated cells using magnetic sorting (Smeal *et al.*, 1996, Polysciences, 2000) The measured response to the factorial study trials was the number of cells recovered, as determined by counting the cells in an Improved Neubauer hemacytometer. The fractional factorial design and coded levels of each variable used are shown in Table 8, and the experimental runs shown in Table 9.

Table 8. Values of coded levels used in the fractional factorial design

Factor	Factor Setting	
	Low (-1)	High (+1)
Biotin (mg)	1	2
Mixing time and type of mixing	15 min, slow vortex, room temperature	60 min, slow rotation, 4 °C
Removal of unreacted biotin	Glycine buffer and centrifugation (washing)	Glycine buffer and dialysis
Magnetic bead to cell ratio	30 beads/cell	70 beads/cell
Incubation time and type of incubation	20 min, 4°C	120 min, ice

Table 9. Coded experimental runs used in the fractional factorial design

Run #	Quantity of biotin	Biotin/cell mixing time and type	Removal of unreacted biotin	Magnetic bead:cell ratio	Bead/cell incubation time and type
1	+1	+1	+1	+1	+1
2	-1	-1	-1	+1	+1
3	-1	+1	-1	-1	+1
4	-1	-1	+1	+1	-1
5	+1	-1	-1	-1	-1
6	-1	+1	+1	-1	-1
7	+1	-1	+1	-1	+1
8	+1	+1	-1	+1	-1

3.2.3.1. Experimental procedure

Cells from the stock solution were enumerated in an Improved Neubauer hemacytometer and appropriate dilutions made to obtain aliquots of approximately $5x10^6$ of mixed-age yeast cells/mL. The yeast were centrifuged, washed with cold sterile 1 x PBS, and resuspended in this solution. The cells recentrifuged and resuspended in 950 μL of 10 x PBS at room temperature in 2.0 mL capacity microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). Depending upon the experimental run, either 1 or 2 mg/mL of NHS-biotin, dissolved in 50 μL dimethyl sulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ), was added immediately to the cells. The mixture was either shaken for 15 min at ambient temperature using a vortex mixer or the mixture was rotated slowly for 60 min at 4 °C.

To terminate the biotinylation reaction, 1 mL 10 mM glycine in 1x PBS at 4 °C was added to the centrifuge tubes and the tubes were incubated for 30 min using slow rotation at 4 °C. Removal of the glycine was accomplished by either washing the cells twice with PBS or, dialysizing the yeast against sterile PBS for 3 h with 2 buffer changes during this time. In the dialysis, Spectra/Por Cellulose ester (CE) membranes with a molecular weight cut-off of 3500 were used (Spectrum Medical Industries, Houston, TX) and runs were performed in a laminar flow cabinet to minimize contamination.

The biotinylated yeast cells were then added to sterile test tubes containing 10 mL of YNB medium. Streptavidin superparamagnetic beads (300 or 700 μl) were washed, as described previously, and added to the tubes. The cells were either incubated for 20 min at 4 °C or put on ice for 2 h. In both cases cells were gently shaken every 10 min. The cells were then sorted magnetically at 4 °C until a clear supernatant was visible, which usually occurred after 5 minutes. Cells were sorted once and cells adhering to the magnets were resuspended in PBS and enumerated in an Improved Neubaur hemacytometer.

3.2.4. Method developed for the biotinylation of S. cerevisiae followed by magnetic cell sorting with streptavidin superparamagnetic beads

Based on the results obtained from the fractional factorial design study, further tests were performed to optimize the biotinylation and recovery of the yeast cells. Optimization focused on: (i) biotin and organic solvent concentration, (ii) biotin/yeast mixing time, (iii) removal of unreacted biotin, (iv) magnetic bead to cell ratio, and (v) magnetic bead/cell incubation conditions. The experimental runs are outlined in Table 10.

The methodology involved preparation of mixed-age cell suspensions, as previously described, with the majority of runs using a cell concentration of ~5 x 10^6 yeast cells/ml. The cells were first centrifuged at 500 x g and 4 °C for 6 min (IEC Refrigerated Centrifuge, Needham Heights, MA) in 2 ml capacity microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). Following removal of the supernatant, 1 ml of 1x PBS was added to each tube. A biotin derivative, initially NHS-biotin but later NHS-LC-biotin (Sigma, St-Louis, MO) was dissolved in DMSO, and added to the cells. Approximately 0.5 ml of 10 mM glycine (Fisher Scientific, Fair Lawn, NJ) dissolved in either 1 x PBS or 10 x PBS was then added to each tube. Cells were then centrifuged using similar conditions, 1 ml of the supernatant was removed and replaced with 1 ml of 1 x PBS. Again, the cells were centrifuged, the supernatant removed and 0.5 ml PBS added to each tube.

Measured quantities of BioMag streptavidin superparamagnetic beads (Qiagen, Valencia, CA) were prepared, as described previously, and added to tubes. The cells were incubated and sorted once using the magnetic sorting device. Cells from all fractions, including those adhering to the magnets, those in the supernatant from the magnetic sorting, and those collected from all centrifugation steps, were enumerated in an Improved Neubauer hemacytometer and cell viability was tested using methylene violet dye, as previously described.

Table 10. Summary of conditions for optimization of biotinylation and recovery of *S. cerevisiae*

Run #	Test Conditions							
	Cell conc. (cells/ml) 10^6	Type of Biotin[a]	Biotin (mg)	DMSO (µl)	Mixing type, temperature, and time	Removal of unreacted biotin	Approximate bead to cell ratio	Bead/cell incubation
1	5.5	NHS-Biotin	1	25	Slow rotation, 4 °C, 30 min	10mM glycine + 1 x PBS (pH 7.2); slow rotation, 4°C, 15 min	10:1	4 °C, 30 min
2	5.5	"	1	25	"	"	10:1	Ice, 1 h
3	4.6	NHS-LC-Biotin	2	25	Slow rotation, 4 °C, 45 min	"	30:1	4 °C, 30 min
4	5.0	"	1	50	Slow rotation, 4 °C, 1 h	"	30:1	4 °C, 20 min
5	5.0	"	2	125	"	"	30:1	"
6	2.3	"	1	50	"	"	30:1	"
7	4.6	"	1.8	100	Slow rotation, room temp., 30 min	"	30:1	"
8	5.4	"	2.4	100	Slow rotation, 8 °C, 30 min	10mM glycine + 1 x PBS (pH 7.2); slow rotation, 4°C, 10 min	50:1	4 °C, 30 min
9	5	"	1.4	100	Slow rotation, room temp., 30 min	"	30:1	"
10	4.5	"	1.8	75	Slow rotation, room temp., 45 min	10mM glycine + 10 x PBS (pH 7.5); slow rotation, 4C, 10 min	30:1	4°C, 35 min

Table 10. Summary of conditions for optimization of biotinylation and recovery of *S. cerevisiae* (cont'd)

Run #	Test Conditions							
	Cell conc. (cells/ml) 10^6	Type of Biotin[a]	Biotin (mg)	DMSO (µl)	Mixing type, temp. and time	Removal of unreacted biotin	Approximate bead to cell ratio	Bead/cell incubation and separation time
11	4.1	"	1.6	50	Slow rotation, room temp., 30 min	"	30:1	"
12	4.1	NHS-LC-Biotin	1.3	50	Slow rotation, room temp., 30 min	10mM glycine + 10 x PBS (pH 7.5); slow rotation, 4C, 10 min	30:1	4°C, 35 min
13	5.0	"	0.08	10	"	10mM glycine + 10 x PBS (pH 7.5); slow rotation, 4C, 10 min	20:1	"
14	5.0	"	0.4	40	"	"	20:1	"
15	4.6	"	0.2	20	Slow rotation, 4°C, 30 min	10mM glycine + 1 x PBS (pH 7.5); slow rotation, 4C, 10 min	30:1	4°C, 25 min
16	4.6	"	0.2	20	"	"	20:1	"
17	4.6	"	0.2	20	"	"	20:1	"

3.3. Results and discussion

3.3.1. Preliminary experiments involving biotinylation of S. cerevisiae and streptavidin superparamagnetic cell sorting

The number of cells recovered from the preliminary biotinylation and magnetic cell sorting study are shown in Table 11. Cell recovery was measured as the absolute number of labelled cells in the positive fraction (those adhering to magnets) divided by the absolute number of labelled cells in the original sample (Radruch *et al.*, 1984). Overall, cell recovery was poor, with fewer than 6% and 7% of cells being recovered in these two tests, respectively. When examined microscopically, small clumps of magnetic beads were observed with very few cells attached. This was attributed to NHS-biotin being present in the YNBD medium and the beads binding to this biotin. Another reason may have been that there was an excess of biotin and/or that the biotin had not bound to the yeast.

Although this protocol was selected since it had been shown previously to successfully biotinylate and magnetically sort *S. cerevisiae*, it did not prove effective in this particular study. Solubility problems were observed with the NHS-biotin as it did not dissolve in the aqueous buffer and therefore could not react with the lysine in the yeast cell walls to form a stable amide bond. Although NHS-biotin was employed in this study, instead of biotinamidocaproate *N*-hydroxysuccinimide ester (NHS-LC-Biotin), which the original protocol used, both chemicals have low solubilities in aqueous environments and must first be dissolved in suitable organic solvents, such as dimethyl sulfoxide (DMSO) or anhydrous dimethylformamide (DMF) (Hermanson, 1996). This was confirmed in separate tests (data not shown) with biotin and buffer (10 x PBS), distilled water, and DMSO. Only the DMSO was successful in dissolving the biotin and the subsequent addition of buffer did not cause precipitation of the biotin.

To date, the only reported method for isolating yeast cells using the biotin-avidin interaction and magnetic cell sorting has been that published by Smeal *et al.*, 1996. While several methods have been published for the isolation of other cells types, such as leukocytes and lymphocytes (Wormmeester *et al.*, 1990) optimization of key parameters, such as biotin concentration, incubation times, bead to cell ratios, etc., must be performed regardless of the cell type (Polysciences, 2000).

Table 11. Visible cells counts and cell recoveries from preliminary yeast biotinylation and magnetic sorting experiments

Test No.	Initial Cell Count (total cells)	Recovered Cell Count (total cells)	Percent recovery [a] (%)
1	~ 1×10^7	5.9×10^5	5.9
2	~ 1×10^7	6.5×10^5	6.5

[a] Percent recovery = (No. of wanted recovered cells/ No. of wanted cells in original sample) x 100%

3.3.2. Combined effect of biotin concentration, mixing conditions, magnetic bead concentration, and bead/cell incubation conditions on the recovery of S. cerevisiae

3.3.2.1. Variable selection for the fractional factorial design

Several factors were taken into account in selecting the variables and levels for the factorial design. In choosing the biotin concentration, although it has been found that excessive biotinylation can inactivate a biologically active molecule, a certain level of excess biotin is necessary to ensure that the reaction is complete (Diamandis and Christopoulos, 1991). However, this amount is best determined empirically, with optimal quantities dependent on such factors as the number and accessibility of lysine in a given protein (Bayer and Wilchek, 1990; Hermanson, 1996).

To date, the amount of lysine available in *S. cerevisiae* for the covalent attachment by the biotin moiety remains largely uncharacterized. In *S. cerevisiae*, the cell wall is composed mostly of mannoprotein and fibrous β1,3 glucan. There is also branched β1,6 glucan that links the other components of the wall, and chitin, which contributes to the insolubility of the fibres. The β1,3 glucan-chitin complex is the major constituent of the inner wall and β1,6 glucan links the components of the inner and outer walls. On the outer surface of the wall are mannoproteins, which are extensively O and N glycosylated (Lipke and Ovalle, 1998). However, studies on the cell wall linkages in *Schizophyllum commune*, revealed that a large portion of the glucosamine residues linking chitin to β-glucan were amino acids, particularly lysine and citrulline (Sietsma and Wessels, 1979).

Concerning the mixing conditions for biotin and yeast, a vortex had been used in the preliminary studies. However, it was believed that this mixing method may be inadequate compared to gentle rotation, resulting in a greater interaction between the cells and the biotin.

The temperatures, either ambient (22 °C) or 4°C, were chosen based on the characteristics of NHS-biotin, since higher temperatures have been found to result in an increased biotinylation of membrane proteins but much of the increase may be due to endocytic membrane turnover (Current Protocols in Protein Science, 1997). Furthermore, since the NHS ester has been shown to hydrolyze in the presence of water, thus rendering the molecule ineffective for biotinylation reactions (Current Protocols in Protein Science, 1997), limiting its exposure to water was considered important.

The removal of unreacted biotin was another parameter to control, since unreacted biotin had the potential to subsequently bind to components in buffers or growth media that contained primary or secondary amino groups. In the methodology of Smeal *et al.*, (1996), excess biotin was removed through several washing steps. To minimize the potential loss of cells during the numerous washing and centrifugation steps, glycine was used. Glycine, which contains a primary amino group, was added to stop the reaction, and removal of the glycine and free biotin was carried out, since it could later compete with the biotinylated yeast for binding sites on the streptavidin magnetic beads. Washing by centrifugation and dialysis were selected as alternative methods to remove the glycine-biotin compound. A small pore size dialysis membrane was selected since it could exclude the glycine-biotin molecule (molecular weight ~400 Da) but would have no effect on the much larger yeast cells.

In selecting the number of magnetic beads, Smeal *et al.*, (1996) used 50 beads/cell. However, this ratio is dependent on the size of the cell and the level of biotinylation, and is best determined empirically (Polysciences, 2000). Therefore, It was decided to investigate both lower and higher numbers of magnetic beads.

Finally, regarding the incubation of the magnetic beads with the biotinylated yeast, a period of 15 - 30 min, either on ice or at 4° C, had been recommended (Polysciences, 2000). Although long incubations may cause magnetic beads to detach from the target cells due to cell surface changes over

time (Polysciences, 2000), Smeal *et al.* (1996) incubated the cells on ice for 2 h, and therefore verification of this procedure was considered necessary.

3.3.2.2. Results from the fractional factorial experiments

The combined effects of biotin concentration, mixing conditions, removal of unreacted biotin, magnetic bead to cell ratio, and bead/cell incubation conditions on the recovery of *S. cerevisiae* are shown in Table 12. Cell recovery varied from 0.42% to 12%, which was well below expectations, but not inconsistent with results obtained in preliminary experiments.

The statistical software Stat-Ease (Stat-Ease, Inc., Minneapolis, MN) was used to calculate parameter estimates and perform a statistical analysis of the data. The data analysis gave an estimate for each main effect and two-factor interaction, as shown in Table 13. Parameter estimates represent the expected change in response, in this case cell recovery, per unit change in a variable when all factors are held constant. The *P* value is the probability that the magnitude of a parameter estimate is due to random process variability. A low *P* value indicates a significant effect and provides a baseline for deeming some factors critical and others less important. Traditionally, a *P* value of <0.05 has been used as a cut-off point for significance (Strobel and Sullivan, 1999). Based on the *P* values obtained, there were no significant model terms in these experiments.

In general, mathematical models are generated to determine the optimal levels of the most significant factors to give a desired response (Box *et al.*, 1978). Since there were no significant terms, no model could be generated to adequately predict cell recovery. Despite the model's lack of significance, it provided a valuable means of understanding the biotinylation and magnetic sorting procedure.

Table 12. Total number and percentage of cells recovered from fractional factorial studies

Run #	Factors					Cell recovery	
	Biotin (mg)	Mixing[a]	Biotin removal[b]	Bead: cell ratio	Bead incubation[c]	Total cells	Percent recovery (%)
1	2	60	dialysis	70	120	1.2×10^5	2.40
2	1	15	washing	70	120	2.1×10^4	0.42
3	1	60	washing	30	120	5.6×10^5	11.2
4	1	15	dialysis	70	20	3.4×10^5	6.80
5	2	15	washing	30	20	6.0×10^5	12.0
6	1	60	dialysis	30	20	4.0×10^4	0.80
7	2	15	dialysis	30	120	6.0×10^5	12.0
8	2	60	washing	70	120	1.4×10^5	2.80

[a] "15" refers to mixing for 15 min at ambient temperature, using a vortex on a slow setting; "60" refers to mixing at 4 °C using slow rotation.
[b] 10 mM glycine buffer plus either washing by centrifugation or dialysis.
[c] "20" refers to incubation of biotinylated cells with streptavidin magnetic beads for 20 min at 4 °C, whereas "120" refers to the same incubation for 120 min on ice.

Table 13. Parameter estimates and *P* values for fractional factorial biotinylation and magnetic sorting of *S. cerevisiae* study

Term	Estimate	*P* value	Level of significance
Mixing conditions	-0.12	0.6158	N.S.[a]
Removal of biotin	0.0037	0.9866	N.S.
Bead:cell ratio	-0.27	0.3762	N.S.
Bead/cell incubation conditions	-0.0037	0.9866	N.S.
Mixing conditions – Removal of biotin	-0.29	0.3510	N.S.
Mixing conditions – Bead/cell incubation Conditions	0.29	0.3510	N.S.

[a] N.S. = Non-significant at $P > 0.05$

3.3.3. Method developed for the biotinylation of yeast cells followed by magnetic cell sorting with streptavidin superparamagnetic beads

Based on the statistical analysis of the fractional factorial design, no terms were significant. Therefore, it was decided to hold some variables constant and to modify others. The variables held constant included: (i) glycine with washing to remove free biotin, (ii) the bead to cell ratio and, (iii) the bead/cell incubation conditions. The conditions that were modified included: (i) the biotin derivative and organic solvent concentration, (ii) the biotinylation reaction conditions, (iii) the pH, and (iv) the buffer concentration.

The results from the optimization experiments are shown in Table 14. The data is reported as the percentage of the total number of cells recovered and includes cells adhering to magnets, cells from the magnetic sorting, cells collected from all centrifugation steps, as well as the total number of cells recovered.

From the results, it can be observed that in runs # 1-14, the recovery of biotinylated cells ranged from 2.7% to 31%. However, between 27 and 70% of total cells were consistently observed in the supernatant following magnetic cell sorting, suggesting insufficient biotinylation. Furthermore, losses from centrifugation varied between 6 and 25% but stabilized at ~10% once the reaction conditions were fully established.

Early on in these experiments, it was decided to use NHS-LC-biotin instead of NHS-biotin. NHS-LC-biotin is similar in composition to NHS-biotin in that it has NHS ester group to increase reactivity with primary amines. However, it also incorporates a long chain (LC) 7-atom spacer arm built off the valeric acid side chain, to extend the biotin from the protein surface, thus improving binding of subsequent reagents, such as streptavidin. NHS-biotin is ~15 Å in length, whereas NHS-LC-biotin is ~22 Å in length. Since the binding sites for biotin and streptavidin are pockets buried ~9 Å beneath the surface of the protein, a spacer

Table 14. Results from cell biotinylation and magnetic sorting optimization experiments

Test No.	Cell Recovery (% of initial cell number)			
	A[a]	B[b]	C[c]	Total
1	6	54	25	85
2	11	70	13	94
3	3	31	25	59
4	17	67	15	99
5	31	40	6	77
6	8	60	15	83
7	24	47	12	83
8	21	14	14	50
9	7	50	17	74
10	27	57	13	97
11	12	27	14	52
12	17	40	9	66
13	14	60	10	84
14	12	60	10	82
15	**80**	**2**	**15**	**96**
16	**90**	**1**	**8**	**99**
17	**77**	**7**	**12**	**96**

[a] Cells adhering to magnets
[b] Cells in the supernatant following magnetic cell sorting
[c] Cells collected from supernatants following all centrifugation steps

can affect the binding accessibility of biotinylated compounds (Hermanson, 1996).

During these experiments, it was also decided to increase the pH from 7.2 to 7.5 since a higher pH could result in increased labelling but at the risk of hydrolysis of the NHS ester (Current Protocols in Protein Science, 1997). The pH was maintained within the lower end for biotinylation reactions, which typically ranges from pH 7.2 – 9.1 (Diamandis and Christopoulos, 1991). Although yeast can tolerate a wide range of pH, their physiological limit is ~pH 8 (Walker, 1998a).

The bead to cell ratio was also maintained fairly constant at 30 beads/cell. This was done throughout the experiments, since visual inspection of the bead-cell mixture in a microscope revealed a large excess of beads. In addition, beads obtained directly from the manufacturer (Polysciences, 2000), were found to be of a superior quality to those obtained from other sources. Although BioMag streptavidin superparamagnetic beads have a shelf-life of approximately two years, they must be stored at between 0 - 8° C immersed in a preservative-containing buffer. Failure to maintain these conditions results in dehydration and loss of protein function. One possibility for the poorer performance of beads from other sources is that they may have degraded during storage and/or transportation.

A high level of cell death was observed in runs # 9 and 10, with death rates of ~40% and ~30%, respectively. The organic solvent, dimethyl sulfoxide (DMSO) was suspected as the cause of this high death rate. Although it is recommended to limit the concentration of organic solvents to 10% in order to avoid precipitation problems (Hermanson, 1996; Current Protocols in Protein Science, 1997), the levels of organic solvent in these runs were 10% and 7.5%, respectively. Tests were also performed to determine if the DMSO affected cell viability. Yeasts were mixed with two different concentrations of DMSO (50 and 100 μL), and with two different concentrations of biotin, 1.6 and 1.3 mg, dissolved in 50 μL DMSO (Tests # 11 and 12). The results revealed that neither 50 nor 100

μL of DMSO influenced cell viability (data not shown). However, cell viability decreased to ~75% and ~88% in runs # 11 and 12, respectively. Furthermore, it was observed that only ~60% of total cells were recovered in these two tests, suggesting perhaps that the biotin was causing the cells to die or lyse.

In runs # 13 and 14, both the biotin and the organic solvent concentrations were reduced. However, biotinylated cell recovery did not improve and cell viability was ~80% and ~65% for these runs, respectively. Another contributing factor to the cell death could have been the high salt concentration in the 10 x PBS. In run # 15, the concentration of biotin was reduced to 20 μL (obtained from a stock solution of 1 mg biotin/ 0.1 mL DMSO) and the 10 x PBS was replaced with 1 x PBS. Cell recovery improved dramatically, with 80% biotinylated cells being recovered. When the experimental conditions were repeated (runs # 16 and 17) the bead to cell ratio was reduced from 30:1 to 20:1 biotinylated yeast cell recovery was 90% and 77%, respectively, indicating that the method was reproducible. In Tests # 15-17, cell viability was >85% and these results are in agreement with those obtained by Smeal *et al.* (1996), who reported a cell loss of 20% for biotinylated cells.

3.4 . Conclusion

In conclusion, a method for the biotinylation and recovery of *S. cerevisiae* cells using magnetic cell sorting was optimized. It was found that ~90% of biotinylated cells could be recovered using magnetic sorting. Cell viability was >85%, using standard cytological staining techniques.

Problems with biotin precipitation were evident throughout the experiments since biotin will often form a colloidal suspension in water, rather than a true solution (Current Protocols in Protein Science, 1997). To avoid this in future, a compound similar to NHS-LC-Biotin, that contains a sulfur group could be used. Sulfo-NHS-LC-Biotin contains a negatively charged sulfonate group on its NHS ring structure. The presence of the negative charge creates sufficient

polarity within the molecule to allow direct solubility in aqueous reaction mediums and will not hydrolyze in water as rapidly as NHS-LC-Biotin (Hermanson, 1996).

It may have also been advantageous to determine the extent of biotinylation. A standard assay for measuring biotin incorporation into macomolecules, such as proteins, is the HABA dye assay. However, this assay is not suitable for use on cells (Hermanson, 1996). Instead, a technique that involves adding fluorescein-avidin to biotinylated cells and then examining the cells with a fluorescence microscope could be tried. This technique was used by Smeal *et al.* (1996) to verify the effectiveness of their biotinylation procedure.

Chapter 4

Continuous Phased Culture and Magnetic Cell Sorting of *Saccharomyces cerevisiae*

4.1. Introduction

In conventional batch and continuous yeast cultivation systems, cell populations are randomized with respect to individual cell cycles. Non-random, or synchronous, yeast cultures are characterized by cells in the population dividing more or less in unison (Walker, 1999). Continuous phased culture has been used previously by Dawson (1970, 1972) as a means of producing a synchronous culture for the study of microbial metabolism. This system delivers a key growth-limiting nutrient to the cell population, at a concentration sufficient to complete one cell cycle (Walker, 1998a). Living cells are comprised of carbon, nitrogen, phosphorus, sulfur, potassium, magnesium, and other elements, each of which can be made the growth-limiting nutrient (Dawson, 1985). Since yeasts require fixed, organic forms of carbon for growth (Walker, 1998a), limiting carbon, in the form of glucose, while maintaining all other nutrients in excess, would restrict the supply of carbon for biosysnthesis or energy (Dawson, 1985).

In the continuous phased culture technique, one half of the culture broth is removed and replaced with fresh nutrients each time the population doubles. During doubling, the limiting nutrient is completely consumed. Therefore, successive generations of cells are subjected to an identical nutrient environment and an absolute nutrient limitation (Sheppard and Cooper, 1991). Using biotinylated virgin yeast, the continuous phased culture technique could also be used as a convenient means to obtain aged cells. After one cell cycle, the biotinylated virgin cells will have reproduced, leaving a bud scar on their surface.

Since one half of the culture broth is removed, this first harvest would include one half of the original biotinylated virgin cell population that are now one generation older, leaving the other half in the culture broth. Successive harvestings would remove half of the remaining virgin cells that are becoming progressively older. Sorting these biotinylated cells with streptavidin magnetic beads would then yield cells of similar age.

4.1.1. Objectives

The objectives of this study were to biotinylate virgin *S. cerevisiae* cells, in conjunction with continuous phased culturing to separate cells with a specific age based on number of cell divisions using strepatavidin magnetic cell sorting.

4.2. Materials and methods

4.2.1. Yeast propagation

Stock suspensions of *S. cerevisiae* (NCYC 1239) were prepared as described in section ***2.2.2.*** Appropriate dilutions of the stock solutions were made using sterile distilled water to obtain working suspensions of the desired cell concentrations.

4.2.2. The continuous phased culturing technique and S. cerevisiae

4.2.2.1. Phased growth media selection

4.2.2.1.1. Preliminary phasing studies using Malt Yeast Peptone Dextrose (MYPD) broth

A working suspension of ~2.5 x 10^7 mixed-age cells/mL was obtained from a stock solution of *S. cerevisiae* (NCYC 1239), as previously described, and biotinylated with NHS-LC-biotin at a rate of 1 mg/mL, according to the established procedure (section ***3.3.3.***). Following biotinylation, the cells were added to 25 mL of Malt Yeast Peptone Dextrose (MYPD) broth, prepared as described in section ***2.2.1.*** An equivalent number of non-biotinylated cells were added to a control flask of 25 mL MYPD broth and the flasks were incubated in a water-bath shaker at 28 °C and 225 rpm (New Brunswick Scientific, New Brunswick, NJ).

The optical density of the control flask was monitored at 600 nm using a Novaspec II Visible Spectrophotometer (Pharmacia, Cambridge, England). When the absorbance value of the broth in the control flask had doubled, after ~2 h, one half the volume (and cells) from each flask was harvested. An equal volume of fresh cell-free nutrient broth was then added to each flask. Streptavidin superparamagnetic beads were added to the harvested biotinylated

cells at a rate of 20 beads/cell, and the cells were sorted magnetically according to the established method (section ***3.3.3.***). Growth was allowed to proceed in this way for four doubling time intervals. All cells, including those adhering to magnets, those in the supernatant following magnetic sorting, and those from all the centrifugation steps were stored in sterile tubes at 4 °C.

4.2.2.1.2. Yeast Nitrogen Base Dextrose (YNBD) broth

4.2.2.1.2.1. Growth curve of S. cerevisiae in YNBD broth

The inoculum for the growth curve was prepared by transferring a loopful of pure culture from the YNBD plates (1.5% agar) into 100 mL of YNB broth supplemented with 10 g/L glucose, followed by incubation for 24 h in a water-bath shaker at 28 °C and 250 rpm (New Brunswick Scientific, New Brunswick, NJ). After 24 h, cells were stained with methylene violet and enumerated using an Improved Neubauer hemacytometer. Appropriate dilutions were then made from the stock solution using sterile distilled water to give a working suspension of ~4.5 x 10^4 cells/mL in 100 mL of YNB broth supplemented with 3 g/L glucose.

The inoculated flask was then incubated, as described previously, and monitored for growth approximately every hour for 15 hours. Cells were stained with methylene violet and enumerated in an Improved Neubauer hemacytometer. The doubling time and specific growth rate were calculated as outlined in section ***2.2.2.***

4.2.2.1.2.2. Limiting nutrient concentration

In order to find a glucose concentration that would permit the cells to double once and then enter the stationary phase during continuous phased growth, tests were conducted in which varying amounts of glucose were added to flasks containing 25 mL of 10g/L YNB broth buffered with PBS. Final glucose concentrations ranged from 0.1 to 5 g/L, as shown in Table 15. All media were prepared in 150 mL Erlenmeyer flasks and autoclaved at 121 °C and 15 psi for

15 min immediately after preparation. The pH of the broth was 6.1. Mixed-age *S. cerevisiae* cells, obtained from stock solutions, were diluted to obtain ~10^6 cells/mL and the cultures were incubated in a water-bath shaker, as described. Cell density was measured by monitoring the absorbance of the cultures at 600 nm at 1 h intervals.

Table 15. Experimental runs to determine growth limiting concentration for a mixed-age population of *S. cerevisiae*

Run #	Glucose concentration (g/L)
1	5
2	3
3	1
4	0.75
5	0.5
6	0.3
7	0.1
8	0.25
9	0.2

4.2.2.1.3. Continuous phased culture using mixed-age S. cerevisiae cells

The phasing system was tested on a mixed-age non-biotinylated population *of S. cerevisiae* using a chemically-defined nutrient broth. A stock solution of *S. cerevisiae* was diluted, as described previously, to obtain ~10^6 cells/mL in a flask containing 25 mL of broth. The growth medium consisted of Yeast Nitrogen Base (YNB) (Difco), 2% (v/v) 10 x PBS (pH 7.0), and 0.25 g/L glucose (Fisher Scientific, Fair Lawn, NJ). YNB was present in excess at 10 g/L and glucose was used as the growth-limiting nutrient. The 10 x PBS was prepared as described in section ***2.2.3.1***.

The yeast culture was monitored by optical density at a wavelength of 600 nm, as outlined in section ***4.2.2.1.1***. One half the volume of cells (12.5 mL) was harvested when the OD reading had doubled and stabilized. Fresh medium (12.5 mL) was added to the original flask in order to maintain a volume of 25 mL. Harvesting was done at ~7 h intervals and this phasing was allowed to proceed for four doubling time intervals. All harvesting, nutrient additions, and OD readings were performed in a laminar flow cabinet to minimize microbial contamination.

4.2.2.1.4. Continuous phased culture using biotinylated virgin S. cerevisiae cells and streptavidin magnetic sorting

Virgin cells of *S. cerevisiae* (NCYC 1239) were obtained according to the method established for density gradient sedimentation, as described in section ***2.2.4.*** Approximately 2 x 10^7 virgin cells/mL were first centrifuged at 500 x g and 4 °C for 6 min (IEC Refrigerated Centrifuge, Needham Heights, MA) in a 2 mL capacity microcentrifuge tube (Fisher Scientific, Pittsburgh, PA). Following removal of the supernatant, 1 mL of 1 x PBS (pH 7.5) was added to the tube. Approximately 0.8 mg of the biotin derivative, NHS-LC-Biotin (Sigma, St-Louis, MO) was dissolved in 25 μL DMSO, and added to cells. The centrifuge tube was incubated for 30 min using slow rotation at 4 °C.

To halt the biotinylation reaction, 0.5 mL 10 mM glycine in 1 x PBS at 4 °C was added to the centrifuge tube and the tube was incubated for 10 min using slow rotation at 4 °C. The glycine was removed by washing the cells twice with PBS and centrifuging at 500 x g for 6 min at 4 °C between each washing step. Finally, the biotinylated cells were resuspended in 1 mL PBS (pH 7).

Following biotinylation, the cells were enumerated using an Improved Neubauer hemacytomer. A total of ~1.7 x 10^7 biotinylated cells, or ~8.5 x 10^5 cells/mL, were then grown in a medium of similar composition to that described in section ***4.2.2.1.3.*** and incubated in a water-bath shaker. However, the total volume in the flask was reduced from 25 mL to 20 mL. A control flask containing an equivalent number of non-biotinylated mixed-age cells was used to monitor cell density at 600 nm. The mixed-age cells were obtained as described in section ***2.2.2.***

Ten mL of the culture was harvested from each flask when the OD reading had doubled and stabilized. This was followed by the addition of 10 mL of fresh medium to each flask. Biotinylated cells were harvested into sterile 15 mL centrifuge tubes (Fisher Scientific, Pittsburgh, PA) and the tubes were then placed at 4 °C until further treatment. Harvesting was done at approximately 6 h intervals and this was allowed to proceed for 6 intervals or 6 generations of cells. All harvesting, nutrient additions, and OD readings were performed in a laminar flow cabinet to minimize microbial contamination.

Harvested biotinylated cells were sorted magnetically using the method developed in section ***3.3.3.*** Streptavidin superparamagnetic beads were washed, as previously described, and added to the centrifuge tube. The cells were incubated for 25 min at 4 °C and the tube was gently shaken every 10 minutes. The cells were then sorted magnetically at 4 °C until a clear supernatant was visible , which usually occurred after 5 minutes. A ratio of 20 streptavidin magnetic beads/cell was used for generations 1 to 4 and the number of beads per cell was doubled and quadrupled for generations 5 and 6, respectively. Cells were sorted in 2 mL capacity microcentrifuge tubes and this sorting procedure was repeated 3 times to ensure that all biotinylated cells were

recovered. This was accomplished by repeatedly resuspending the yeast in 1 mL of PBS, then applying a magnet to the side of the tube and aspirating the supernatant. Sorted cells were enumerated using an Improved Neubauer hemacytometer.

4.2.2.1.5. Viability testing of biotinylated phased grown S. cerevisiae

Viability testing was carried out on the recovered biotinylated yeast using both vital staining and plate counts. Cells were stained and enumerated using the methodology outlined in section **2.2.2**. Plate counts were made by preparing serial dilutions, using sterile peptone water (0.1%) (Difco, Detroit, MI), followed by surface plating onto Oxytetracycline Glucose Yeast Extract Agar plates (OGYEA) (Difco, Detroit, MI). Plates were incubated at 28 °C for 3 days and colonies were enumerated by visual inspection. Counts were expressed as Colony Forming Units per mL (CFU/mL).

4.3. Results and discussion

4.3.1. Phased growth in different media

4.3.1.1. Malt Yeast Peptone Dextrose (MYPD) broth

The results from the experiments using biotinylated mixed-age *S. cerevisiae* are shown in Table 16. Overall, ~40-50% of biotinylated cells were recovered from each harvesting step. It was observed that the number of cells recovered increased as the number of sorts increased, probably due to non-biotinylated cells becoming less entrapped within the magnetic matrix, which had been shown in previous studies (Smeal *et al.*, 1996).

Cells were harvested approximately every 2 hours, which was consistent with the results obtained for the doubling time of this yeast strain in MYPD broth

(section ***2.3.1.***). However, it was observed that after 2 hours several of the daughter cells were still attached to the mother cells, and in harvests # 3 and 4, some cells had formed chains, with 3 or more cells attached to the biotinylated mother cell. Sonication for ~30 s in a Branson 8200 Ultrasonic Cleaner (Bransonics, Danbury, CT) was used with these cells but without success. Nonetheless, this process confirmed that the biotin did not transfer to the daughter cells since magnetic beads were only observed on the mother cells.

Table 16. Expected versus actual cell recoveries for mixed-age biotinylated *S. cerevisiae*.

Harvest No.	Expected cell recovery (total cells)	Actual cell recovery (total cells)	Percentage of total cells [(Actual/Expected) x 100%] (%)
1	1.25×10^7	4.8×10^6	38
2	6.25×10^6	3.1×10^6	50
3	3.13×10^6	1.4×10^6	41
4	1.56×10^6	7.5×10^5	48

4.3.1.2. Yeast Nitrogen Base Dextrose (YNBD) broth

Since the objective was to obtain populations of cells of similar age, various media were tested to find one that would permit the cells to double only once. Previous work with this strain had demonstrated that up until the stationary phase, both the mother and daughter often remained attached. However, they separated in the early stages of the stationary phase.

Media tested included MYP broth and Yeast-peptone (YP) broth supplemented with between 1 and 5 g/L glucose. Cell density was monitored by absorbance 600 nm. Glucose was not found to be limiting in any of the media tested (data not shown). Since MYP and YP are non-defined media, i.e. their chemical composition is unknown, a chemically-defined medium, Yeast Nitrogen Base (YNB), was selected since it contained no carbon sources. Therefore, glucose consumption by the yeast could be more easily monitored.

4.3.1.2.1. Growth curve

The growth curve of *S. cerevisiae* in YNBD broth is shown in Figure 15. Maximum growth (~6 x 10^5 cells/mL) was reached after 14 h at 28 °C. The specific growth rate (μ) was 0. 279 h^{-1} and the cell doubling time (t_D) was 2.48 h. (calculated using equations ***8.0*** and ***5.0***, respectively). Both values were obtained from the mean of two replicate samples.

Growth was noticeably slower in YNBD broth, compared to MYPD broth, with a doubling time of 1.86 h being found (section ***2.3.1.***). One possible explanation was that the yeast had to first assimilate the necessary elements (e.g. vitamins, nitrogen, etc.) to synthesize the requirements for growth and reproduction. In contrast, both yeast extract and peptone contained a readily available supply of amino acids and thus the yeasts could begin their reproductive cycles more rapidly. A second reason may have been due to the change in pH over the growth period. The initial pH of the broth was 5.4 and the final pH was 3.1. Yeasts can tolerate a pH as low as 2.8, but most prefer a pH

between 4.5 and 6.5 (Walker, 1998a). In subsequent experiments involving YNBD, the pH was adjusted to 6.1 using PBS (pH 7.0).

4.3.1.2.2. Limiting nutrient concentration

Several glucose concentrations were tested to find one that would limit cell reproduction to one cycle (Table 15). For most concentrations (Runs # 1-6), glucose was not a growth-limiting factor. However, in Run # 7 it was found that there was insufficient glucose for all the cells to divide. The responses in Runs # 8 and 9 were quite similar (data not shown), in that the initial OD was ~0.092 and stabilized at ~0.220 and ~0.217, respectively. Cells from these runs were enumerated using a Improved Neubauer hemacytometer, and were found to have doubled to ~2 x 10^6 cell/mL with both mother and daughter cells being well separated. Viability testing using vital staining, as described, indicated >95% viability for both runs.

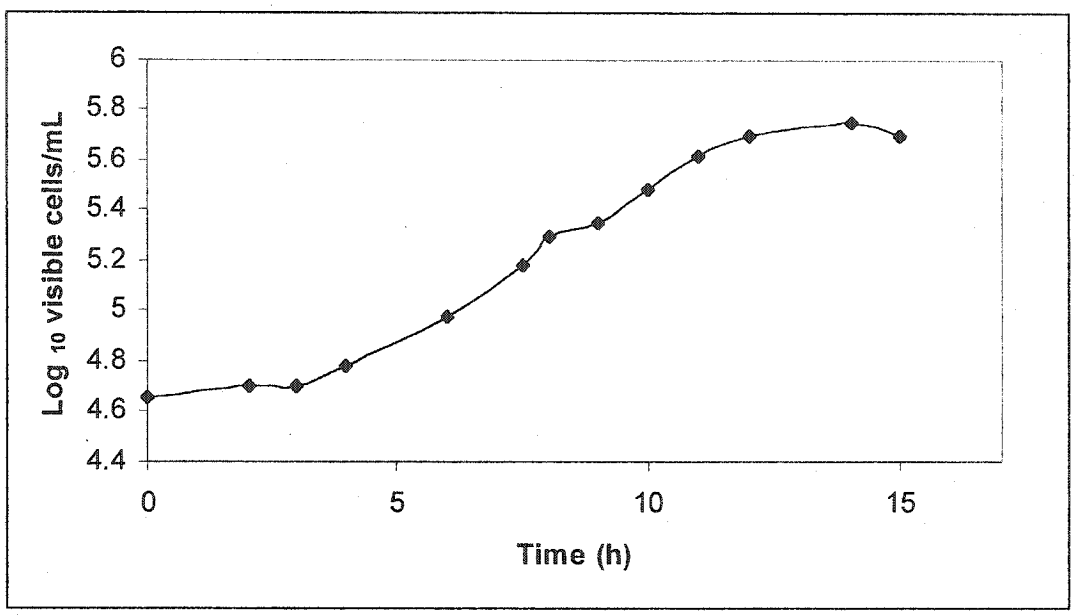

Figure 15. Growth curve of *S. cerevisiae* (NCYC 1239) in YNB broth supplemented with 3 g/L glucose.

4.3.1.3. Mixed-age S. cerevisiae phased growth

The results from the phasing experiment using mixed-age *S. cerevisiae* in a nutrient-limited broth are shown in Figure 16. OD readings represented the mean of two replicate samples. The objective of this test was to determine how well continuous phasing worked with the selected medium.

Cell growth was very slow and with a lag phase of ~6 hours. The initial OD reading was 0.093 and stabilized at ~0.217, at which point cells were harvested. A second harvesting occurred when the OD stabilized at ~0.219, after ~6.5 h. This was in agreement with earlier experiments using a medium of similar composition (section ***4.3.1.2.2.***). An increase in OD was observed between harvests # 3 and 4, which occurred after ~7 h each. These changes in OD could have been due to changes in cell volume during sampling and harvesting.

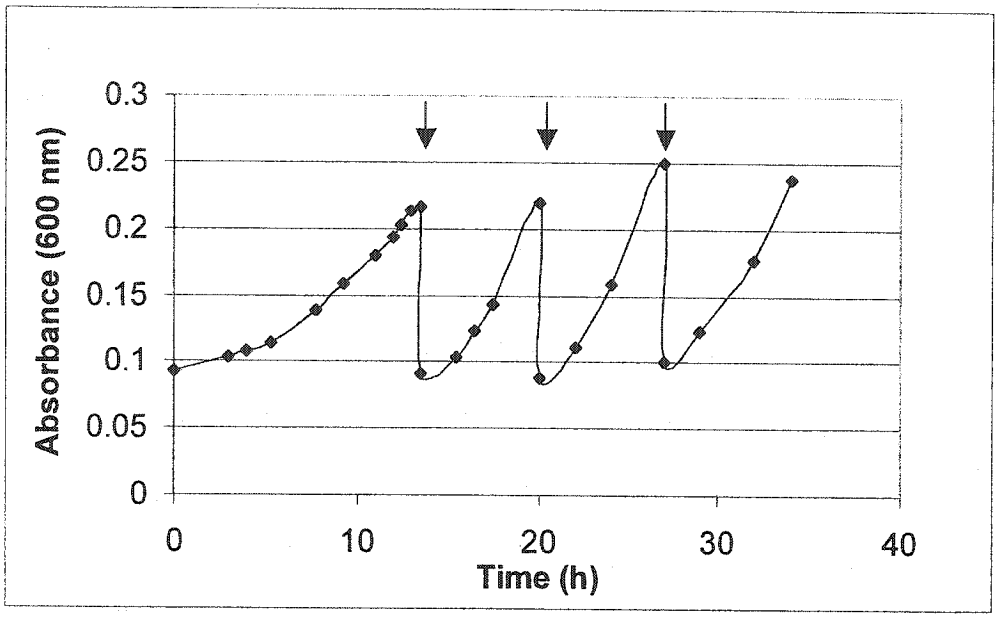

Figure 16. Continuous phased culturing of a non-biotinylated mixed-age population of *S. cerevisiae* (NCYC 1239). Arrows indicate times for harvesting of cells and addition of fresh nutrient broth.

4.3.1.4. Biotinylated S. cerevisiae phased growth and recovery with magnetic cell sorting

The results from the continuous phase culture of biotinylated virgin *S. cerevisiae* cells are shown in Table 17. Overall, approximately 75% of biotinylated cells were recovered. Losses may have been due to centrifugation, insufficient labelling agent, magnetic bead quality and/or quantity, or cell lysis.

The continuous phasing *of S. cerevisiae* through six generations is shown in Figure 17. After an initial lag period of approximately 5 hours, cells were harvested at ~6 h intervals, when the OD of the control flask had doubled and stabilized. Care was taken to ensure that all media and cultures were accurately transferred and harvested cells were immediately sorted magnetically, using the methodology described previously. Prior experiments had demonstrated that with low biotinylated cell concentrations, a higher ratio of magnetic beads to cells was required to increase contact between biotin and streptavidin. Since generations #5 and 6 had low cell concentrations, and increase in beads to 40 and 80 beads per cell, respectively, was warranted. Cells were sorted 3 or 4 times, as described previously. Recovered cells were enumerated using an Improved Neubauer hemacyotmeter. When examined microscopically, cell purity was generally very good but occasionally an unseparated mother and daughter cell was observed. Furthermore, some cell clumping was observed, which was perhaps due to the streptavidin-coated magnetic beads either aggregating or binding to biotin sites on more than one yeast cell.

Cells recovered from the first harvesting are shown in Figure 18. Cells were viewed using phase contrast in a fluorescent microscope, described in section ***2.2.4.3***. Due to the presence of the magnetic beads, it was impossible to observe any bud scars. However, the presence of bud scars may have been revealed by staining the cells with Calcofluor White and viewing the cells in a fluorescent microscope.

Table 17. Expected versus actual cell recoveries for biotinylated *S. cerevisiae*.

Generation Number	Expected cell recovery (total cells)	Actual cell recovery (total cells)	Percentage of total cells [(Actual/Expected) x 100%] (%)
1	8.6×10^6	6.3×10^6	74
2	4.3×10^6	3.2×10^6	74
3	2.1×10^6	1.6×10^6	76
4	1.1×10^6	8.5×10^5	77
5	5.3×10^5	4.0×10^5	75
6	2.6×10^5	2.0×10^5	77
Total cells (Σ generations)	1.68×10^7	1.25×10^7	74.4

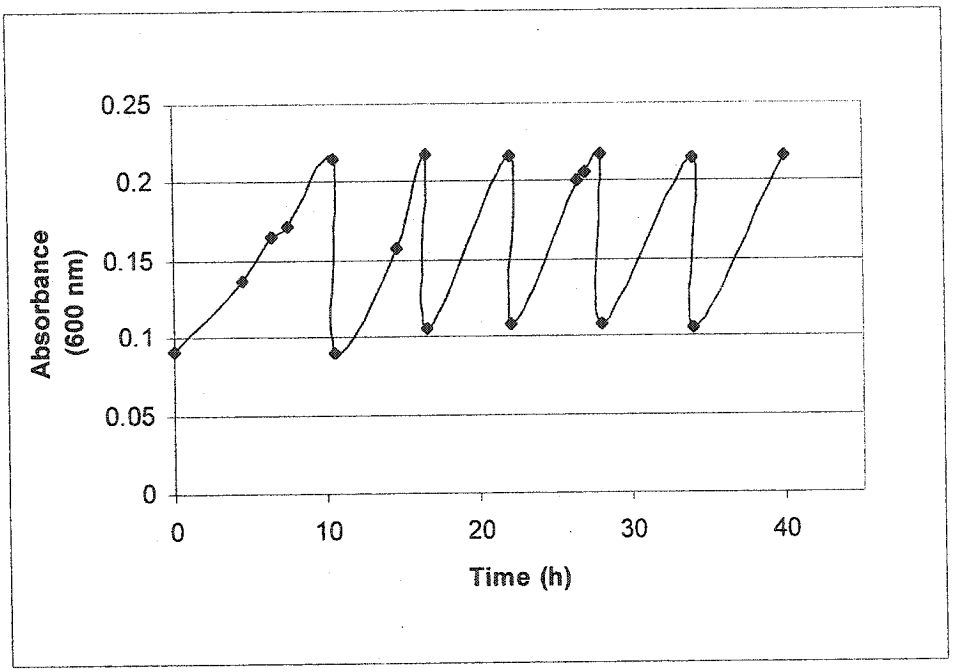

Figure 17. Continuous phase culturing of biotinylated virgin *S. cerevisiae* (NCYC 1239) cells.

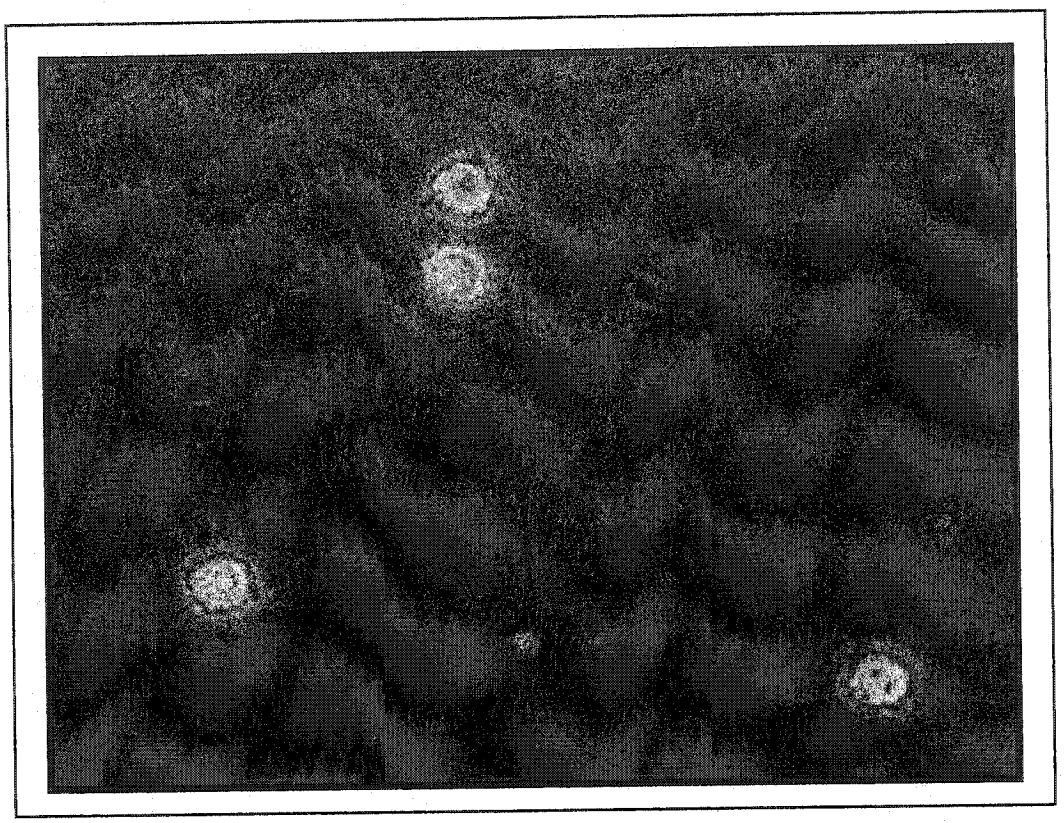

Figure 18. Streptavidin superparamagnetic beads attached to biotinylated virgin *S. cerevisiae* cells.

4.3.1.5. Viability testing

Results from the viability testing are shown in Tables 18 and 19. Cells were stained with methylene violet and the overall cell viability was >98%. However, the recovery of biotinylated cells on agar plates, reported as Colony Forming Units (CFU), ranged from 7-47%.

Results from the viability testing were more difficult to interpret. Cells had been tested to observe whether the magnetic beads had a detrimental effect upon the cell's ability to metabolize nutrients. Also, since the objective was to obtain cells of specific age groups, maintaining cell viability, while discouraging growth through reproduction, was important.

Yeast cells are generally considered to be dead when they irreversibly lose their ability to reproduce. However, cells incapable of division may still be capable of active metabolism. Discrepancies arise in viability assessments based on plate counting and vital staining because the criteria for death differs for each method. Yeast viability can be determined directly by measuring loss of cell reproduction (e.g. plate count) and indirectly by assessing cellular damage (e.g. vital stains) or loss of metabolic activity (e.g. ATP, NADH). The most accurate measures of yeast viability are considered to be the plate count and slide culture methods (Walker, 1998a).

Table 18. Total non-viable cells of biotinylated *S.cerevisiae* as measured by methylene violet staining.

Generation Number	Non-viable cells (total cells)	Percentage of total cells [(Non-viable cells/ actual total cells[a]) x 100%] (%)
1	9.8×10^4	1.6
2	6.0×10^4	1.9
3	2.0×10^4	1.3
4	1.5×10^4	1.8
5	6.3×10^3	1.6
6	2.5×10^3	2.5
Total cells (Σ generations)	2.0×10^5	1.6

[a] actual total cells as shown in Table x for each generation of cells.

Table 19. Number of colony forming units (CFUs) and percentage of cells recovered.

Generation Number	Colony forming units (CFU) (represents total possible CFUs)[a]	Percentage of total cells [(CFU/ actual total cells[a]) x 100%] (%)
1	$2.3x10^6$	37
2	$1.5x10^6$	47
3	$5.7x10^5$	36
4	$1.4x10^5$	16
5	$3.6x10^4$	9
6	$7.0x10^3$	7
Total cells (Σ generations)	$4.6x10^6$	37

[a] the number of CFUs was calculated based on the total volume of sample.

4.4. Conclusion

In conclusion, six different generations of *S. cerevisiae* cells, representing 75% of biotinylated virgin yeast cells, were separated and recovered using continuous phased culturing and magnetic cell sorting. A medium consisting of glucose as the growth-limiting nutrient, with Yeast Nitrogen Base present in excess, and buffered with phosphate buffered saline (PBS, pH 7.0) was used in the phased growth. Although losses may have been due to centrifugation, insufficient labelling agent, magnetic bead quality and/or quantity, or cell lysis, it is generally recognized that perfect synchrony is unlikely, due momentary variations in the size and density of cells of identical cell age, and the natural variability in the duration of mitotic cycles of individual cells (Wheals, 1987; Walker, 1999).

Finally, the presence of the magnetic beads on the cell surface may have prevented cells from obtaining adequate nutrients from the OGYEA plates, thereby reducing cell viability. To date, methods for detaching magnetic particles from cells after separation have included culturing cells for up to 48 hours during which magnetic particles fall away from the cells due to cell surface turnover (Pricop *et al.*, 1993) and by using proteases to release the biotin-streptavidin magnetic complex from the cell surface. However, the protease action may be non-specific and could be potentially damaging to the cells (Polysciences, 2000). Perhaps a further reduction in the number of magnetic beads used could be tried, since fewer magnetic beads would be attached to the cell surface.

Chapter 5

General Conclusion

In order to study age-related changes in yeast, the availability of aged organisms is a prerequisite. Previous investigations on cellular aging in yeast have used haploid and diploid laboratory strains. Futhermore, methods developed to isolate age-synchronized yeast cell populations have used mating pheromones to obtain synchronous cultures and therefore have not been applicable to brewing yeast strains, which generally don't mate. Others have simply used sucrose density gradients to obtain age-fractionated cells. However, high contamination rates from younger or older generations have been observed.

A novel approach to the isolation of same age cells involves the biotin-avidin interaction in conjunction with magnetic beads. This method is based on the observation that the cell surface of a mother cell does not contribute to that of its daughter. The surfaces of young cells were labelled with a biotin derivative, which covalently attaches to primary amines in proteins on the cell surface. Cells are allowed to divide for a fixed period of time and then mixed with avidin-coated magnetic beads. Cells are separated by placing a magnet next to the culture, selectively isolating the biotin-avidin-coated cells. This method was only used to obtain old cells, however, not cells of different ages.

In this study, the approach employed to obtain cells with a specific age based on the number of cell divisions, involved isolating unbudded (virgin) cells using sucrose density gradients, labelling these virgin cells with a biotin derivative, and then synchronizing cell growth using the continuous phased culture technique. Sequential harvests of synchronized cells were treated with streptavidin superparamagnetic beads and the cells recovered were those originally labelled, which had now divided a fixed number of times.

In this procedure, concentrated cells from a brewing yeast strain of *S. cerevisiae* (NCYC 1239) were layered onto non-linear sucrose density gradients

(5-20% w/w) and centrifuged. The upper bands from the gradients, containing the smallest or youngest cells, were collected and the cells were labelled with a biotin derivative.

Preliminary biotinylation experiments were unsuccessful, due in part to the choice of biotin ester (NHS-Biotin). However, the biotinylation and magnetic cell sorting procedure was optimized using biotinamidocaproate-*N*-hydroxysuccimide ester (NHS-LC-Biotin) along with careful modifications to the reaction conditions. The method developed in this study was found to be highly reproducible and easier to perform than that previously published for the isolation of yeast cells using biotin-avidin magnetic cell sorting.

During continuous phased culturing, biotinylated cells were added to a carbon-limited nutrient medium and cell growth was synchronized using the doubling time of the cells. The nutrient medium consisted of 10 g/L Yeast Nitrogen Base and 0.2 g/L glucose, buffered to pH 6.1 with phosphate buffered saline (pH 7.0). Cell density was measured by monitoring the absorbance of a control flask at 600 nm. At each doubling time interval, one half the volume (and cells) from each flask was harvested. An equal volume of cell-free nutrient broth was then added to each flask. Streptavidin superparamagnetic beads were added to the harvested biotinylated cells and the cells were sorted.

In total, six generations of cells of the same age were obtained using these techniques and approximately 75% of the biotinylated cells were recovered. Viability testing was conducted using vital staining and plate counts, with >98% viability reported with the vital stain and 37% viability with the agar plates.

However, during these procedures, factors such as osmotic shock, starvation, and physical manipulation, may have temporarily or permanently injured the cells. Concerning the sucrose density gradients, neither Egilmez *et al.* (1990) nor Barker and Smart (1996), reported any detrimental effects on the yeast cells due to the high sugar content. However, non-osmotic polymers, such as Ficoll or Percoll, could be tried. The main disadvantages to these are the higher centrifugation speeds that are required and the cost.

Problems with biotin precipitation were evident throughout this study. To avoid such problems in future, Sulfo-NHS-LC-Biotin could be tried. This compound contains a negatively charged sulfonate group, which allows direct dissolution in aqueous environments. Therefore, it would eliminate the need for organic solvent.

For the continuous phased culture technique to be useful with magnetic cell sorting, sufficient quantities of biotinylated cells are required initially to ensure that representative numbers of similarly aged cells can be recovered. It has been shown that higher yields of virgin cells can be achieved with a swing-bucket rotor compared of a fixed-angle rotor, which was used in this study. Moreover, virgin non-biotinylated yeast cells could then be used in the control flasks during the continuous phased culturing, which might better reflect the cell size and density of the biotinylated yeast, compared to those of the mixed-age cells.

Another area worth investigating could involve altering the time interval between the addition of fresh medium to the cell culture during the continuous phased growth. In this study, 6 h intervals were used. Dawson (1972) observed that while the appearance of buds occurred after approximately one-third of the doubling time had elapsed, the growth-limiting nutrient was exhausted more quickly as the doubling time lengthened. By shortening the time interval, it may reduce the possibility that the yeast become "starved" while awaiting fresh nutrients. Finally, the effects of the magnetic beads on the cells' abilities to absorb nutrients should be investigated, along with methods to detach the beads.

In conclusion, the objectives of this research were met. Although further experiments should be performed to confirm the reproducibility of the procedure, the techniques developed in this study could potentially be used to study age-related changes in brewing strains of *S. cerevisiae*.

References

Abel, C., F. Linz, T. Scheper, and K. Schugerl. 1994. Transient behaviour of continuously cultivated baker's yeast during enforced variations of dissolved oxygen and glucose concentrations. *J. Biotechnol.* **33**: 183-193.

American Society of Brewing Chemists. 1980. Report of sub-committee in microbiology. *J. Am. Soc. Brew. Chem.* **38**: 109-110.

Austin, G. D., R. W. J. Watson, and T. D'Amore. 1994. Studies of on-line viable yeast biomass with a capacitance biomass monitor. *Biotechnol. Bioeng.* **43**: 337-341.

Austriaco, N. R. 1996. To bud until death: the genetics of ageing in the yeast, *Saccharomyces*. *Yeast.* **12**: 623-630.

Axcell, B. S. and E. S. C. O'Connor-Cox. 1996. The concept of yeast vitality - an alternative approach. *Proc. 24th Conv.Inst.Brew.(Asia Pacific Sect.).* Oxford, UK, IRL Press. pp. 64-71.

Bamforth, C. W. 2000. Brewing and brewing rsearch: past, present and future. *J. Sci. Food Agri.* **80**: 1371-1378.

Barker, M. G. and K. A. Smart. 1996. Morphological changes associated with the cellular aging of a brewing yeast strain. *J. Am. Soc. Brew. Chem.* **54**: 121-126.

Bayer, E.A. and M. Wilcheck. 1990. Protein biotinylation. *In:* E.A. Bayer (ed.), Methods in Enzymology, Avidin-Biotion Technology. Academic Press, Toronto. pp. 138-160.

Box, G.E., W.G. Hunter, and J.S. Hunter. 1978. Statistics for Experimenters. John Wiley & Sons, New York.

Breeuwer, P., J.-L. Drocort, F. M. Rombouts, and T. Abee. 1994. Energy-dependent, carrier-mediated extrusion of carboxyfluorescein from *Saccharomyces cerevisiae* allows rapid assessment of cell viability by flow cytometry. *Appl. Environ. Microbiol.* **60**: 1467-1472.

Bromberg, S. K., P. A. D. G. R. Bower, J. Fehring, L. Ferber, V. K. Lau, and M. Tata. 1997. Requirements for zinc, manganese, calcium and magnesium in wort. *J. Am. Soc. Brew. Chem.* **55**: 123-128.

Cahill, G., D. M. Murray, P. K. Walsh, and D. Donnelly. Effect of the concentration of propagation wort on yeast cell volume and fermentation performance. *J. Am. Soc. Brew. Chem.* **58**: 14-20.

Casey, G. P., C. A. Magnus, and W. M. Ingledew. 1993. High-gravity brewing: nutrient enhanced production of high concentrations of ethanol by brewing yeast. *Biotech. Lett.* **5**: 429-434.

"Consumption and Exports of Beer Made in Canada – 1987, 1996 and 1997". 2001. (*brewers.ca*), Available: http://www.brewers.ca/frames/enter_statisitcs.htm (Accessed: 2001, March).

Coote, P. J., M. B. Cole, and M. V. Jones. 1991. Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH. *J. Gen. Microbiol.* **137**: 1701-1708.

Creanor, J. and J. Toyne. 1993. Preparation of synchronous cultures of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *In*: P. Fantes and R. Brooks (eds.), The Cell Cycle: A Practical Approach. IRL Press, Oxford. pp. 25-44.

Crowe, J. H., L. H. Crowe, and D. Chapman. 1984. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science*. **223**: 701-703.

Cunningham, S. and G. G. Stewart. 1998. Effects of high-gravity brewing and acid washing on brewer's yeast. *J. Am. Soc. Brew. Chem.* **56**: 12-18.

Current Protocols in Protein Science. 1997. Biotinylation of proteins in solution and on cell surfaces. John Wiley & Sons, New York.

Dawson, P. S. S. 1970. Cell cycle and post cycle changes during continuous phased growth of *Candidia utilis*. *Can. J. Microbiol.* **16**: 783-795.

Dawson, P. S. S. 1972. Continuously synchronized growth. *J. Appl. Chem. Biotechnol.* **22**: 79-103.

Dawson, P. S. S. 1985. Continuous cultivation of microorganisms. *Crit. Rev. Biotechnol.* **2**: 315-372.

Deans, K., A. Pinder, B.J. Catley, and J.A. Hodgson. 1997. Effects of cone cropping and serial re-pitch on the distribution of cell ages in brewery yeast. *Proc. 26th Eur. Brew. Conv. Cong.* Oxford, UK, IRL Press. pp. 469-476.

Diamandis, E. P. and T. K. Christopoulos. 1991. The biotin-(strept)avidin system: principles and applications in biotechnology. *Clin. Chem.* **37**: 625-636.

Duboc, P., I. Marison, and V. von Stockar. 1996. Physiology of *Saccharomyces cerevisiae* during cell cycle oscillations. *J. Biotechnol.* **51**: 57-72.

Duca, J. G. 1999. Latest practices of CIP operations in the brewery. *MBAA Tech. Quart.* **36**: 407-411.

Egilmez, N. K. and S. M. Jazwinski. 1989. Evidence for the involvement of a cytoplasmic factor in the aging of the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* **171**: 37-42.

Egilmez, N. K., J. B. Chen, and S. M. Jazwinski. 1990. Preparation and partial characterization of old yeast cells. *J. Geront.* **45**: B9-17.

Esser, C. 1998. Historical and useful methods of preselection and preparative scale sorting. *In*: D. Recktenwald and A. Radbruch (eds.), Cell Separation Methods and Applications. Marcel Dekker, New York. pp. 1-14.

Fargher, J. and N. A. Smith. 1995. Evidence of cold shock sensitivity in brewing yeast strains. *Proc. 25th Eur. Brew. Conv. Congr.* Oxford, IRL Press. pp. 345-352.

Fiechter, A., D. Kappeli, and F. Meussdoerffer. 1987. Batch and continuous culture. *In*: A. H. Rose and J. S. Harrison (eds.), The Yeasts: Vol. 2. Academic Press, Orlando, FL. pp. 99-129.

Fukui, N., A. Kogin, S. Furukubo, H. Kondo, H. Yomo, and Y. Kakimi. 1996. Evaluation of yeast activity and its application to our industrial brewing. *MBAA Tech. Quart.* **33**: 166-169.

Gershon, H. and D. Gershon. 2000. The budding yeast, *Saccharomyces cerevisiae*, as a model for aging research: a critical review. *Mech. Ag. Develop.* **120**: 1-22.

Graepler, F., U. Lauer, and M. Gregor. 1998. Magnetic cell sorting for parietal cell purification using a new monoclonal antibody without influence on cell function. *J. Biochem. Biophys. Meth.* **36**: 143-155.

Grzelak, A., J. Skierski, and G. Bartosz. 2001. Decreased antioxidant defense during replicative aging of the yeast *Saccharomyces cerevisiae* studied using the 'baby machine' method. *FEBS Lett.* **492**: 123-126.

Hammond, J. R. M. 1987. Brewer's yeast. *In*: A. H. Rose and J. S. Harrison (eds.), The Yeasts: Vol. 5. Academic Press, Orlando, FL. pp. 7-68.

Hartwell, L. H. 1970. Periodic density fluctuation during the yeast cell cycle and the selection of synchronous cultures. *J. Bacteriol.* **104**: 1280-1285.

Hayflick, L. 1965. The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell. Res.* **37**: 614-636.

Heggart, H. M., A. Margaritis, H. Pilkington, R. J. Stewart, T. M. Dowhanick, and I. Russel. 1999. Factors affecting yeast viability and vitality characteristics: a review. *MBAA Tech. Quart.* **36**: 409-431.

Hermanson, G. T. 1996. Bioconjugate Techniques. Academic Press, Toronto.

Hornsey, I. S. 1999. Brewing. Royal Society of Chemistry, Cambridge.

Hough, J. S. 1985. The Biotechnology of Malting and Brewing. Cambridge University Press, Cambridge.

Howland, J. L. 1973. Cell Physiology. McMillan, Toronto.

Imai, T., I. Nakajima, and T. Ohno. 1994. Development of a new method for evaluation of yeast vitality by measuring intracellular pH. *J. Am. Soc. Brew. Chem.* **52**: 5-8.

Jareo, P. W., L. C. Preheim, M. U. Snitily, and M. J. Gentry. 1997. Use of magnetic cell sorting to isolate blood neutrophils from rats. *Lab. Ani. Sci.* **47**: 414-418.

Jazwinski, S. M. 1990. Aging and senescence of the budding yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **4**: 337-343.

Jazwinski, S. M. 2001. Metabolic control and yeast aging. *ASM News* **67**: 259-265.

Johnston, G. C., J. R. Pringle, and L. H. Hartwell. 1977. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* **105**: 79-98.

Johnston, J. R. 1966. Reproductive capacity and mode of death of yeast cells. *Antonie van Leeuwenhoek*. **32**: 94-97.

Jones, H. L. 1997. Yeast propagation - past, present and future. *Brew. Guard.* **126**: 24-27.

Kennedy, B. K., N. R. Austriaco, and L. Guarente. 1994. Daughter cells of *Saccharomyces cerevisiae* from old mothers display a reduced life span. *J. Cell Biol.* **127**: 1985-1993.

Kennedy, B. K., N. R. Austriaco, J. Zhang, and L. Guarente. 1995. Mutation in the silencing gene *SIR4* can delay aging in *S. cerevisiae*. *Cell.* **80**: 485-496.

Korpela, J. 1984. Avidin, a high affinity biotin-binding protein as a tool and subject of medical research. *Med. Biol.* **62**: 5-26.

Lech, K. and R. Brent. 1988. *Escherichia Coli*, plasmids, and bacteriophages, *In*: F. M. Ausubel (ed.), Current Protocols in Molecular Biology. John Wiley & Sons, Toronto. p.1.2.1.

Lentini, A. 1993. A review of the various methods available for monitoring the physiological status of yeast: yeast viability and vitality. *Ferment.* **6**: 321-327.

Liberti, P. A. and B. P. Feeley. 1991. Analytical- and process-scale cell separation with bioreceptor ferrofluids and high-gradient magnetic separations. *In*: D. S. Kompala and P. Todd (eds.), Cell Separation Science and Technology. American Chemical Society. pp. 268-279.

Linko, M., A. Haikara, A. Ritala, and G. Penttila. 1998. Recent advances in the malting and brewing industry. *J. Biotechnol.* **65**: 85-98.

Lloyd, D., L. John, C. Edwards, and A. H. Chagla. 1975. Synchronous cultures of microorganisms: large scale preparation by continuous flow size selection. *J. Gen. Microbiol.* **88**: 152-158.

Maca, H. W., M. Barney, G. Goetzke, D. Daniels, and D. Ryder. 1994. The use of radio frequency capacitance for the measurement of yeast viable biomass and its use in the automatic pitching of fermentations. *MBAA Tech. Quart.* **31**: 146-148.

Mager, W. H. and P. Moradas Ferreira. 1993. Review Article: Stress response of yeast. *Biochem. J.* **290**: 1-13.

Mager, W. H. and J. C. S. Varela. 1993. Osmostress response of the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **290**: 1-13.

Majara, M., E. S. C. O'Connor-Cox, and B. C. Axcell. 1996. Trehalose - an osmoprotectant and stress indicator compound in high and very high gravity brewing. *J. Am. Soc. Brew. Chem.* **54**: 149-154.

Manson, D. H. and J. C. Slaughter. 1986. Methods for predicting yeast fermentation activity. Campbell, I. and Priest, F. G. (eds). *Proc. Sec. Aviemore Conf. Malt. Brew. Dist.* London, Institute of Brewing. pp. 295-297.

Markx, G. H. and D. B. Kell. 1995. The use of dielectric permittivity for the control of the biomass level during biotransformation of toxic substrates in continuous culture. *Biotechnol. Prog.* **11**: 64-70.

McCaig, R. 1990. Evaluation of the fluorescent dye 1-anilino-8-naphthalene sulfonic acid for yeast viability determination. *J. Am. Soc. Brew. Chem.* **48**: 22-25.

Mitchison, J. M. and W. S. Vincent. 1965. Preparation of synchronous cultures by sedimentation. *Nature*. **205**: 987-989.

Mitchison, J. M. and J. Creanor. 1971. Induction synchrony in the fission yeast, *Saccharomyces pombe*. *Exp.Cell Res.* **67**: 368-374.

Mitchison, J. M. and B. L. A. Carter. 1975. Cell cycle analysis. *In*: D. M. Prescott (ed.), Methods in Cell Biology. Academic Press, New York. pp. 209-219.

Mitchison, J. M. 1988. Synchronous cultures and age fractionation. *In*: I. Campbell and J. H. Duffus (eds.), Yeast: A Practical Approach. IRL Press, Washington, DC. pp. 51-63.

Mochaba, F., P. A. Torline, and B. C. Axcell. 1993. Resorufin-labelled casein as a sustrate for protease activity in beer. *Proc. Eur. Brew. Conv.* **24**: 533-541.

Mochaba, F. M., P. A. Torline, and Axcell B.C. 1994. A novel and rapid method for the determination of glycogen in pitching yeasts. *J. Am. Soc. Brew. Chem.* **52**: 145-147.

Mochaba, F., E. S. C. O'Connor-Cox, and B. C. Axcell. 1997. A novel and practical yeast vitality method based on magnesium ion release. *J. Inst. Brew.* **103**: 99-102.

Moonsamy, N., F. M. Mochaba, M. Majara, E. S. C. O'Connor-Cox, and B. C. Axcell. 1995. Rapid yeast trehalose measurement using near infrared reflectance spectrometry. *J. Inst. Brew.* **101**: 203-206.

Mortimer, R. K. and J. R. Johnston. 1959. Life span of individual yeast cells. *Nature*. **183**: 1751-1752.

Munch, T., B. Sonnleitner, and A. Fiechter. 1992. New insights into the synchronization mechanisms with forced synchronous cultures of *Saccharomyces cerevisiae*. *J. Biotechnol.* **24**: 299-314.

Munroe, J. H. 1995. Fermentation. *In*: J. H. Hardwick (ed.), Handbook of Brewing. Marcel Dekker Inc., New York. pp. 323-253.

Nestelbacher, R., P. Laun, D. Vondrakova, A. Pichova, C. Schuller, and M. Breitenbach. 2000. The influence of oxygen toxicity on yeast mother cell-specific aging. *Exp. Geront.* **35**: 63-70.

O'Connor-Cox, E. S. C., F. M. Mochaba, E. J. Lodolo, M. Majara, and B. C. Axcell. 1997. Methylene blue staining: use at your own risk. *MBAA Tech. Quart.* **34**: 306-312.

O'Connor-Cox, E. S. C. 1998. Improving yeast handling in the brewery. Part 3: yeast pitching and measurement of yeast quality. *Brew. Guard.* **127**: 20-26.

Oliver, S. G. 1991. "Classical" yeast biotechnology. *In*: M. F. Tuite and S. G. Oliver (eds.), *Saccharomyces*. Plenum Press, New York. pp. 213-225.

Opekarova, M. and K. Sigler. 1982. Acidifation power: indicator of metabolic activity and autolytic changes in *Saccharomyces cerevisiae*. *Folia Microbiol.* **27**: 395-403.

Owen, C. S. and P. A. Liberti. 1987. Magnetite-protein conjugates for the separation of cells by high gradient magnetic filtration. *In*: T. G. Pretlow and T. P. Pretlow (eds.), Cell Separation: Methods and Selected Applications. Academic Press Inc., New York. pp. 259-275.

Pierce, J. S. 1970. Institute of Brewing: Analysis Committee measurement of yeast viability. *J. Inst. Brew.* **76**: 442-443.

Piper, P. W. 1993. Molecular events associated with acquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **11**: 339-356.

Pirt, S. J. 1975. Principles of Microbe and Cell Cultivation. John Wiley & Sons, New York.

Polysciences Technical Data Sheets. 2000. Polysciences Inc., Valencia, CA, USA.

Pretlow, T. G. and T. P. Pretlow. 1991. Separation of cells by sedimentation. *In*: D. S. Kompala and P. Todd (eds.), Cell Separation Science and Technology. American Chemical Society, Washington, DC. pp. 90-102.

Quain, D. E. 1988. Studies in yeast physiology - impact of fermentation performance and product quality. *J. Inst. Brew.* **95**: 315-323.

Radbruch, A., B. Mechtold, A. Thiel, S. Miltenyi, and E. Pfluger. 1994. High-gradient magnetic cell sorting. *In*: Z. Darzynkiewicz, J. P. Robinson, and H. A. Crissman (eds.), Methods in Cell Biology. Academic Press, Toronto. pp. 387-403.

Reed, G. and T. K. Nagodawithana. 1991. Yeast Technology. Van Nostrand Reinhold, New York.

Rickwood, D. 1978. Isopycnic centrifugation in non-ionic media. *In*: G. D. Birnie and D. Rickwood (eds.), Centrifugal Separations in Molecular and Cell Biology. Butterworths, Toronto. pp. 219-250.

Ridge, D. 1978. Practical aspects of rate-zonal centrifugation. *In*: G. D. Birnie and D. Rickwood (eds.), Centrifugal Separations in Molecular and Cell Biology. Butterworths, Toronto. pp. 36-49.

Russell, I. 1995. Yeast. *In*: W. A. Hardwick (ed.), Handbook of Brewing. Marcel Dekker Inc., New York. pp. 169-202.

Seidl, J., R. Kneuchel, and L. A. Kunz-Schughart. 1999. Evaluation of membrane physiology following fluorescence activated or magnetic cell separation. *Cytometry*. **36**: 102-111.

Sheppard, J.D. and D. G. Cooper. 1991. The response of *Bacillus subtilis* ATCC 21332 to manganese during continuous-phased growth. *Appl. Microbiol. Biotechnol.* **35**: 72-76.

Sietsma, J. M. and J. G. H. Wessels. 1979. Evidence for covalent linkages between chitin and beta-glucan in a fungal wall. *J. Gen. Microbiol.* **114**: 99-108.

Sinclair, D. A., K. Mills, and L. Guarente. 1998. Molecular mechanisms of yeast aging. *TIBS*. **23**: 131-134.

Sinclair, D. A. 1999. Yeast aging research: recent advances and medical relevance. *Cell. Mol. Life Sci.* **56**: 807-816.

Skutelsky, E. and E. A. Bayer. 1979. The ultrastructural delineation of cell growth and division processes using the avidin-biotin complex. *Exp. Cell Res.* **121**: 331-336.

Smart, K. A. and S. Whisker. 1996. Effect of serial repitching on the fermentation properties and condition of brewing yeast. *J. Am. Soc. Brew. Chem.* **54**: 41-44.

Smart, K. 1999. Ageing in brewing yeast. *Brew. Guard.* **128**: 19-24.

Smart, K. A., K. M. Chambers, I. Lambert, and C. Jenkins. 1999. Use of methylene violet staining procedures to determine yeast viability and vitality. *J. Am. Soc. Brew. Chem.* **57**: 18-23.

Smeal, T., J. Claus, B. Kennedy, F. Cole, and L. Guarente. 1996. Loss of transcriptional silencing causes sterility in old mother cells of *S. cerevisiae*. *Cell.* **84**: 633-642.

Soares, E. V. and M. Mota. 1996. Flocculation onset, growth phase and genealogical age in *Saccharomyces cerevisiae*. *Can. J. Microbiol.* **42**: 539-547.

Stewart, G. G. and I. Russell. 1986. One hundred years of yeast research and development in the brewing industry. *J. Inst. Brew.* **92**: 537-558.

Stewart, G. G. and I. Russell. 1995. Brewer's yeast. *In*: Y. H. Hui and G. G. Khachatourians (eds.), Food Biotechnology. VCH Press, New York. pp. 847-871.

Strobel, R. J. and G. R. Sullivan. 1999. Experimental design for improvement of fermentations. *In*: A. L. Demain and J. E. Davies (eds.), Manual for Industrial Microbiology and Biotechnology, 2nd ed. ASM Press, Washington, D.C. pp. 80-93.

Thomas, K. C., S. H. Hynes, and W. M. Ingledew. 1994. Effects of particulate materials and osmoprotectants on very high-gravity ehtanolic fermentation. *Appl. Environ. Microbiol.* **60**: 1519-1524.

Tiligada, E., V. Miligkos, E. Ypsilantis, K. Papamichael, and A. Delltheos. 1999. Molybdate induces thermotolerance in yeast. *Lett. in Appl. Microbiol.* **29**: 77-80.

Vallee, L. 1996. Profil de l'industrie brassicole. Ministère de l'Agriculture, des Pêcheries et de l'Alimentation, Québec.

Van Vlasselaer, P., V. C. Palathumpat, G. Strang, and M. H. Shapero. 1998. New approaches in density gradient separation using colloidal silica gradients in the processing of human hematopoietic progenitor cells. *In*: D. Recktenwald and A. Radbruch (eds.), Cell Separation Methods and Applications. Marcel Dekker, New York. pp. 15-41.

Vettese-Dadey, M. 1999. Going their separate ways - a profile of products for cell separation. *The Scientist.* **13**: 21-30.

Waggoner, A. S. 1990. Fluorescent probes for cytometry. *In*: M. R. Melamed, T. Lindmo, and M. L. Mendelson (eds.), Flow Cytometry and Sorting. Wiley-Liss, New York. pp. 209-226.

Walker, G. M. 1998a. Yeast Physiology and Biotechnology. John Wiley & Sons, Toronto.

Walker, G. M. 1998b. Magnesium as a stress-protectant for industrial strains of *Saccharomyces cerevisiae*. *J. Am. Soc. Brew. Chem.* **56**: 109-113.

Walker, G. M. 1999. Synchronization of yeast cell populations. *Meth. Cell Sci.* **21**: 87-93.

Watson, K. 1990. Microbial stress proteins. *Adv. Microbiol. Physiol.* **31**: 183-223.

Weissler, H. E. 1995. Brewing calculations. *In*: W. A. Hardwick (ed.), Handbook of Brewing. Marcel Dekker Inc., New York. pp. 643-705.

Werner-Washburne, M., E. L. Braun, M. E. Crawford, and V. M. Peck. 1996. Stationary phase in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **19**: 1159-1166.

Wheals, A. E. 1987. Biology of the cell cycle in yeasts. *In*: A. Rose and J. S. Harrison (eds.), The Yeasts: Vol. 1. Academic Press , Orlando, FL. pp. 283-390.

Wormeester, J., F. Stiekema, and C. De Groot. 1990. Immunoselective cell separation. *In*: E. A. Bayer (ed.), Methods in Enzymology, Avidin-Biotin Technology. Academic Press, Toronto. pp. 314-319.