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**Preservation, Inoculum Development and Quality  
Management of Yeasts in the Brewing Industry**

by

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Submitted in accordance with the requirements for the  
degree

**PHILOSOPHIAE DOCTOR**

in the

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*NB: Chapters two and three of this dissertation are presented as a compilation of papers sent for publication and are written according to the style of the journals in which they are to be published.*

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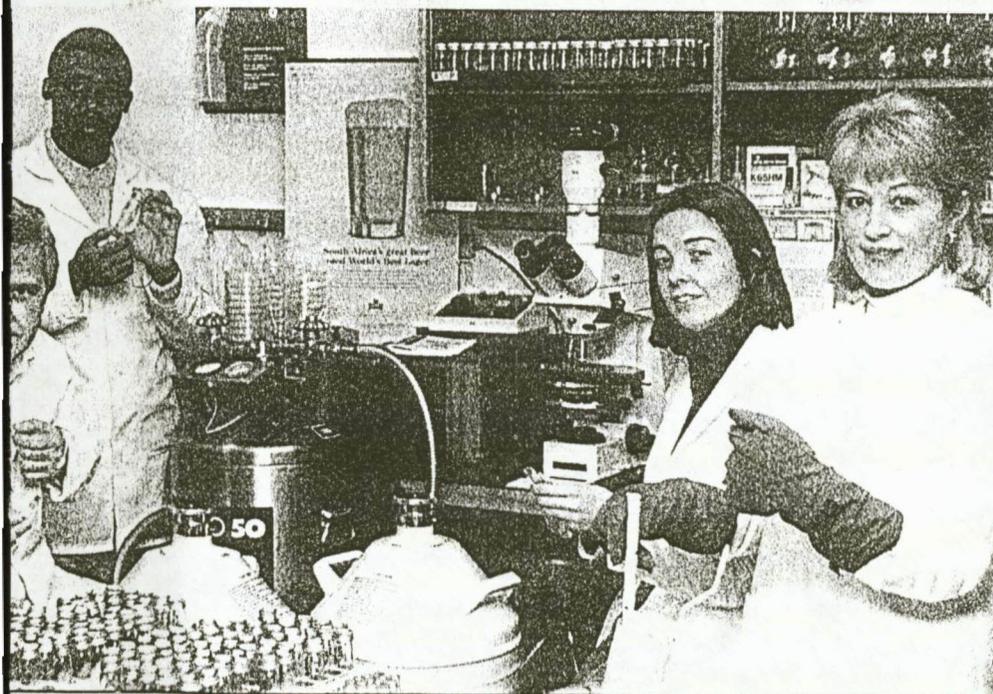
Finally to the **Lord, The Almighty**.

## Preface

This research is in the process of application at the factories of the South African Breweries. Furthermore, this study has contributed in the brewing of the champion beer "Castle Lager" that was awarded first prize at the International Championship held at Burton-on-Trent in April 2000.

*Excerpt from Technology and Human Resources for Industry Programme (THRIP) annual report 1999/2000*

## BREWING THE BEST



Gontse Morakile, whose PhD forms an extension of the yeast technology project, is seen with Professor Kock, Dr Carlien Pohl and Elma Pretorius (far right), the team whose achievements are improving on "the taste that's stood the test of time".

The many drinkers of South African Breweries (SAB) beers will soon reap the benefits of a new yeast culture storage technology being implemented in all SAB breweries in South Africa. The technology was developed by the Department of Microbiology and Biochemistry at the University of the Orange Free State, and SAB's Yeast & Ferment Research Department. The implementation of the technology is the culmination of four years of SAB/THRIP-funded research and concentrated skills transfer.

Project leader **Professor Lodewyk Kock** explains that the new yeast preservation process will be applied in the maintenance of yeasts and in producing starter cultures for the seeding of beer production processes at SAB. "This technological know-how gives our local industry the edge when it comes to consistent processes and quality."

At the recent Brewing Industry International Awards at Burton-on-Trent (known as the "Oscars" of brewing), SAB's flagship brand Castle Lager was adjudged the champion beer in the bottled and canned lager category. The new yeast storage technology was applied in the brewing of the champion beer and could be said to have contributed to this world-class achievement.

**4** "Ongoing workshops have ensured that SAB staff members are fully equipped to take advantage of this technology, while international presentations and publication of part of an MSc thesis on the process, have sparked the interest of global beer producers." — Professor Lodewyk Kock, University of the Orange Free State

# Chapter 1

# Introduction

## 1.1 Motivation

Culture maintenance and preservation techniques have become the centre of broad scientific interest since the 1930's and are applied in many institutions and processes. Maintenance of yeasts originates hundreds of years ago where ancient brewers used a portion of the brewage as common seed for the next brewing. Such pioneer maintenance traditions still exist in South Asian countries. An example is the use of *Aspergillus* spores, which are cultured and sold as brewing material, a Japanese tradition that spans over 300 years. It is also common practise for many years in breweries to maintain yeasts through sub-culturing which are eventually used to produce inocula. This method may however induce mutations through replication errors that may result in an inconsistent brewing process with development of off-flavours in the beer produced. With this as a background, one of the aims of this study became to develop a maintenance procedure whereby a brewing inoculum can be stored for a prolonged time without loss of characteristics and in such a way that it can be transported and handled with ease. Special consideration will also be placed on the exploration of rigorous and efficient microbiological quality control systems pertaining to the inoculum maintainance. Currently the methods employed in most breweries are tedious and sometimes not accurate. Therefore another aim of this study became the evaluation of rapid, inexpensive quality control methods, capable of determining contamination of preserved yeast.

## **1.2 Maintenance of microorganisms**

Preservation of microorganisms for routine or future use is a fundamental requirement in microbiology. However, many times preservation of microorganisms poses a problem, especially when the main purpose is to preserve the homogeneity of a culture. Homogeneity can be ensured under conditions that retain strain viability and prevent loss of strain characteristics (Rhodes & Fletcher, 1966). The need to maintain cultures is important especially to laboratories in teaching institutions which require culture collections for research and educational purposes, while reference strains are required by pathological laboratories for routine testing and research (Kirsop, 1991; Hasegawa, 1996). Similarly a number of industries have to preserve their cultures for use in the manufacturing of their product whether it be beer, wine, antibiotics or milk products (Ghera, 1981). Hereunder an attempt shall be made to give a general view of the various methods used in the preservation of microorganisms.

### **1.2.1 Preservation through serial transfer**

Serial transfer is one of the most common preservation methods for maintaining working cultures. The method involves sub-culturing or transferring cultures from one medium to another using the most suitable growth conditions (Smith & Onions, 1983). Cultures to be stored are usually prepared on agar slants in culture bottles or tubes. Stab or broth cultures are also used particularly for anaerobic cultures. Because of its ease of use, serial transfer is often the first to be used by many microbiologists. The method however poses a major problem since it can induce mutations leading to loss of genetic and phenotypic characteristics with each sub-culturing step (Smith and Kolkowski, 1996). Furthermore, loss of viability and contamination are constant hazards of this method. However despite all of this, in some organisms serial transfer is the only proven preservation technique as is the case with algae (Acreman, 1994).

### 1.2.2 Preservation in distilled water

This is a simple preservation method that involves transferring a culture from the growth medium to a cryovial containing approximately 2ml sterile distilled water (Castellani, 1939). Alternatively, glass vials with rubber caps at room temperature may be used.

This method has been extensively tested on fungal strains. In a study conducted by Hartung de Capriles *et al.* (1989) on the use of this method to preserve 594 fungal strains, it was found that 62% of the strains tested on agar blocks in water, grew well and maintained their original morphology. In another study conducted by McGinnis *et al.* (1974), 389 of 417 fungal cultures or 93% survived storage for four years at room temperatures using this method. It was observed that the fungi that failed to survive were poor sporulators. Figueiredo & Pimentel (1975) successfully maintained viability and pathogenicity in fungal plant pathogens while Ellis (1979) reported further successes on the use of this method on species of *Phytophthora* and *Pythium*. Studies were also conducted on other organisms. The bacterium *Pseudomonas solanicearum* was reported to survive for more than ten years at room temperature (Heckly, 1978), while Tanguay and Bogert (1974) found that both *Saccharomyces cerevisiae* and *Sarcina lutea* survived well when suspended in a dilute phosphate buffer at 4°C for four months but deteriorated drastically after a year storage. The advantage of water storage is that the cultures remain stable and viable. The method is cheap and requires no expensive equipment (Smith and Onions, 1983). It is however important to note that many microorganisms such as *Enterobacteriaceae* and yeasts do not survive suspension in distilled water especially over a prolonged storage period (Malik, 1991a).

### 1.2.3 Preservation under oil

One of the pioneer methods for maintenance of organisms is the use of agar slants covered with oil (Buell & Weston, 1947). Reports on the use of this method indicate that many organisms survive reasonably well and that the

method is applicable across a wide range of different organisms (Dade, 1960; Smith & Onions, 1983). Nardirova and Zemlyakov (1970) reported that *Pseudomonas*, *Bacillus* and *Escherichia* could survive up to 3 years under oil.

#### 1.2.3.1 Protocol for preservation under oil

Mature and healthy cultures to be preserved are grown on agar medium and covered by 1cm of sterilized mineral oil. Cultures are then stored upright at room temperature or lower temperatures of 10-15°C. To revive, a small piece of culture colony is removed using a culture needle or a loop, excess oil is drained off and the cells are then streaked onto agar plates (Buell & Weston, 1947). A wide range of organisms in particular fungi survived this method (Reischer, 1949). The use of oil overlay has been found to prevent evaporation from the culture and to decrease the metabolic activity by limiting supply of oxygen (Monaghan *et al.* 1999). The method is also used to maintain sensitive cultures that do not survive lyophilization (Hasseltine & Haynes, 1974). Cultures remain stable for many years provided they remain covered with oil. The disadvantage of the method is that, although the use of oil overlay has been found to decrease metabolic activity of the culture by limiting the supply of oxygen, mutations during prolonged storage time is possible. It is believed that bacteria can continue to reproduce under these conditions. Perhaps the greatest disadvantage of using oil covered slants is that it is a messy method. Oil may sputter when sterilizing the inoculation needle and in so doing produces aerosols that can be dangerous when handling pathogens. It is noteworthy to consider that this method allows survival of species that do not survive other method of maintenance, it is cheap and does not require expensive or specialised equipment.

#### 1.2.4 Dehydration of cultures

One of the most effective methods for prolonged culture preservation is drying or preservation by induced dormancy. The technique involves removal of water and prevention of rehydration. The method is suitable for microbial structures such as the fungal spores that are capable of surviving very dry conditions (Kirsop, 1991). Since these structures can only be revived under

moist condition, prevention of rehydration therefore prevents changes in metabolic activity of these structures. Usually spores lie dormant in dust, in soil or in many stored products. Dormancy may also be induced by low temperature which also reduces metabolic activity (Smith & Onions, 1983).

#### 1.2.4.1 Soil

For many microorganisms soil is a natural habitat. A preservation method in soil is therefore favoured. Survival of more than one year in soil has been reported for plant pathogens (Naumann & Griesbach, 1993) while in another study, Vela (1974) reported survival of up to 13 years for *Azotobacterium*. Sterile soil has also been used to induce sporulation on both aerobic and anaerobic bacilli (Heckley, 1978). The disadvantage with this method is the difficulty of quantification. Furthermore, since soil is a variable commodity not easily defined, applicability of the method appears not to be suitable for general use with all cultures. The method however is cheap, does not require expensive equipment and is not labour intensive. Furthermore, cultures are unlikely to be infected by mites (Smith & Onions, 1983).

#### 1.2.4.2 Silica gel

The method has been used successfully across a wide range of sporulating fungi. Fungi with thin walled spores or spores with appendages do not survive well (Onions, 1977; Smith, 1983a). Survival often differs from isolate to isolate and often depends on healthy sporulating cultures. A protocol for drying on silica gel involves oven sterilisation of half filled desiccant activated silica gel in screw cap tubes. When the tubes are cooled, a spore suspension of conidia is dispersed into the tubes. These are then quickly cooled to reduce heat generated as the liquid is absorbed and vortexed to break-up the lumps. Tubes are then dried with the caps loose from 10-14 days at 25°C until the silica gel crystals readily separate. After being dried the caps are screwed tight and then stored at 4°C in airtight containers with desiccants to absorb moisture (Monaghan *et al.* 1999). The method has several advantages. It is cheap, simple and requires no expensive apparatus. It produces stable cultures and because of the dry conditions, penetration by mites is less likely to occur. The disadvantage of silica gel storage is that it is

mostly limited to sporulating fungi, survival of other fungi such as mycelial fungi or fungi with delicate or complex spores is poor and so is *Pythium*, *Phytophthora* and other Oomycetes (Smith, 1991).

#### 1.2.4.3 Preservation by drying on filter paper

Another preservation method suitable for spore forming fungi is drying on filter paper. The method has also been successfully used with some yeasts (Kirsop, 1991). Fruiting bodies of myxobacteria containing myxospores can also be preserved on pieces of sterile filter paper (Reichenbach & Dworkin, 1991). The method is also suitable for actinomycetes and unicellular bacteria. The protocol for drying on filter paper involves placing pieces of agar containing fruiting bodies on sterile filter paper, which are then dried in a desiccator under vacuum and stored at room temperature. Another way is to transfer the vegetative cells from the growth medium to the small pieces of sterile filter paper on water agar and incubate these until fruiting bodies develop. The fruiting bodies are then allowed to mature for eight days and then placed in sterile screw cap tubes, which are dried over silica gel in an evacuated desiccator. When dry, the containers are tightly closed and stored (Reichenbach & Dworkin, 1991).

#### 1.2.4.4 Beads

The preservation method on beads, developed by Ledderberg, is one of the most successful for bacteria. Cell suspensions are prepared from 24-48 hrs culture slants in sucrose solutions. The sterile beads are transferred to a sterile petri dish and inoculated with the cell suspension. The beads are returned to the vial with sterile forceps and the vial is loosely capped and dried in a vacuum desiccator for 72-96 hrs. Vials are then stored at 25°C in a closed metal cabinet containing Drierite (Hunt *et al.* 1958; Jones, 1991).

#### 1.2.4.5 Liquid drying

Microorganisms sensitive to the damage that freezing can cause, are generally preserved by liquid drying. The method has been used to preserve specifically large collections of sensitive anaerobic and aerobic microorganisms that fail to survive freezing (Malik, 1991b). Preservation of

unicellular algae using the liquid drying technique was found to be markedly successful compared to lyophilisation (Malik, 1993). The protocol for liquid drying involves preparation of solutions of protective agents e.g. myo-inositol 5% (w/v) along with activated neutral charcoal in distilled water in a screw cap bottle. The cultures to be preserved are suspended in a protective medium to yield a heavy suspension of at-least  $10^8$  cells/ml. The cell suspension is then added to the vials and the vials are subjected to liquid drying by dehydrating the liquid in the cell suspension under vacuum in a metallic jar maintained at 20°C. Initial drying is continued for 1 hr at 10-30 mbar and a second step drying at approximately 0.01 to 0.001 mbar vacuum for 2 hrs while maintaining the temperature at 20°C. At the end, vacuum is replaced with sterile nitrogen or argon gas. The ampoules are then transferred to soft glass tubes and stored under vacuum (Lang & Malik, 1996). The method is rapid, economical and is especially useful for small laboratories since many sensitive microorganisms can be successfully preserved using simple apparatus. The dried cultures in small ampoules are economical for storage and for mailing (Malik, 1991b). Although survival of various anaerobic phototrophic and other sensitive microorganisms after liquid drying is high, many cases were observed where a loss of viability during prolonged storage at high temperatures were encountered.

### **1.2.5 Lyophilization**

Lyophilization, also referred to as freeze drying, is the process of preserving microorganisms by freezing and drying under vacuum from the frozen state through the sublimation of ice. Sublimation occurs when a frozen liquid changes directly to the gaseous state without passing through the liquid phase (Cabasso & Regamy, 1977). Many physiologically diverse organisms have been successfully preserved by this technique. Whereas drying at ambient temperatures from the liquid phase usually results in changes in the product, in freeze drying however the material does not go through the liquid phase thus allowing preparation of a stable product that is easy to use (Meryman, 1966).

At the beginning of the protocol the material to be lyophilized is frozen. Freezing causes separation of the water as ice from the solids. It is important to freeze the product to below the eutectic temperature before beginning the freeze-drying process (<http://www.freezedry.com>). This is vital since most of the samples are eutectics, which are a mixture of substances that freeze at lower temperatures than the surrounding water. Eutectics in biological systems occur as a result of removal of free water by freezing. This removal of water increases the concentration of the solutes remaining in the aqueous phase, thus lowering the freezing point. Formation of eutectics therefore, exposes the cells to high concentration of solutes in the aqueous phase (Morris *et al.* 1988). Furthermore, small amounts of unfrozen material remaining in the product tends to expand and may compromise the structural stability of the freeze dried product. In the second stage of the process, conditions are established that remove ice from the frozen product via sublimation resulting in a dry intact product. To accomplish this requires careful control of two parameters: energy in the form of heat and pressure (Perry, 1995). The difference in vapour pressure of the product to the vapour pressure of the ice collector is the factor that determines the rate of sublimation of ice from a frozen state. Since vapour pressure is related to temperature, it is therefore essential to ensure that for sublimation to occur at the ice interphase there should exist a temperature gradient between the heat source and the interface (<http://www.freezedry.com>). The balancing of the energy input is essential to ensure that the temperature that maintains the frozen integrity of the product and the one that maximises the vapour pressure of the product is controlled to avoid conditions that may tend to contribute to structural breakdown, a phenomenon called collapse (Perry, 1995). After formation of the vapour at the interface, a mechanism must be established that transports it to the condenser surface. The condensing surface typically colder than  $-40^{\circ}\text{C}$  and usually colder than the interface, helps attract the ice vapour (<http://www.freezedry.com>). After the removal of ice from the product, a concentrated solid phase remains that will become, at the end of the process, freeze-dried material. In general, the cultures to be freeze-dried should be viable and depending on the type of organism, late logarithmic phase cultures generally proves suitable for preservation (Smith &

Onions, 1983; Snell, 1991). The suspending medium should be chosen to provide protection during the freezing and subsequent drying process. The most commonly used suspending media include skimmed milk, serum, peptone, various sugars or mixtures of these (Redway & Lapage, 1974; Mackenzie, 1977). The rate of freeze-drying is also an important factor. Optimum results have been recorded with slow rates of freezing at approximately 1°C/min (Heckly, 1978). It may be useful to measure the moisture content of the finished product since over-drying tends to kill the cells and in some cases causes mutations by damaging the DNA (Ashwood-Smith & Grant, 1976). The residual moisture content for survival during storage should be between 1 to 2% (Smith, 1983b). Storage of the freeze-dried product should exclude the presence of both oxygen and water as these can cause rapid deterioration. Greater longevity may be achieved by storage at low temperatures (Heckly, 1978). Rehydration of cultures should be carried out slowly to allow time for absorption of the moisture before plating on a suitable medium. Freeze-drying has an advantage of being suitable for batch production and distribution, and requires undemanding storage requirements (Snell, 1991). The disadvantage however is that some selection of cells may occur during freeze-drying through loss of viability and consequently disqualifies the use of the method for starter cultures. It is important to note that loss of viability may approach 1000 fold with more delicate organisms (Ashwood-Smith & Grant, 1976). Furthermore, freeze-dried cultures are time consuming to open and to resuscitate and several subcultures may be needed before organisms regain their usual morphological and physiological characteristics (Snell, 1991).

### **1.2.6 Freezing**

During freezing, water is made unavailable to microorganisms and consequent dehydrated cells are then stored at low temperatures (Perry, 1995). Methods for freezing can be broadly classified according to the storage temperatures; -20 to -30°C is achievable with standard laboratory freezers, -70°C with ultra low temperature freezers and -140 to -196°C in liquid nitrogen. Generally, temperatures above -20°C give poor results due to

the formation of eutectic mixtures and hence exposure of cells to high salt concentration. Temperatures in the range of  $-60^{\circ}\text{C}$  to  $-70^{\circ}\text{C}$  often result in good viability, however the most preferred storage temperature is at  $-196^{\circ}\text{C}$  in liquid nitrogen. It is believed that at this temperature cellular viability is almost independent of the period of storage and biological systems are believed to be genetically stable (Perry, 1995).

#### 1.2.6.1 Preservation: frozen agar plug

The cultures to be preserved are grown on solid media containing agar. Ten to 15% glycerol is used as a cryoprotectant and 2 ml of this is dispersed in 4 ml capacity vials. Several plugs are cut from the agar medium and deposited into each of the vials, which are then frozen at  $-70^{\circ}\text{C}$ . This type of preservation is most suited for short-term preservation and intermediate term preservation of the *Actinomycetes* and fungi (Hwang, 1968).

#### 1.2.6.2 Preservation in liquid nitrogen

Preservation in liquid nitrogen provides the lowest practical temperature for storing microorganisms. The method is used for maintenance of a wide variety of microorganisms without loss in viability. To preserve the cultures this way, coloured propylene-drinking straws (4 mm diameter) are used as ampoules (Challen & Elliot, 1986). Usually a 10% (v/v) aqueous solution of glycerol or DMSO is used as a cryoprotectant. Some researchers have used other cryoprotectants such as ethanol, methanol, YM broth and hydroxyl starch (Kirsop, 1991; Bond, 1995). The advantages of liquid nitrogen storage are that cultures are maintained in a stable condition over a very long period, viability remains high and the cultures can be completely sealed free from contamination. The disadvantages however are that the apparatus are expensive and that liquid nitrogen requires continuous supply. When this fails, cultures may be lost (Smith & Onions, 1983).

### **1.3 Inoculum development**

Preparation of a microbial culture from its preserved state to a state where it is ready for inoculation to illicit microbial activity of interest is referred to as inoculum development (Hunt & Steiber, 1986). Preparation may vary in scale depending on the purpose of inoculum development. Small inocula may be required for the production of vitamins, while large inocula may be required for the final production stage in the beer fermentation (Hunt & Steiber, 1986). The objective of inoculum development is to produce sufficient biomass in good physiological state in the shortest time possible that is suitable for performance in the final production stage (Voight & Walla, 1995). To achieve this, it is vital that care is taken to ensure that recovery from dormancy minimises loss of viable cells and that a culture is obtained of similar genetical makeup as the population that was stored. Following revival, the process for inoculum development is undertaken by growing microbial cells in a stepwise sequence using increasing volumes of media. In each step approximately 0.5 to 5% of inoculum is transferred from the preceding step in the sequence (Casida, 1968). There should be no product accumulation during the inoculum preparation stage. To ensure this, the incubation period in each step should be short, yet sufficient to obtain biomass that is adequate in terms of quantity and quality. Cells should also be transferred to bigger volumes of media in their logarithmic growth phase in order to avoid product formation.

#### **1.3.1 Media**

The choice of medium for inoculum preparation is important for the recovery of the organism and for the synthesis of the product (Underkofler & Hickey, 1956). The growth medium is a source of energy and is also essential for the synthesis of the cell components. To establish nutritional requirements that will permit inoculum growth, a series of media may be screened for their effect on cell biomass. Alternatively, the medium composition may be obtained from literature. Bioassay procedures may also be used to determine the exact concentrations of the growth factors needed (Atlas, 1984). Once established,

the ideal medium is optimised and tested for product formation on large-scale production (Song *et al.* 1987). In most growth media the important constituents required by most microorganisms are carbon and nitrogen sources. Other components may be added to enhance productivity and these include vitamins, inorganic salts, buffers, dissolved oxygen, etc. (Atlas, 1984). The composition of the medium may be simple or complex depending on the nutrient requirements of the particular organism (Casida, 1968).

### **1.3.2 Sterilisation**

The choice of sterilisation is an important factor that may alter the quality of the medium if a wrong choice is made. There are various ways of sterilisation and these include exposure to elevated temperatures, radiation and filtration (Prescott *et al.* 1990). The most common method involves the use of an autoclave for sterilisation which permits exposure to high temperatures (Kent *et al.* 1990). Both the intensity and duration of heat application are critical factors for some nutrients. Care should always be exercised when deciding on the heat sterilisation program on a particular type of a medium. Synthetic media, for example, require a relatively shorter sterilisation program, while complex or crude media may require a longer sterilisation program due to the viscosity of the media (Lee, 1951). Some nutrients are not amenable to heat sterilisation and may be destroyed by high temperatures. Others are volatile and may be lost from the medium by heat sterilisation. In some instances heat sterilisation causes interaction between phosphates, amino acids, sugars and ammonia creating an environment which can be growth inhibiting. In such cases, sterilisation by filtration or by other means may be required (Meyrath & Suchanek, 1972).

### **1.3.3 Inoculum source**

Cultures from preserved stocks may be reactivated by transferring directly to liquid medium or by growing on solid medium, depending on the type of storage the culture was initially placed under (Parton & Willis, 1990). From the reactivation medium, cultures are usually sub-cultured to provide a set of

working cultures. If the inoculum is to be used for small-scale production, a first generation culture may be used. It is important to note that cultures to be re-activated from storage, especially the spores, may present special problems as regards to the initiation of the inoculum build-up. Both vegetative cells and spores are handled differently before transferring to an inoculation medium (Casida, 1968). Spores of *Clostridium* require heat treatment to allow germination of a high percentage of spores. In contrast, spores of the *Actinomycetes* or fungi are heat sensitive and are prepared by adding a diluent such as sterile water, to sporulated agar growth (Casida, 1968; Monaghan *et al.* 1999). With some organisms, however, spores do not become wetted by water and tend to remain floating on the liquid surface. In such a case, a special wetting agent such as sodium lauryl sulphate is added in small quantities to the dilution medium (Rose, 1961).

#### 1.3.4 Acclimatisation

Some microbial processes are different from others in that the inoculum used is recovered from the previous production phase. Here, a fresh inoculum is usually required when the quality of the inoculum has deteriorated or when it is contaminated. The re-use of the inoculum provides advantages such as a decreased lag period and sufficient biomass build-up since the inoculum is already acclimatised to the conditions of the production process (Monaghan *et al.* 1999). During the re-use of inocula in the brewing industry, yeast cells from previous fermentations are washed with phosphoric acid, tartaric acid or ammonium persulphate that reduces the pH and removes bacterial contamination if present. Following this, cells are re-employed for fermentation. Van Ginkel *et al.* (1995) reported on an improved operation of a sewerage sludge plant with high degradation rates and low retention rates when acclimatised inocula were used. The same concept was used to improve operational efficiency for xylitol production by *Candida guilliermondii* grown on hydrolysates of sugar cane (Filepe *et al.* 1996).

### **1.3.5 Immobilisation**

In some microbial processes, the method of producing a product employs microorganisms adsorbed onto solid surfaces such as agarose, alginate or fibre matrix (Pirt, 1975). Media containing chemicals are then passed through the adsorbed organisms which in-turn secretes the product into the surrounding medium. All environmental conditions including temperature, pH and oxygen concentration are set at optimum to ensure maximum product formation. This technique provides the inoculum with unique characteristics such as UV resistance, enhanced inoculum activity, protection from stress during manufacture and increased metabolite production (Monaghan *et al.* 1999). An immobilised inoculum also makes an industrial process more economical by avoiding the expense of continuously growing microorganisms and discarding unwanted biomass. Mixed culture inocula may also be used (McLoughlin, 1994).

### **1.3.6 Process scale-up**

The experimental conditions as determined in flasks in a laboratory, are employed on a more grand scale for the operation of a large production. Thus, determination of incubation conditions to be employed with large-scale production based on information obtained with various smaller experimental production is known as scale-up (Oosterhuis & Kossen, 1981). To scale up the process, the results obtained at experimental or small-scale level must be reliable and reproducible. In practice effective process scale up involves coordinated use of production and pilot plants in a series of systemic scale-up experiments. Operating conditions in the pilot process are adjusted until the process behaves the same way as the production process (Aiba *et al.* 1973; Parton & Willis, 1990).

## 1.4 Quality management

### 1.4.1 Maintenance of properties

It is essential that in any industrial microbiological process, the culture to be used for inoculation is kept in a healthy active state, in a suitable morphological form, free of contamination in order to retain its original product forming capabilities (Meyrath & Suchanek, 1972). An important factor to consider for obtaining an inoculum fulfilling these criteria, is the choice of the culture conditions such as growth medium, pH, oxygen and temperature (Granade *et al.* 1985). Among these, the choice of a suitable growth medium is the most important criterion that eventually determines the subsequent performance of the inoculum in the production stage. This is because the inoculum medium is compounded to quickly yield large numbers of microbial cells in their proper physiological and morphological states without sacrificing genetic stability of the cells. Studies on the synthesis of macromolecules in cells have indicated that a change in growth medium results in a change in the regulated response which determines the final growth rate and physiological state of the culture in the medium (Bull & Trinci, 1977). Such a change occurs since some molecules, like enzymes, are produced only in response to the presence of a substrate that an organism is capable of metabolising for energy and growth. If such a substrate is omitted from the medium or become exhausted, the microorganism in succeeding generations will decrease its output of this adaptive molecule and growth will cease.

Maintenance of properties refers to those properties an inoculum possesses that correlates with its performance (Calam, 1969). These properties include among others strain stability, viability, respiration, resistance to phage attack and morphological forms (Granade *et al.* 1985).

### 1.4.2 Genetic stability

Even though a culture may produce a very high level of a desired product, it would be unsuitable, if the production of the product is unstable. It is therefore important to select a stable and genetically uniform culture that produces large amounts of the desired product and a minimum of unwanted metabolites (Elander & Chang, 1979). The ability of a culture to maintain a high productivity while remaining stable is an important quality since the degeneration of culture stability in most cases results in a decrease in productivity. Degeneration of culture stability is normally due to the occurrence of spontaneous revertants, which often have a higher growth rate than the parent strain. This problem of reversion can be serious if the inoculum is a mutant strain. Mutations are not always stable and the frequency of back mutation or loss of a particular mutation can be high (Bu'Lock, 1979; Hasegawa, 1996). The frequency of back mutations may however be controlled by applying selective procedures to increase the inoculum stability. For example, a mutant strain requiring L-Lysine for growth should not be allowed to exhaust the supply of this amino acid during production (Nakayama, 1972). Should this occur, the mutant would not be able to compete with the non-lysine requiring back mutants which will eventually become dominant in the inoculum. Stability may also be controlled by the introduction of more than one mutation giving the same phenotype. Generally however, factors affecting the stability of a strain should be determined and steps taken to minimise them. Such factors include medium composition, incubation temperature and the shock of transfer of inocula from one stage to the next (Stanbury & Whitaker, 1984).

### 1.4.3 Viability

The viable count of microbial populations is the absolute concentration of viable organisms present, while viability is the ratio of viable counts to the total concentration of microbes, dead or alive (Brock *et al.* 1994). Generally an organism is viable only if it is capable of multiplying to form progenies under optimal conditions. In industrial microbiology the viability of an inoculum is a

critical factor that has a direct bearing on the manufacturing of the product. This is because only viable cells are capable of optimum and vigorous growth under required conditions. Also, only viable cells can utilise the substrate in the medium for maximal output of the product and give consistent results (Postgate, 1969).

#### *1.4.3.1 Determination of viable numbers*

Microbial populations to be examined are diluted in a non-toxic diluent, usually the growth medium. Care should be taken to avoid cold shock or dilution shock of susceptible organisms. Also, growth should not take place while the dilution is being made. A dilution series is then prepared and aliquots of the diluted suspension spread over replicate agar plates for aerobes or mixed with molten agar and allowed to set for oxygen sensitive microorganisms. After incubation, the colonies are counted and the number of viable cells or colonies deduced from the colony count and dilution (Belt, 1996).

#### *1.4.3.2 Determination of viability*

Procedures for assessing viability include staining methods, assessment of dehydrogenase activity (Steponkus & Lanphear, 1967), measurements of dye up-take (Vasil, 1984), immersion refractometry (Sowa & Towill, 1991), observing leakage of purines and selective staining of fluctuating RNA (Postgate, 1969). Furthermore, estimates of viability can be obtained by observing the proportion of a population capable to grow and multiply when incubated under suitable conditions.

### **1.4.4 Contamination**

Growth of contaminating organisms is an ever-present risk in inoculum preparation. The presence of contaminating organisms may change the chemical nature of the nutrients in the inoculum medium. They may also cause changes in pH and produce metabolic products that may inhibit or slow the growth rate of the inoculum. Furthermore, the presence of contaminating organisms may alter the oxidation-reduction potential of the medium and racemize or even destroy the product. Consequently, efforts must be made to

monitor culture purity through every step of inoculum development (Casida, 1968).

#### *1.4.4.1 Phage contamination*

Phage contamination is a constant threat in industrial processes. Phages are viruses that attack bacteria and not fungi or yeasts. Fermentation of dairy products is especially susceptible to phage contamination. Phages are not capable of replication and require a host to survive. Because of their small size, phages are difficult to detect and count (Mathews & Van Holde, 1990; Schlegel, 1992). Phages are usually detected by the use of phage plaque plating procedures (Mathews & Van Holde, 1990). The impact of phage attack on industrial production processes depends on the nature of infection of the phage. Phage contamination is not a problem if the phage do not lyse the organism. However if the contaminating phage infects a rapidly growing sensitive host, it may lyse and kill many of these organisms in a relatively short time. This will drastically decrease the production rate. The surviving cells, which are naturally resistant to this phage, will slowly multiply and eventually repopulate the inoculum and restore the production rate. (Hongo *et al.* 1972). A possible method for reducing phage contamination is to select a culture from the production site that is resistant to the phage. However, a culture resistant to a particular phage is still subject to the possibility of infection by new contaminating phages. Plant hygiene remains the most essential to minimise risk of phage contamination (Bader, 1986).

#### *1.4.4.2 Contamination by mites*

Culture infestation by mites is one of the most frequent causes of contamination. Mites may consume the culture thereby killing all cells. They can also introduce other microorganisms which are carried on their bodies thereby causing severe cross-contamination. This may eventually overgrow the whole culture inoculum (Smith & Onions, 1983). Mites are usually detected as small light-coloured objects producing eggs of similar size. Cultures growing on agar plates and suspected of infection should be observed for random-cross walk patterns across the plate with bacterial and fungal tracks forming (Smith & Kolkowski, 1996). Mite infected cultures can

be freed from contamination by subculturing two or three times on an agar medium containing Kelthane (1.1-Bischlorophenyl-trichloroethanol) which is toxic to mites (Rhodes & Fletcher, 1966; Smith, 1984).

#### **1.4.5 Verification of purity**

##### *1.4.5.1 Conventional microbiological methods*

The purity of the production culture is usually checked microscopically for the presence of contaminants. Occasionally serial dilutions on nutrient agar will demonstrate morphological variants in the culture (Dietz & Churchill, 1985). Purity may also be verified using the membrane filter method, whereby a known volume of the fluid is passed through a porous membrane filter which retains the microorganisms present. The membrane is then allowed to absorb the culture and is incubated to enable the colonies to develop (Collins & Lyne, 1976). The choice of the medium and conditions for filter incubation, are determined to a large extent by the amount of information required. A non-specific medium capable of supporting the growth of most microorganisms is adequate in most cases. However, if the aim is to detect a specific organism, then a selective growth medium should be employed. Generally, cultures which are to be used in the manufacturing plant for metabolite production, are tested in shake flasks for authenticity of the expected productivity (Collins & Lyne, 1976).

##### *1.4.5.2 DNA fingerprinting*

DNA fingerprinting techniques are based on the analysis of DNA fragments obtained by treatment with restriction endonuclease. The resulting fragments are electrophoretically separated and transferred from the gel by southern blotting and detected by labelled probes or non-radioactive methods. Probes can be prepared against genes encoding tRNA, various enzymes, transposable elements and other metabolic functions (Deak & Beuchat, 1996). Restriction enzymes generate numerous fragment lengths of variable sizes resulting in characteristic banding patterns. DNA fingerprinting techniques have been used extensively to discriminate between closely

related yeast strains and consequently it has been applied as a reproducible and efficient quality control tool (Lavallee *et al.* 1994).

#### *1.4.5.3 Lipid and sterol analysis*

Lipid analysis, particularly fatty acids and sterols, can be used reliably for characterisation of microorganisms and has become a promising technique for industrial application. Cellular fatty acids have been used for characterisation of brewery (Oosthuizen *et al.* 1987) and wine yeast (Tredoux *et al.* 1987; Augustyn & Kock, 1989), while sterol analysis, particularly ergosterol, has been used as an important indicator of brewery yeast expected performance and vitality. Fatty acids can be measured in their esterified and free form using gas chromatography while ergosterol and other sterols can be quantified using high pressure liquid chromatography (HPLC).

### **1.5 Growth rate measurement techniques**

The objective of growth rate measurement is to develop a reliable and reproducible indicator of inoculum performance during production. Growth measurement is essential to the manufacturing of the product as an on-line application to effectively monitor growth and perhaps to measure inoculum quality and transfer timing (Dietz & Churchill, 1985).

#### **1.5.1 ATP measurements**

Measurement of ATP depends upon reacting ATP extracts from the cells, with luciferase enzyme from the firefly to form a bioluminescent complex, which, upon oxidation, yields an amount of light proportional to the ATP present. Measurement of the cellular ATP level has however not been widely used as an indicator of cell growth because of variations in ATP concentrations in cells, depending upon carbon source, growth conditions, strain of the cell and growth phase (Prior *et al.* 1988). Cellular ATP levels may however be utilised for inoculum development purposes as a marker of cell status and perhaps as

a measure of the cell capacity to support energy requiring biosynthesis process (Monaghan *et al.* 1999).

### **1.5.2 Calorimetry**

Microbial growth is usually accompanied by the release of metabolic heat into the medium. This evolution of heat depends on the type and utilisation efficiency of the carbon source. It correlates well with oxygen uptake rate and carbon dioxide production rate of the culture. Together with culture fluorescence, microcalorimetry can provide suitable information to monitor growth and serve as a valuable measure of inoculum quality and transfer timing (Cooney *et al.* 1969; Luong & Voleskey, 1983; Beaubien & Jolicoeur, 1985; Andlid-Larsson *et al.* 1995).

### **1.5.3 Capacitance**

Capacitance measured over a frequency range from 100 KHZ to 1 MHZ may be used to measure microbial cell concentrations in a bioreactor (Mashima *et al.* 1991a). Such a value shows linearity between the capacitance value and living cell concentrations of microorganisms as well as of animals and plants. The relation between biomass and capacitance may lead to the use of the latter as an interesting on-line measurement of monitoring inoculum development and timing of seed transfer (Mashima *et al.* 1991b).

### **1.5.4 Viscosity**

The viscosity of the liquid phase reflects complex functions or influence of the biomass present in the medium (Richards *et al.* 1978). Morphology of the cells plays an important role in determining viscosity. The viscosity is measured by determining the pressure drop for a known flow through capillary tubes from which a value may be calculated using the Poiseville equation (Onken & Buchhotz, 1982). Viscosity has been used to monitor growth in antibiotic and polysaccharide fermentation processes (Onken & Buchhotz, 1982).

### 1.5.5 Fluorescence

Measurement of NADH as a component of the metabolic machinery of viable cells has been shown to correlate with the biomass present and may also be used to estimate cell growth (Zabriskie & Humphrey, 1978a). Fluorescence involves the absorption of light at a given energy, followed by emission of that light at lower energy or larger wavelength (Mathews & Van Holde, 1990). The technique for NADH dependent culture fluorescence measurement is therefore based upon the observation that microbial cultures irradiated with UV at 366 nm fluoresce at 460 nm because of the NADH present (Beyeler *et al.* 1981). In some cases however, measurement of NADH culture fluorescence may be ineffective as an estimator of cell growth because of the physiology of the organism or the presence of fluorophores such as chlorophylls and antibiotics, which can distort the results. Nonetheless, depending upon the culture, the NADH measurement technique under standard conditions, permits on-line *in situ* determination within bioreactors. This measurement may provide information relevant to optimum inoculum and optimum transfer time (Monaghan *et al.* 1999).

### 1.5.6 Gas composition

This type of measurement, concerns almost exclusively oxygen and carbon dioxide. Oxygen may be measured by using a mass spectrometer and the rate of its uptake can provide an estimate of the growth rate of microorganisms. Similarly, carbon dioxide may be measured using a mass spectrometer and the rate of its production can also be used to measure growth (Boyles, 1977; Cooney, 1982). The ratio between the two measurements, the respiratory quotient (RQ) is used to determine the characteristic metabolic condition of a cell. An RQ of 1 indicates glucose supported metabolism while a change to an RQ of 0.7 indicates oil-supported metabolism for *Nocardia ladamdurans* during production of efrotomycin (Buckland *et al.* 1985). In *Saccharomyces cerevisiae*, a change in RQ has been shown to accurately simulates diauxic aerobic growth on glucose

(Zabriskie & Humphrey, 1978b), while in *Streptomyces cattleya* change in RQ is associated with the production of multiple products (Bushell & Fryday, 1983).

### **1.5.7 Optical density**

Optical density of microbial suspensions exhibits a very close correlation with the amount of biomass present. Because of its simplicity combined with speed and convenience, the optical density measurement technique or turbidimetry is widely used to determine growth and can be a reproducible indicator for inoculum timing and quality (Mellet, 1971). Instrumentation for optical density varies in complexity. Klett-Sommerson units are designed to measure optical densities directly in shake flask side arms. Most often optical density is measured at 600nm. The other method to measure optical density involves the use of spectrophotometers designed to work with special cuvettes at a fixed or adjustable wavelength (Koch, 1981). The wavelength used is usually around 420 or 660 nm. Continuous on-line turbidimetric systems have also been devised for use in fermentation processes (Lee, 1981; Metz, 1981).

### **1.5.8 Specific growth rate**

The aim of specific growth rate measurement is to establish the biomass at successive times at maximal growth conditions. Such measurement can be used as a key factor for the timing of inoculum transfer into the next stage of growth or into a production medium. The specific growth rate can be estimated from oxygen uptake rate or carbon dioxide evolution rate. However, measurements of these values require expensive equipment or equipment that requires frequent calibration (Monaghan *et al.* 1999). Specific growth rate may also be deduced by determining a slope from a straight line plot of natural logarithmic of biomass concentration against time in an optimum environment with excess nutrient supply (Stanbury & Whitaker, 1984).

### **1.5.9 DNA**

DNA, unlike protein, is found extensively in cells and its determination may be used to indicate biomass even in complex medium containing proteins. Although the ratio of DNA to dry weight shows some variations during growth, fluctuations however are much less than that for other macromolecular components such as carbohydrates, lipids and RNA (Dean & Hinshelwood, 1966). Measurement techniques for DNA include colorimetric measurement of the diphenylamine reaction of deoxyribose residues in a perchloric acid extract of the cells and a more sensitive fluorometric method for determining deoxyribose using diaminobenzoic acid which may be applied to whole cells (Hinegardener, 1971). Recent techniques however employ fluorescent dyes such as ethidium bromide and propidium iodide which intercalate with the double stranded nucleic acids to yield highly fluorescent, conjugated forms (Dean & Hinshelwood, 1966; Lepecqs & Paleotti, 1987).

### **1.6 Statistical quality control**

Statistical quality control is an approach used to maintain and improve quality and hence improvement of productivity. Primarily, statistical quality control, emphasises the constant use of data and control charts to monitor the production process and make timely modifications to process variables to improve or maintain quality and increase productivity (Badavas, 1993). Ideally, in a microbiological industrial process the product produced should be exactly the same from one production batch to another. However some variability is unavoidable. The amount of variability depends on various elements of the production process such as inoculum storage and recovery methods and materials such as medium, pH, temperature etc. The variability of the process can be determined utilising different control charts that are specified for different quality characteristics (Grant & Leavenworth, 1988).

## **1.7 Maintenance of yeasts in the brewing industry**

The South African Breweries have historically used sub-culturing as the basis for culture storage and from which inocula were prepared to be used in the brewing process. However it was found that the yeast recovered from this method of preservation exhibited poor viability, increased number of mutants and as such, resulted in poor fermentation performance (Hulse *et al.* 1999).

Of importance is also the type of microbiological quality control methods employed to evaluate the yeast in the brewing process. These usually include the use of Wallerstein Laboratory Nutrient Medium (WLN) for estimation of mutants and bacteria, Wort Agar (WA) overlaid with Triphenyl-Tetrazolium-Chloride (TTC) for estimation of respiratory deficient (RDs) yeasts, detection of the wild yeasts using the Swartz-Differential Medium (SDM), estimation of the non-*Saccharomyces* species using Lysine-Medium (LYS), detection of the lactose assimilating and lactose fermenting microorganisms using the Lactose-Peptone-Broth (LP) and detection of brewery bacteria using the Universal Liquid Medium (ULM) (Analysis Committee of the Institute of Brewing, 1997). Generally these methods are time consuming and sometimes not accurate. Furthermore some of these methods such as the WA-beer based media are variable in quality and are not optimised for growth of microorganisms (Lewis & Young, 1996). Addition of inhibitory agents such as actidione in ULM may affect the recovery of the desired microbe and so result in an underestimation of the level of contamination with dire consequences when carried over to the fermentation process (Lewis & Young, 1996).

## **1.8 Purpose of study**

With this as background the aims of this study became:

- 1) To develop a viable yeast maintenance programme over a prolonged period of time for implementation by the brewing industry in initiating fermentation.

- 2) To develop relevant alternative quality control techniques in order to determine viability and stability of the yeast after preservation and during inoculum preparation.

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## Chapter 2

## **Preface**

This research is in the process of application at the factories of the South African Breweries. Furthermore, this study has contributed in the brewing of the champion beer "Castle Lager" that was awarded first prize at the International Championship held at Burton-on-Trent in April 2000.

# Comparison of Preservation Methods in the Maintenance of Brewing Yeast

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*(Style according to South African Journal of Science)*

## Abstract

Preservation methods (cryopreservation at  $-196^{\circ}\text{C}$  in liquid nitrogen, freezing at  $-70^{\circ}\text{C}$  and lyophilization) were compared in the maintenance of brewing inocula over a period of two years. Interestingly, a decrease in the percentage of variants and respiratory deficient yeasts (RDs) was generally found when preserved through cryopreservation ( $-196^{\circ}\text{C}$ ) and freezing ( $-70^{\circ}\text{C}$ ). In contrast, the percentage variants when revived yeasts were grown in wort, increased when maintained through lyophilization. A high percentage viable cells ( $>95\%$ ) was recorded for yeast cultures maintained at  $-196^{\circ}\text{C}$  and at  $-70^{\circ}\text{C}$  while viability was low ( $<50\%$ ) when maintained through lyophilization. Consequently, the maintenance methods of choice are cryopreservation and freezing. The developed cryopreservation method was successfully implemented in the brewing process that produced the champion lager beer "Castle Lager" at Burton-upon-Trent in the UK (April, 2000).

Key words: *Brewing yeast, mutants, preservation, viability*

## Introduction

In beer fermentation, the yeast strain used is responsible for the production of ethanol, carbon dioxide and a range of metabolic products which contribute to the flavour and drinkability of the finished product.<sup>1</sup> Many brewers therefore use yeast that has been carefully selected to suit their particular needs. In order to preserve these characteristics, it is important to the brewer to use effective yeast maintenance procedures in order to ensure that the yeast of choice remains stable and viable.

Several methods are available to preserve microorganisms and include drying, lyophilization, cryopreservation and sub-culturing.<sup>2,3</sup> Many of these methods<sup>4,5</sup> however, may lead to population change through selection, loss of viability as well as poor survival rates.

A major South African brewery has used sub-culturing for many years as the basis for culture preservation.<sup>6</sup> However, it was found that the yeast recovered from this method showed considerable variation and was inconsistent in terms of biomass produced and physiological conditions. This in-turn influenced fermentation consistency and performance.

In order to rectify this problem, the preservation of yeasts in liquid nitrogen at  $-196^{\circ}\text{C}$ , freezing at  $-70^{\circ}\text{C}$  and lyophilization was investigated in this study with the purpose of producing more reproducible, stable and viable yeast inocula to be used in the brewing process.

## Materials and Methods

### *Maintenance at -196°C in liquid nitrogen*

A part of this study i.e. cryopreservation is an extension of the work of Morakile *et al.*<sup>7</sup>

### *Yeast used*

A *Saccharomyces cerevisiae* strain used by The South African Breweries in beer production was used in the study.

### *Preparation of primary inoculum for the brewing process*

This was performed as reported by Morakile *et al.*<sup>7</sup> The brewing strain was cultivated on YM agar slants at 21°C for 3 days. The cells were resuspended in 9 ml saline solution, after which 1 ml was inoculated in 15 ml wort contained in a McCartney bottle. These were then grown at 25°C for 24 h. This was followed by decanting the wort culture aseptically into a round bottom flask containing 200 ml wort and cultivating further for 72 h at 20°C while shaking at 100 rpm until stationary phase was reached. These cells served as primary inoculum in the preparation of a 5 l inoculum to be used in the brewing process.

### *Cultivation and ampoule preparation*

One millilitre of the saline solution containing the culture as described above, was inoculated into 500 ml conical side-arm flasks (each containing 50 ml YM broth) and incubated at 25°C while shaking at 160 rpm. The cells were grown until logarithmic growth phase. Following this, 7.5 ml of sterile 70% (w/w) glycerol was added to each flask as cryoprotectant and thoroughly mixed. Of this mixture, 1.15 ml was pipetted aseptically into 1.8 ml ampoules (Sigma Nalgene Cryogenic Vials, Sigma, South Africa) with the final concentration of glycerol in ampoules being 9.1%. The ampoules were individually sealed with NuncCryoFlex (Nunc, South Africa) and inserted into aluminium cane ladders

(Nunc, South Africa) which were covered with cane sleeves (Sigma PVC Cryosleeve, Sigma, South Africa). These were cooled at  $-70^{\circ}\text{C}$  for two hours in order to allow sufficient dehydration of the cells. The ladders with ampoules were then immediately immersed in liquid nitrogen at  $-196^{\circ}\text{C}$ . Three ampoules per flask were revived after 30 h, 17 days, 87 days 136 days, 365 days and 728 days by immersing each ampoule in water at  $35^{\circ}\text{C}$  until completely thawed. These were then analyzed as described below.

#### *Determination of viability, stability and contamination*

The saline yeast suspension prepared from the YM slant as well as samples from the wort cultures (i.e. after 72 h) present in round bottom flasks, were analysed according to brewery protocol.<sup>8</sup> This include quantification of the following: (1) variants through the Wallerstein Nutrient Medium (WLN)- a protocol for detecting mutants and bacteria, (2) quantifying respiratory deficient (RDs) yeasts using the Wort-Agar-with-Triphenyl-Tetrazonium-Chloride-Overlay method, (3) quantifying wild yeasts with the Schwartz-Differential-Medium (SDM) protocol, (4) quantifying the non-*Saccharomyces* species with the Lysine-Medium (LYS) method, (5) quantifying lactose fermenting and lactose assimilating microorganisms by using the Lactose-Peptone-Broth (LP) method and (6) quantifying brewery bacteria by using the Universal-Liquid-Medium (ULM) protocol. In addition, total cell counts were obtained in all cases by haemocytometer reading and viable cell count by methylene blue staining.<sup>8</sup>

This procedure was also repeated on ampoules at each revival stage. One ampoule per flask was directly analysed as above while the other two ampoules per flask were first grown in wort to stationary phase as previously described.

#### *Estimation of the variance components*

A  $6 \times 3 \times 3 \times 2$  nested design (Fig. 1) (i.e. 6 different liquid nitrogen storage times [St]; 3 separate cultivations, [Cult]; 3 ampoules per cultivation flask [Cryo]; 2 analysis per ampoule [Ana]) was performed in order to determine the sources

of variation in yeast viability, stability and contamination after preservation in liquid nitrogen.<sup>9</sup> Before cryopreservation was conducted, the yeast culture was cultivated in triplicate flasks (i.e. culturing phase = Cult) after which 30 ampoules were prepared from each flask as described (i.e. cryopreservation phase = Cryo). Three ampoules per flask were then revived (still cryopreservation phase = Cryo) after six different storage times (St) i.e. 30 h, 17 days, 87 days, 136 days, 365 days and 728 days and processed as described. Therefore, a total of nine ampoules were revived per culture phase (Cult) at a given revival period which amounts to a total of  $6 \times 9 = 54$  ampoules over the six revival periods. Consequently, the influence of the variance components (i.e. cryopreservation, culturing and storage time) on the variation in yeast viability, stability and contamination was determined.<sup>9</sup> As far as possible, the influence of the analytical techniques (Ana) on the process variation was also determined. In order to achieve this, a particular analytical technique was performed in duplicate for each sample tested.

#### ***Maintenance at $-70^{\circ}\text{C}$ in a freezer***

A similar experimental design and yeast strain were used as described for cryopreservation. However, the ampoules containing the yeast suspension with glycerol were preserved in a freezer at  $-70^{\circ}\text{C}$  and were revived at different intervals i.e. 30 h, 17 days, 180 days, 360 days and 728 days and analysed as described above.

#### ***Maintenance through lyophilization***

The above experimental design was again followed with the same yeast. In this case 20% skimmed milk and 10% sodium glutamate were used as lyoprotectant.<sup>10</sup> The yeasts in ampoules were cooled at  $-70^{\circ}\text{C}$  for 12 h and then lyophilized in a freeze drier (FTS systems, USA). Ampoules under vacuum were stored at room temperature and revived at different time intervals i.e. 30 h, 22 days, 99 days, 226 days, 401 days and 728 days.

## Results and Discussion

### *Maintenance at -196°C in liquid nitrogen*

A decrease in the percentage variants and RDs was experienced in the revived ampoules of the yeast maintained through cryopreservation for two years (Fig. 2). The variants and RDs determined before cryopreservation were recorded at 8.3% and 3.1% respectively. They however decreased to 4.7% and 1.3% after 30 h of cryopreservation and further decreased to 4% to 0.5% respectively after two years of cryopreservation. In all cases a high cell viability (>95%) was recorded in the revived ampoules. A similar trend was experienced when the yeasts from the agar slants and the yeasts subjected to cryopreservation were cultivated in wort for 72 h (data not shown).

Four main process components in the development of the ampoules for cryopreservation i.e. storage time in liquid nitrogen, culturing in YM broth, cryopreservation and the analytical tests were selected and statistically evaluated for their contribution towards process variation.<sup>9</sup>

The results obtained for variants and RDs indicate that the largest source of variation was the error arising from the analytical tests (Table 1a). Cryopreservation also influenced variation in the number of RDs, though to a lesser extent. Considering  $\mu_{\max}$ , the largest source of variation was the error arising from cryopreservation protocol. The analytical test, measuring cell concentration, might have contributed significantly to this value, however this could not be determined as the test has not been separated from cryopreservation protocol. Culturing in YM broth also influenced variation in  $\mu_{\max}$  though to a lesser extent. Interestingly, storage time did not have an effect on either the variants, RDs or  $\mu_{\max}$ .

### ***Maintenance at -70°C in a freezer***

There was a significant decrease in the percentage variants and RDs observed in the revived ampoules of the yeast maintained through freezing at -70°C (Fig. 3). Before freezing, the variants and RDs were recorded at 5.2% and 1.5% respectively. These decreased to 2.5% and 0.8% after 30 h of freezing and then further decreased to 1.0% and 0.8% after 728 days respectively. Similar to cryopreservation, a high cell viability (>95%) was recorded for all ampoules tested at -70°C. The variants and RDs also decreased when the yeasts from the agar slant and the yeast subjected to freezing were cultivated in wort for 72 h (data not shown).

The results from the estimate of the components of variance showed that the largest source of variation in both the variants and RD results was the error arising from the analytical test (Table 1b). When considering  $\mu_{\max}$ , the source of variation was the error arising from freezing protocol and culturing in YM broth. Storage time did not have any influence on the variants, RDs or  $\mu_{\max}$ .

### ***Maintenance through lyophilization***

Similar to the other two methods, there was a decrease in the percentage of the variants and RDs in the revived ampoules maintained through lyophilization (Fig. 4). Before lyophilization, the variants and RDs were recorded at 3% and 1.7% respectively. They decreased to 0.3% and 0.7% respectively after 30 h of lyophilization and remained more or less constant on subsequent revivals. Interestingly, viability was found to be low (<50%) upon all revivals. When the yeast from the agar slant and the yeasts subjected to lyophilization were cultivated in wort (round bottom flasks), the variants decreased from 3.5% before lyophilization to 1.2% after 99 days (Fig. 5). Subsequent revivals, however, showed a steady increase in these mutants up to 728 days of preservation (Fig. 5). Under these conditions, the RDs decreased drastically after 30 h of lyophilization (i.e. 3.5% to 0.1%) and then remained more or less stable.

Consistent with the other two methods, the largest source of variation for both the variants and RDs was the error arising from the analytical test (Table 1c). However, the lyophilization process as well as culturing in YM broth also influenced the variation in the number of variants, RDs and  $\mu_{\max}$ . Storage time did not have an effect on the variants but influenced variation in the number of RDs as well as  $\mu_{\max}$ .

In conclusion, both cryopreservation methods at  $-196^{\circ}\text{C}$  in liquid nitrogen and at  $-70^{\circ}\text{C}$  in a freezer proved successful for the maintenance of the brewing inocula. The mutants of the brewing yeast (i.e. variants and RDs) decreased when maintained through these methods while the viability of the cells remained high. This may be attributed to a higher sensitivity of these mutants towards lower temperatures. The results of the estimate of the components of variance showed that the largest source of variation in all three methods tested, was the error arising from the analytical test. Studies should therefore be conducted to develop alternative and more accurate analytical methods.

Maintenance through cryopreservation was successfully implemented by The South African Breweries. This preservation method contributed to the crowning of "Castle Lager", the flagship of The South African Breweries, as champion beer in the Lager Division at Burton-upon-Trent, UK. (2000). Other international breweries have also shown interest in applying this technology.

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## List of figures

- Fig. 1 A  $6 \times 3 \times 3 \times 2$  hierarchical (nested) design. **St**, storage time; 1, 30h; 2, 17 days; 3, 87 days; 4, 136 days; 5, 365 days; 6, 728 days; **Cult**, culturing in YM broth; **Cryo**, cryopreservation; **Ana**, analytical tests
- Fig. 2 A comparison between the percentage variants and RDs present in culture stored on YM agar slant (before cryopreservation) and in cryopreserved ampoules at  $-196^{\circ}\text{C}$  in liquid nitrogen. The percentage variants and RDs was determined at different storage times directly upon revival of the ampoules.
- Fig. 3 A comparison between the percentage variants and RDs present in culture stored on YM agar slant (before freezing) and in ampoules frozen at  $-70^{\circ}\text{C}$  in a freezer. The percentage variants and RDs was determined at different storage times directly upon revival of ampoules.
- Fig. 4 A comparison between the percentage variants and RDs present in culture stored on YM agar slant (before lyophilization) and in ampoules maintained through lyophilization. The percentage variants and RDs was determined at different storage times directly upon revival of ampoules.

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Fig. 5

A comparison between the percentage variants and RDs present in culture stored on YM agar slant (before lyophilization) and in ampoules maintained through lyophilization. The percentage variants and RDs was determined at different storage times after the yeasts, subjected to lyophilization, were cultivated in round bottom flasks containing wort for 72 h.

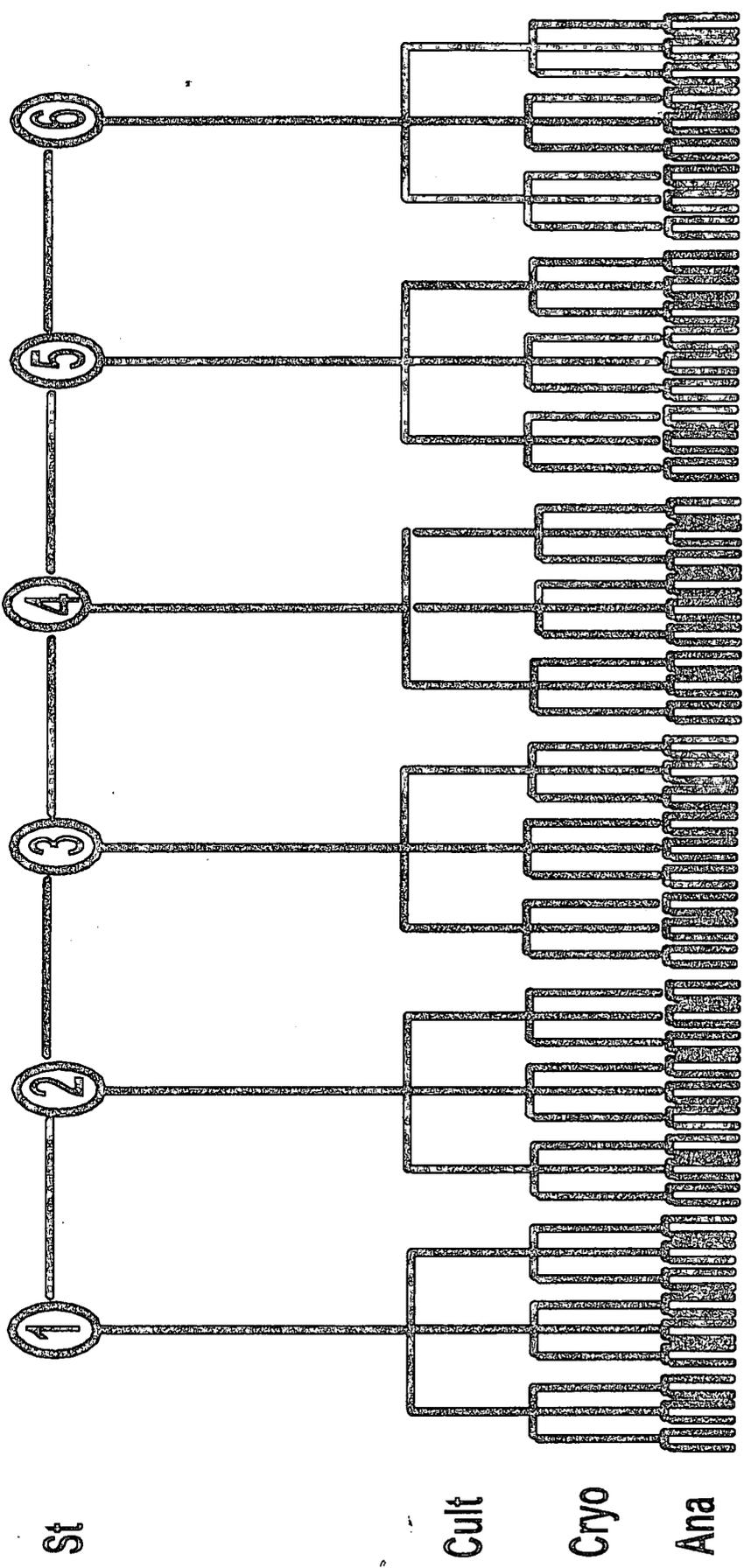


Fig. 1



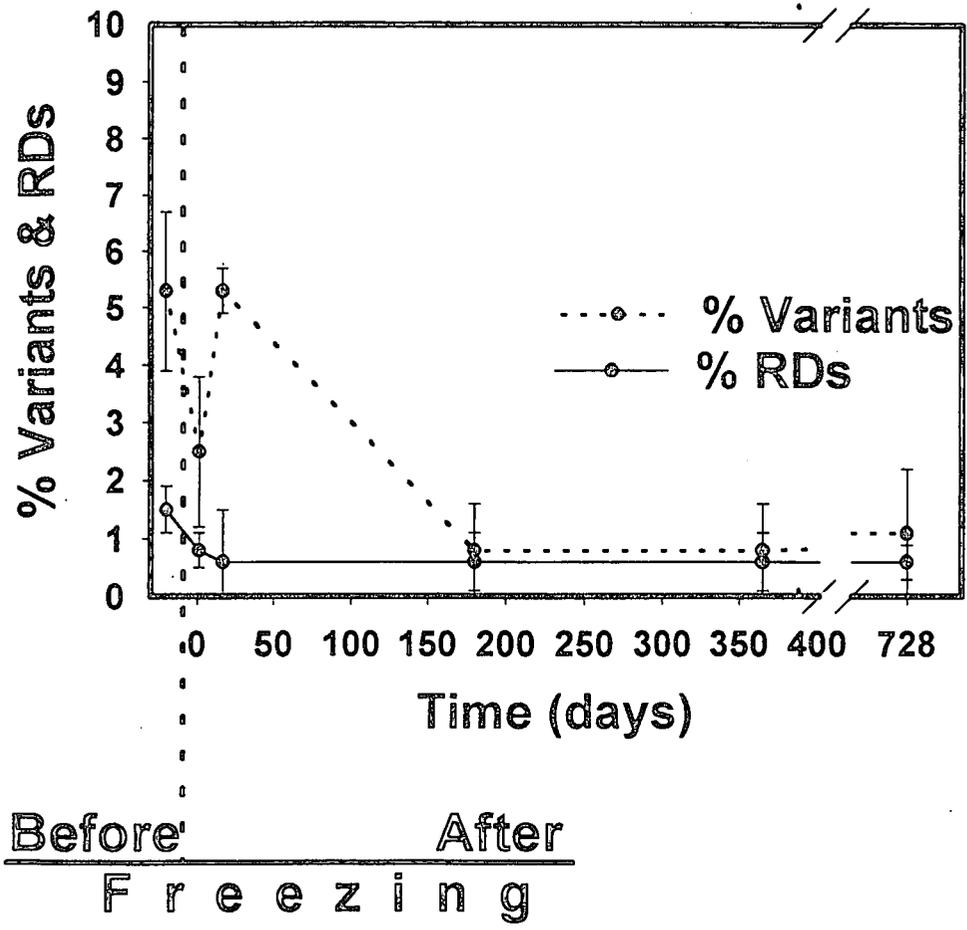
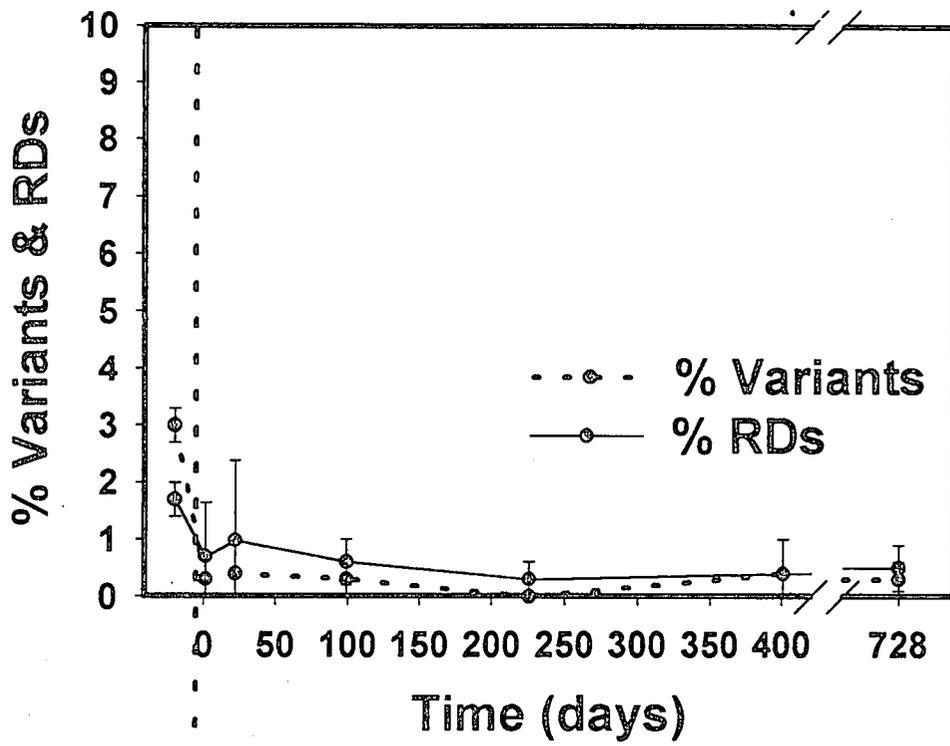


Fig. 3



Before                      After  
 —————  
 L y o p h i l i z a t i o n

Fig. 4



Table 1a Influence of different variance components on yeast maintenance through cryopreservation at  $-196^{\circ}\text{C}$ .

VARIANCE COMPONENTS	VARIANTS	RDs	$\mu_{\text{max}}$
$V_{\text{ana}}$	9.8	1.0	-
$V_{\text{cryo}}$	0.0	0.4	0.004
$V_{\text{cult}}$	0.0	0.0	0.003
$V_{\text{st}}$	0.0	0.0	0.000
<b>Grand average</b>	6.7	1.2	0.33
<b>Grand Standard Dev.</b>	2.7	1.2	0.07

- $V_{\text{ana}}$  - Variance component (analytical test) estimate.
- $V_{\text{cryo}}$  - Variance component (cryopreservation at  $-196^{\circ}\text{C}$  methodology) estimate.
- $V_{\text{cult}}$  - Variance component (culturing in YM) estimate.
- $V_{\text{st}}$  - Variance component (storage time under liquid nitrogen) estimate.

Table 1b Influence of different variance components on yeast maintenance through freezing at  $-70^{\circ}\text{C}$ .

VARIANCE COMPONENTS	VARIANTS	RDs	$\mu_{\text{max}}$
$V_{\text{ana}}$	4.7	1.1	-
$V_{\text{fre}}$	0.0	0.0	0.003
$V_{\text{cult}}$	0.0	0.0	0.003
$V_{\text{st}}$	0.0	0.0	0.000
<b>Grand average</b>	4.1	1.0	0.25
<b>Grand Standard Dev.</b>	2.8	1.0	0.08

- $V_{\text{ana}}$  - Variance component (analytical test) estimate.
- $V_{\text{free}}$  - Variance component (freezing at  $-70^{\circ}\text{C}$  methodology) estimate.
- $V_{\text{cult}}$  - Variance component (culturing in YM) estimate.
- $V_{\text{st}}$  - Variance component (storage time in a freezer) estimate.

Table 1c Influence of different variance components on yeast maintenance through lyophilization.

VARIANCE COMPONENTS	VARIANTS	RDs	$\mu_{max}$
$V_{ana}$	1.9	1.3	-
$V_{lyo}$	0.1	0.6	0.002
$V_{cult}$	0.2	0.4	0.006
$V_{st}$	0.0	2.3	0.001
<b>Grand average</b>	2.0	1.2	0.28
<b>Grand Standard Dev.</b>	1.7	1.5	0.07

- $V_{ana}$  - Variance component (analytical test) estimate.
- $V_{lyo}$  - Variance component (lyophilization methodology) estimate.
- $V_{cult}$  - Variance component (culturing in YM) estimate.
- $V_{st}$  - Variance component (storage time in vacuum at room temperature) estimate.

## Chapter 3

## Chapter 3

(Style according to: *Journal of the Institute of Brewing*)

### Development of Alternative Quality Control Methods for Yeasts in the Brewing Industry

#### 3.1 Introduction

Presently, differentiation and characterisation of yeasts in the brewing industry are based on a small number of characteristics such as growth properties and morphology. In addition, a variety of inhibitory and selective media have been developed for the detection of wild yeasts and bacteria<sup>5</sup>. In the case where inhibitory agents are used, growth of the culture yeast is suppressed while only contaminating organisms are supported. The addition of 1ppm actidione in a non-selective medium inhibits the growth of brewery yeast or fungi but will support the growth of bacteria from the brewery<sup>3</sup>. Other inhibitors such as crystal violet and fuchsin-sulphite have also been used for detection of wild yeasts<sup>1</sup>. Lysine agar is another selective medium used in most breweries for the detection of non-*Saccharomyces* wild yeasts<sup>6</sup>. Another selective medium i.e. Lactose Peptone Broth contains the carbohydrate lactose and supports growth of lactose assimilating species of bacteria and yeast but not the strains of *Saccharomyces cerevisiae*<sup>6</sup>.

For differentiation based on colony morphology, Wallerstein Laboratory Nutrient Medium which contains a pH indicator bromocresol green, is used<sup>2</sup>. Culture yeasts vary slightly on this medium and will grow as smooth white, pale green or pale bluish, raised colonies whereas wild yeasts are markedly different (e.g. wrinkled, raised and rough) and their colour also differs ranging from intense blue to green.

Results from Chapter 2 and Morakile *et al.*<sup>4</sup> illustrate that most of these conventional differentiating tests were variable and not completely accurate. Consequently, it became the aim of this project to develop new and more reliable identification methods that can be used to detect contamination and mutations in the starter yeast cultures that will be used in seeding the brewing process. To achieve this, PCR based RFLP, fatty acid and sterol profiles were evaluated.

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### 3.2 Differentiation of Brewing and Related Yeasts Based on PCR Amplification and Restriction Fragment Length Polymorphism of Ribosomal DNA

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#### Summary

A study to differentiate brewing yeasts from related yeasts using PCR amplification and RFLP of the internal transcribed spacers region was conducted. Differentiation was dependent on the restriction enzymes used to digest the amplified rDNA. Digestion with Hae 111, Cfo 1, Sau 3A1 and Msp 1 divided representatives of the genus *Saccharomyces* into several unique groups. With Msp 1 the DNA patterns for two brewing strains were similar, but could be differentiated from *Sacch. cerevisiae* and other species tested. It was also possible to distinguish some members of the *Saccharomyces sensu stricto* group i.e. *Sacch. bayanus* and *Sacch. pastorianus* from *Sacch. cerevisiae* and *Sacch. paradoxus* using Hae 111 as well as *Sacch. paradoxus* from the other *sensu stricto* members using Msp 1 digestion.

Key words: *Differentiation, rDNA, RFLP, yeast.*

## Introduction

Beer production is one of the oldest biotechnologies in the world. In this industry one thing that should not change and which is one of the pillars of this industry is the yeast that is used. In order to ensure that any changes in the yeast population applied in the brewing process is monitored, a variety of methods have been used that examine for instance contamination, mutation and viability. Generally, differentiation of yeasts relies on a number of criteria that include among others the mode of sexual reproduction, carbohydrate utilisation, cell wall composition and serology. Many of these methods, even though they have been used for a long time, suffers from the lack of sensitivity and specificity to distinguish between the brewing strains and other wild yeasts or to differentiate one production strain from the other<sup>1,8</sup>.

Recently, we reported on a yeast cryopreservation method for use in the brewing process<sup>7</sup>. As this was an improvement on the previous preservation method used, we also attempted to improve on the current conventional identification and characterisation methods. Previously, we found that these tests were subject to variation and contributed most to the variation of our preservation protocol<sup>7</sup>.

In recent times, the use of molecular genetics gained momentum particularly in the differentiation and characterisation of yeasts used in industrial fermentation<sup>2</sup>. Data from previous studies show that RFLP patterns allow recognition of individual species as well as individual strains within a species<sup>4</sup>. Consequently this method has considerable diagnostic value which may have use in the microbial quality control of biotechnological processes and in medicine.

The PCR products of rDNA, originating from several medically important yeasts from the genus *Candida* were treated with a variety of restriction

endonucleases in order to differentiate between species<sup>6</sup>. These authors found that they could distinguish between *C. albicans*, *C. guilliermondii* and *C. tropicalis* since these species produced sufficiently different digestion patterns. Similar results were obtained by Vilgays & Hester<sup>9</sup> for several species of *Cryptococcus*. In addition, Lachance<sup>5</sup> used RFLPs to map the genetic profiles of 125 isolates of *Clavispora opuntiae* which were collected across the world.

In this paper we are reporting on a DNA fingerprinting method which involves PCR amplification of rDNA and subsequent digestion with restriction enzymes. This method was evaluated as a possible quality control method that can be used in the brewing industry to determine contamination with closely related yeasts.

## Materials and Methods

### *Strains used*

Fourteen type strains representing the genus *Saccharomyces* and obtained from the Centraalbureau voor Schimmelcultures, The Netherlands (CBS), were used in this study (Table 1). Two brewing strains i.e. UOFS Y-0494 and UOFS Y-0532, were also included. These strains are held in our culture collection at the University of the Free State.

### *DNA extraction and amplification*

Yeast strains were grown in 5 ml YPD medium at 30 °C for 20 h. The yeast cells were transferred to microfuge tubes and harvested by centrifuging for 15 min at 13000 rpm. DNA was isolated using CTAB according to Innes<sup>3</sup> method and diluted to 1-50 ng/μl. The rDNA region was amplified in a Hybaid thermal cycler. The primers ITS 4 and ITS 5 described by White *et al.*<sup>10</sup> were used to amplify the ITS region. The thermal cycling parameters were initial denaturing at 96 °C for 2 min., followed by 35 cycles of denaturation at 96 °C for 1 min, primer annealing at 55 °C for 1 min and extension at 72 °C

for 2 min. A final extension was at 72 °C for 10 min. Products of each amplification reaction were resolved on 2 % agarose gel, which contained 2.5 µl ethidium bromide (10 mg/ml) and visualised under Ultra Violet light. Negative controls (no DNA templates) were used in every reaction to test for the presence of contamination in reagents. The sizes of the DNA were compared to a commercial size standard (100 bp ladder) obtained from Promega.

#### *Restriction digestion and RFLP analysis*

PCR products were digested with restriction endonucleases Hae 111, Cfo 1, Sau 3A1 and Msp 1 according to the supplier's instructions. The restriction fragments were electrophoresed on 2 % agarose gel at 70 V for 90 min. The reaction fragment sizes were measured in base pairs calculated from comparison to a commercial standard (100 bp ladder).

### **Results and Discussion**

By using the primers ITS 4 and ITS 5, the extracted DNA from all strains listed in Table 1 was successfully amplified and showed length variation (Fig. 1). Four restriction endonucleases, which recognise four nucleotide bases, were used to digest the PCR products. Well-developed restriction bands that exhibited a high level of length polymorphism were obtained. (Figs. 2-6).

On the basis of DNA band profiles obtained through Hae 111 digestion (Fig. 2), the yeast species representing the genus *Saccharomyces* can be divided into nine separate groups, each with similar band patterns within each group. Group 1: This group comprises of *Sacch. bayanus* (Fig. 2-b) and *Sacch. pastorianus* (Fig. 2-e) which are characterised by three bands each of approximately 468, 219, 139, base pairs and 460, 217, 145 base pairs respectively. Group 2: *Sacch. cerevisiae* (Fig. 2-c) and *Sacch. paradoxus* (Fig. 2-d) characterised by four bands each of approximately 275, 217, 162,

142 base pairs and 279, 215, 159, 140 base pairs respectively. Group 3: *Sacch. castellii* (Fig. 2-f) and *Sacch. dairenensis* (Fig. 2-g) characterised by one band of approximately 676 base pairs. Group 4: *Sacch. exiguus* (Fig. 2-h) characterised by two bands of approximately 478, 239 base pairs. Group 5: *Sacch. transvaalensis* (Fig. 2-i) characterised by two bands of approximately 480, 312 base pairs. Group 6: *Sacch. unisporus* (Fig. 2-j) characterised by two bands of approximately 485, 119 base pairs. Group 7: *Sacch. kluyveri* (Fig. 2-k) and *Sacch. spencerorum* (Fig. 2-m) characterised by three bands each of approximately 380, 204, 114 base pairs and 389, 190, 119 base pairs respectively. Group 8: *Sacch. servazzii* (Fig. 2-l) characterised by three bands each of approximately 285, 220, 206 base pairs. Group 9: *Sacch. barnettii* (Fig. 2-n) and *Sacch. rosinii* (Fig. 2-o) characterised by two bands each of approximately 457, 208 base pairs and 476, 219 base pairs respectively.

DNA band patterns obtained through Cfo 1 endonuclease (Fig. 3) divided the genus *Saccharomyces* into eight groups. Group 1: Comprises strains of *Sacch. barnettii* (Fig. 3-n), *Sacch. bayanus* (Fig. 3-b), *Sacch. cerevisiae* (Fig. 3-c), *Sacch. paradoxus* (Fig. 3-d), *Sacch. pastorianus* (Fig. 3-e), *Sacch. castellii* (Fig. 3-f) and *Sacch. unisporus* (Fig. 3-j) characterised by three bands of approximately 328, 270, 42; 367, 337, 151; 367, 327, 156; 384, 323, 146; 355, 327, 156; 327, 309, 120 and 327, 309, 111 base pairs respectively. Group 2: *Sacch. dairenensis* (Fig. 3-g) characterised by two bands of approximately 347, 125 base pairs. Group 3: *Sacch. exiguus* (Fig. 3-h) characterised by three bands of approximately 355, 260, 50 base pairs. Group 4: *Sacch. transvaalensis* (Fig. 3-i) characterised by three bands of approximately 457, 298, 112 base pairs. Group 5: *Sacch. kluyveri* (Fig. 3-k) characterised by three bands of approximately 298, 176, 36 base pairs. Group 6: *Sacch. servazzii* (Fig. 3-l) characterised by four bands of approximately 309, 179, 136, 100 base pairs. Group 7: *Sacch. spencerorum* (Fig. 3-m) characterised by three bands of approximately 260, 119, 38 base

pairs. Group 8: *Sacch. rosinii* (Fig. 3-o) characterised by four bands of approximately 292, 221, 100, 30 base pairs.

Digestion with *Sau* 3A1 (Fig. 4) yielded five groups. Group 1: *Sacch. bayanus* (Fig. 4-b), *Sacch. cerevisiae* (Fig. 4-c), *Sacch. paradoxus* (Fig. 4-d) and *Sacch. pastorianus* (Fig. 4-e) characterised by two bands of approximately (396, 347; 410, 388; 397, 370 and 415, 387 base pairs respectively. Group 2: *Sacch. castellii* (Fig. 4-f), *Sacch. unisporus* (Fig. 4-j), *Sacch. kluyveri* (Fig. 4-k), *Sacch. servazzii* (Fig. 4-l), *Sacch. spencerorum* (Fig. 4-m) *Sacch. barnettii* (Fig. 4-n) and *Sacch. rosinii* (Fig. 4-o) characterised by two bands of approximately 397, 260; 401, 257; 392, 245; 421, 257; 392, 271; 427, 240 and 398, 253 base pairs respectively. Group 3: *Sacch. dairenensis* (Fig. 4-g) characterised by three bands of approximately 320, 240, 154 base pairs. Group 4: *Sacch. exiguus* (Fig. 4-h) characterised by two bands of approximately 421, 174 base pairs. Group 5: *Sacch. transvaalensis* (Fig. 4-i) characterised by two bands of approximately 533, 210 base pairs

With *Msp* 1 (Fig. 5) four groups were differentiated. Group 1: *Sacch. bayanus* (Fig. 5-b), *Sacch. cerevisiae* (Fig. 5-c), *Sacch. pastorianus* (Fig. 5-e) and *Sacch. exiguus* (Fig. 5-h) characterised by two bands each of approximately 676, 98; 610, 98; 620, 98 and 522, 86 base pairs respectively. Group 2: *Sacch. paradoxus* (Fig. 5-d), *Sacch. transvaalensis* (Fig. 5-i), *Sacch. unisporus* (Fig. 5-j), *Sacch. kluyveri* (Fig. 5-k), *Sacch. servazzii* (Fig. 5-l), *Sacch. spencerorum* (Fig. 5-m), *Sacch. barnettii* (Fig. 5-n) and *Sacch. rosinii* (Fig. 5-o) characterised by one band each of approximately 741; 767; 630; 501; 653; 550; 526 and 630 base pairs respectively. Group 3: *Sacch. castellii* (Fig. 5-f) characterised by three bands of approximately 422, 86, 81 base pairs. Group 4: *Sacch. dairenensis* (Fig. 5-g) characterised by four bands of approximately 320, 112, 98, 84 base pairs.

Application of Msp 1 on the PCR products of the two brewing strains showed no difference between them (Fig. 6, c and d). Neither could the application of Cfo 1 (Fig. 3), Hae 111 (Fig. 2) and Sau 3A1 (Fig. 4) differentiate between the two brewing strains (results not shown). The profiles of the two brewing strains could be differentiated from the profile of *Sacch. cerevisiae* (Fig. 6), i.e. the appearance of two extra bands with a molecular weight of approximately 116 and 12 base pairs.

## Conclusions

According to our results it was possible to differentiate the closely related *Sacch. bayanus* (Fig. 2-b) and *Sacch. pastorianus* (Fig. 2-e) (the latter two producing similar band patterns) from *Sacch. cerevisiae* (Fig. 2-c) and *Sacch. paradoxus* (Fig. 2-d) (the latter two producing similar band patterns) using Hae 111. It was also possible to distinguish *Sacch. paradoxus* from the *sensu stricto* members using Msp 1 digestion (Fig. 5). In addition, the *Saccharomyces sensu stricto* group (i.e. *Sacch. bayanus*, *Sacch. cerevisiae*, *Sacch. paradoxus* and *Sacch. pastorianus*) could easily be differentiated from the *Saccharomyces sensu lato* group (i.e. *Sacch. barnettii*, *Sacch. castellii*, *Sacch. dairenensis*, *Sacch. exiguus*, *Sacch. kluyveri*, *Sacch. rosinii*, *Sacch. servazii*, *Sacch. spencerorum*, *Sacch. transvaalensis*, *Sacch. unisporus*) when comparing band patterns after Sau 3A1 digestion (Fig. 4). It was also possible to distinguish the brewing strains from all the other *Saccharomyces* species tested on the basis of the two extra bands that differentiated the brewing yeasts from *Sacch. cerevisiae* (Fig. 6). *Sacch. cerevisiae* could also be differentiated from *Saccharomyces sensu lato* using the same restriction enzyme (Fig. 5). These results show promise in the construction of a quality control system where it is necessary to distinguish brewery strains from closely related yeasts. However, more strains from other yeast genera known to be contaminants should be analysed to make a thorough evaluation if the brewing strains remain unique on the basis of this characteristic.

*Acknowledgements.* The authors wish to thank the National Research Foundation in South Africa for funding.

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M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J., (eds.). PCR protocols: *A guide to methods and application*. New York: Academic Press. 1990.

## List of abbreviations and some terminology used

CBS	-	Centraalbureau voor Schimmelcultures
Cfo 1	-	Restriction enzyme obtained from <i>Clostridium formicoaceticum</i> .
CTAB	-	Hexadecyl trimethyl ammonium bromide
DNA	-	Deoxyribose nucleic acid
rDNA	-	Ribosomal deoxyribose nucleic acid
EDTA	-	2-ethylene-diamine-tetra-acetate
Hae 111	-	Restriction enzyme obtained from <i>Haemophilus aegyptius</i>
ITS	-	Internal transcribed spacer
Msp 1	-	Restriction enzyme obtained from <i>Moraxella sp.</i>
dNTP	-	Deoxyribose nucleoside triphosphate
PCR		Polymerase chain reaction
ppm	-	Parts per million

- RFLP - Restriction fragment length polymorphisms
- Sau 3A1 - Restriction enzyme obtained from *Staphylococcus aureus*
- T E buffer - Tris, 2-ethylene-diamine-tetraacetate buffer
- YPD medium - Yeast extract 1%, Peptone 2%, Glucose 2%

Table 1 A list of *Saccharomyces* species and brewing strains used in this study

Organism	Strain no.
<i>Saccharomyces barnettii</i>	CBS 5648T
<i>Saccharomyces bayanus</i>	CBS 0380T
<i>Saccharomyces castellii</i>	CBS 4309T
<i>Saccharomyces cerevisiae</i>	CBS 1171NT
<i>Saccharomyces dairenensis</i>	CBS 0421T
<i>Saccharomyces exiguus</i>	CBS 0379T
<i>Saccharomyces kluyveri</i>	CBS 3082T
<i>Saccharomyces paradoxus</i>	CBS 0406T
<i>Saccharomyces pastorianus</i>	CBS 1397T
<i>Saccharomyces rosinii</i>	CBS 7127T
<i>Saccharomyces servazzii</i>	CBS 4311T
<i>Saccharomyces spencerorum</i>	CBS 3019T
<i>Saccharomyces transvaalensis</i>	CBS 2186T
<i>Saccharomyces unisporus</i>	CBS 0398T
Brewing strain 1	UOFS Y-0494
Brewing strain 2	UOFS Y-0532

CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; T = Type strain; NT = New Type strain; UOFS = University of the Orange Free State, Bloemfontein, South Africa

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- Fig. 2 Restriction pattern of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with Hae 111 restriction enzyme.
- Fig. 3 Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with Cfo 1 restriction enzyme.
- Fig. 4 Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with Sau 3A1 restriction enzyme. Broad bands between 300 bp and 400 bp that appear as one, were found to be two upon closer inspection (see text).
- Fig. 5 Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with Msp 1 restriction enzyme.
- Fig. 6 Restriction pattern of the PCR-amplified rDNA region of *Saccharomyces cerevisiae* and the two brewing strains digested with Msp 1 restriction enzyme.

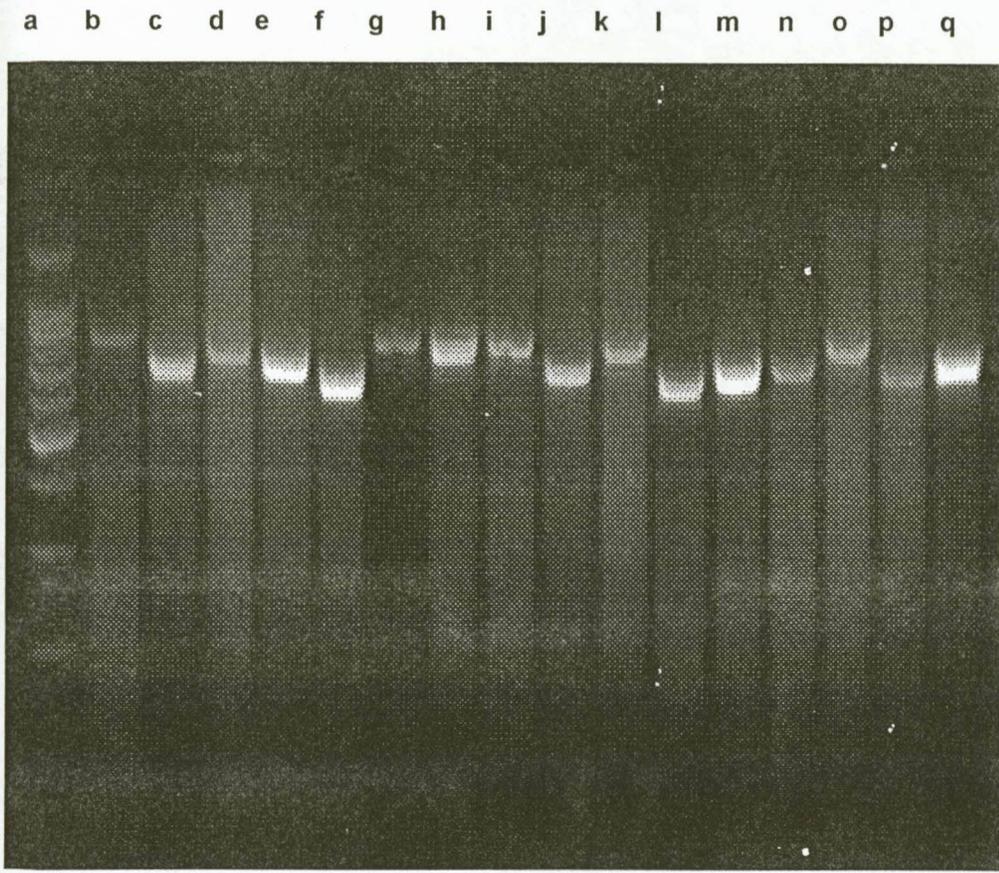


Fig. 1, Profile for the PCR based amplification of rDNA region of *Saccharomyces* species and the two brewing strains.

Lane a=Molecular size standard marker (100 pb DNA ladder), b=*Sacch. bayanus*, c=*Sacch. cerevisiae*, d=*Sacch. paradoxus*, e=*Sacch. pastorianus*, f=*Sacch. castellii*, g=*Sacch. dairenensis*, h=*Sacch. exiguus*, i=*Sacch. transvaalensis*, j=*Sacch. unisporus*, k=*Sacch. kluyveri*, l=*Sacch. servazii*, m=*Sacch. spencerorum*, n=*Sacch. barnettii*, o=*Sacch. rosinii*, p= Brewing strain 1 UOFS Y-0494, q= Brewing strain 2 UOFS Y-0532

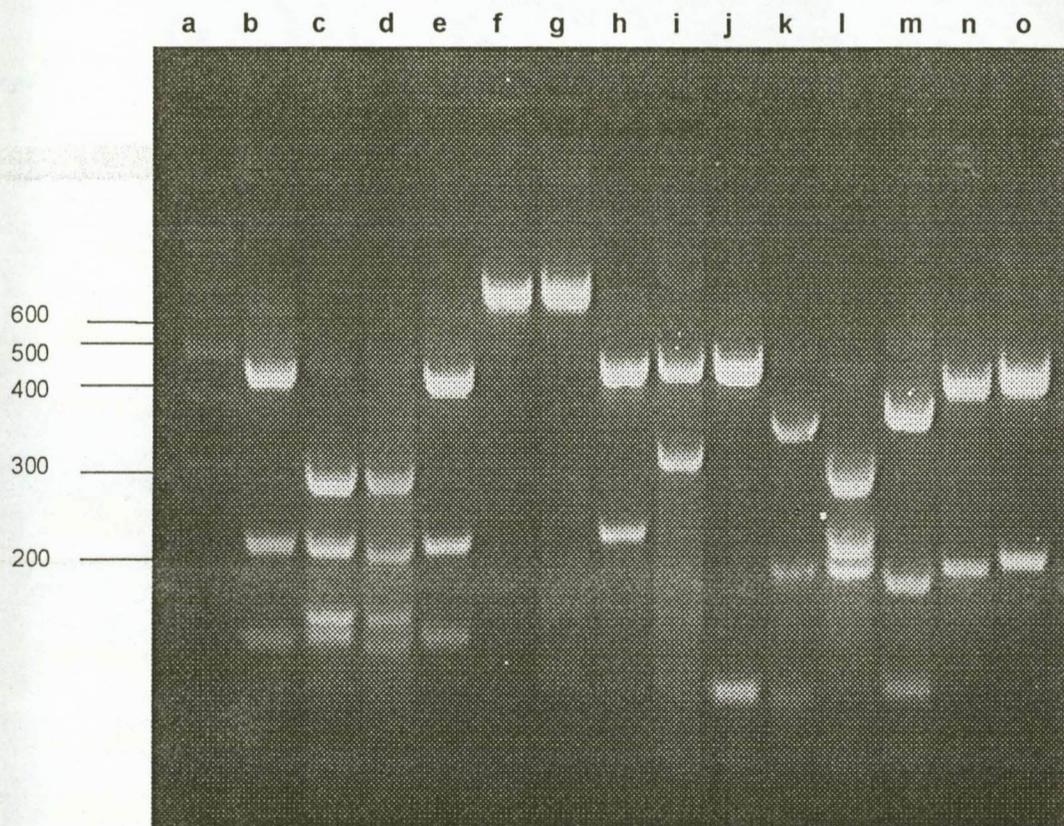


Fig. 2, Restriction pattern of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with Hae 111 restriction enzyme. Lanes a=Molecular size standard marker (100 pb DNA ladder), b=*Sacch. bayanus*, c=*Sacch. cerevisiae*, d=*Sacch. paradoxus*, e=*Sacch. pastorianus*, f=*Sacch. castellii*, g=*Sacch. dairenensis*, h=*Sacch. exiguus*, i=*Sacch. transvaalensis*, j=*Sacch. unisporus*, k=*Sacch. kluyveri*, l=*Sacch. servazii*, m=*Sacch. spencerorum*, n=*Sacch. barnettii*, o=*Sacch. rosinii*.

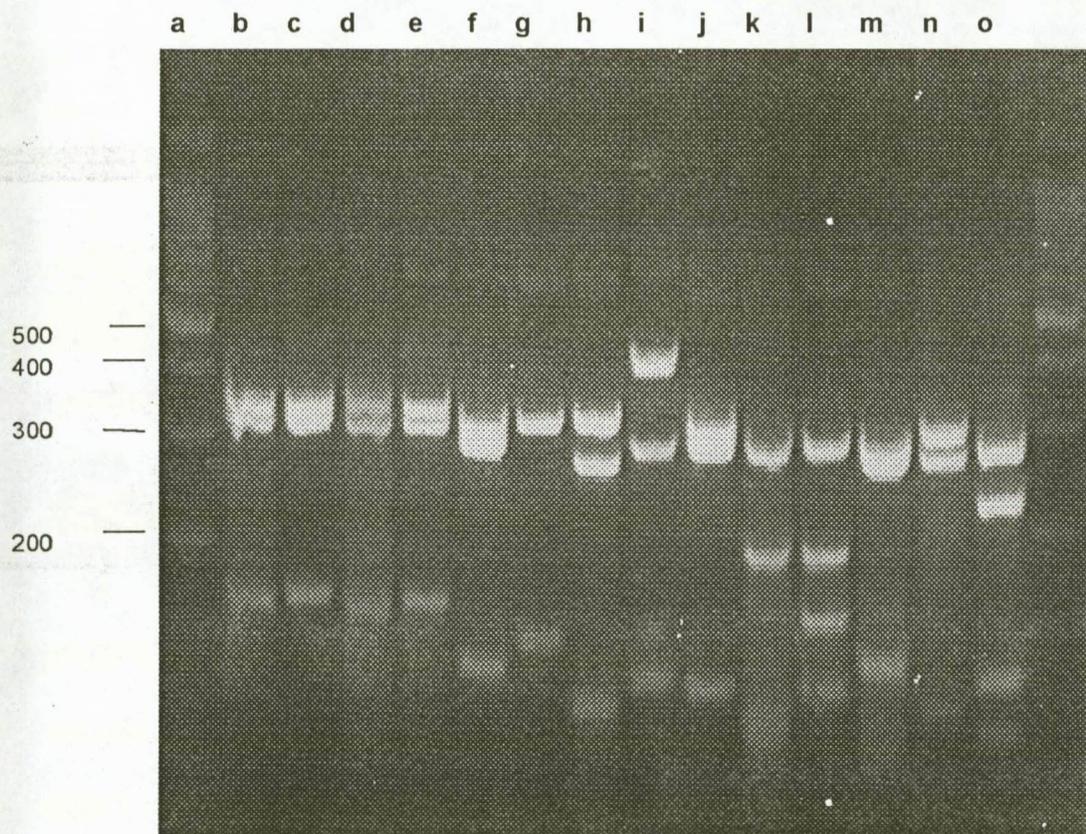


Fig. 3, Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with Cfo 1 restriction enzyme.

Lanes a=Molecular size standard marker (100 pb DNA ladder), b=*Sacch. bayanus*, c=*Sacch. cerevisiae*, d=*Sacch. paradoxus*, e=*Sacch. pastorianus*, f=*Sacch. castellii*, g=*Sacch. dairenensis*, h=*Sacch. exiguus*, i=*Sacch. transvaalensis*, j=*Sacch. unisporus*, k=*Sacch. kluyveri*, l=*Sacch. servazii*, m=*Sacch. spencerorum*, n=*Sacch. barnettii*, o=*Sacch. rosinii*.

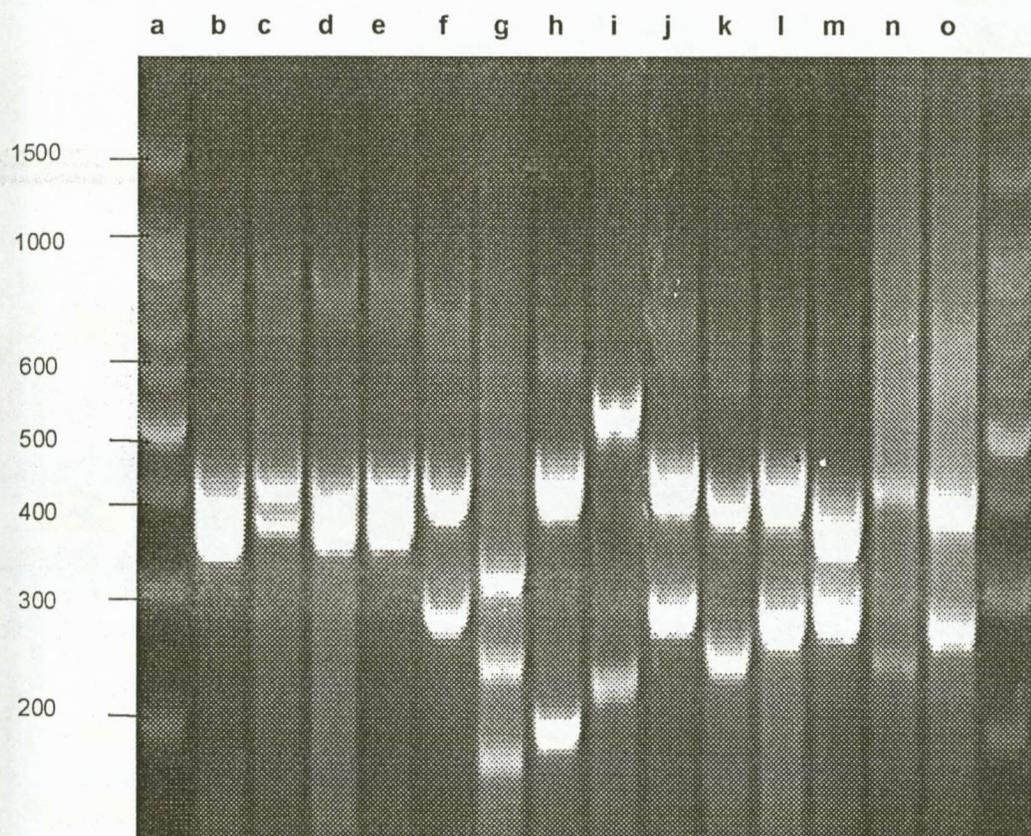


Fig. 4, Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with *Sau* 3A1 restriction enzyme. Lanes a=Molecular size standard marker (100 pb DNA ladder), b=*Sacch. bayanus*, c=*Sacch. cerevisiae*, d=*Sacch. paradoxus*, e=*Sacch. pastorianus*, f=*Sacch. castellii*, g=*Sacch. dairenensis*, h=*Sacch. exiguus*, i=*Sacch. transvaalensis*, j=*Sacch. unisporus*, k=*Sacch. kluyveri*, l=*Sacch. servazii*, m=*Sacch. spencerorum*, n=*Sacch. barnettii*, o=*Sacch. rosinii*.

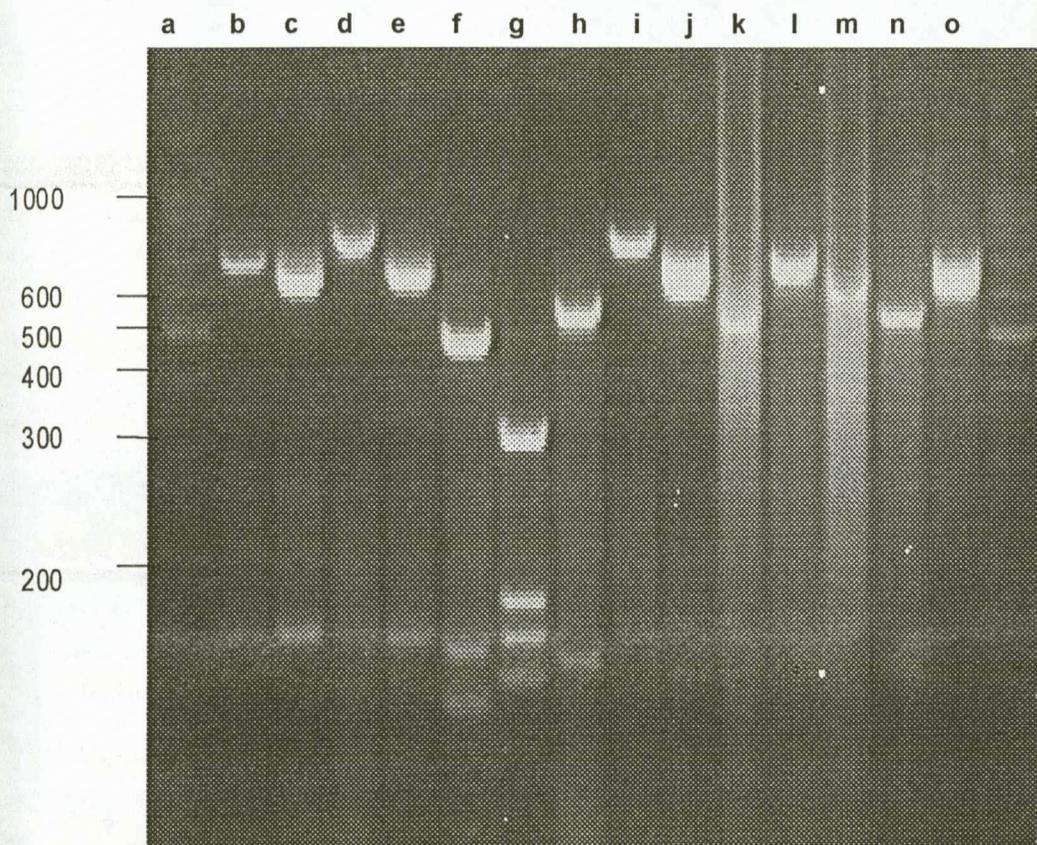


Fig. 5, Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with Msp 1 restriction enzyme.

Lanes a=Molecular size standard marker (100 pb DNA ladder), b=*Sacch. bayanus*, c=*Sacch. cerevisiae*, d=*Sacch. paradoxus*, e=*Sacch. pastorianus*, f=*Sacch. castellii*, g=*Sacch. dairenensis*, h=*Sacch. exiguus*, i=*Sacch. transvaalensis*, j=*Sacch. unisporus*, k=*Sacch. kluyveri*, l=*Sacch. servazii*, m=*Sacch. spencerorum*, n=*Sacch. barnettii*, o=*Sacch. rosinii*.

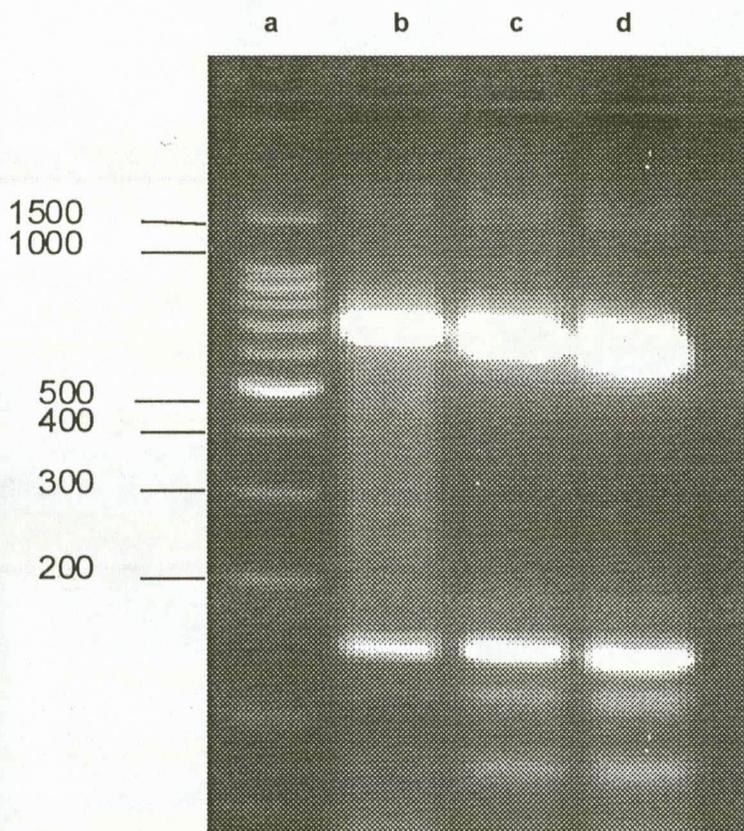


Fig. 6, Restriction patterns of the PCR amplified rDNA region of *Saccharomyces cerevisiae* and the two brewing strains digested with Msp 1 restriction enzyme.

Lanes a= marker, b= *Sacch. cerevisiae*, c=Brewing strain 1, d=Brewing strain 2

### 3.3 The Use of Fatty Acid and Sterol Analyses as Quality Control Methods in the Brewing Industry

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#### Summary

Fatty acid and sterol analyses were evaluated as alternative quality control methods to conventional differentiation and characterisation systems in the brewing industry. The presence of linoleic acid (18:2) in brewing yeast could be used to distinguish these from closely related yeast species. Furthermore, the absence of lanosterol and stigmasterol enabled differentiation of the brewing yeast from the rest of the closely related species tested. However, both fatty acid and sterol methods were not sensitive enough to detect mutants (variants) of brewing yeast. Conventional brewing identification tests proved sensitive enough to detect variants at low concentrations.

*Key words: Brewing yeasts, fatty acids, quality control, sterols.*

## Introduction

Yeast identification using cellular long-chain fatty acids (FAs) spans over 30 years of research. Abel and co-workers<sup>1</sup> pioneered the application of FAs for taxonomic purposes by differentiating between various bacteria. Many researchers have since followed. In 1979, Gangopadhyay *et al.*<sup>8</sup> used cellular long-chain FAs to differentiate between 38 isolates of anamorphic genera of *Candida*, *Cryptococcus* and *Torulopsis*. In 1980, Gunasekaran & Hughes<sup>9</sup> used FA profiles to characterise 85 strains of *Candida* based on the presence or the absence of certain FAs. Successive studies on cellular FA composition of yeast from various taxa<sup>10</sup> showed that many yeast strains have characteristic FA profiles. Furthermore, the potential of this method for industrial application has been evaluated<sup>3</sup>. Subsequently, FA fingerprinting based on the presence or absence of especially linoleic (18:2) and linolenic (18:3) acids has been used for the characterisation of brewing yeasts<sup>14</sup>. It has also been reported that sterol composition together with FA profiles show promise as a taxonomic marker in fungi<sup>13</sup>. Both these lipid types are critical to membrane structure and function and are also associated with brewing yeast vitality and fermentation performance<sup>16</sup>.

Apart from studies involving the chemotaxonomic status of FAs and sterols in differentiating yeasts from closely related species, it is also important to evaluate the sensitivity of these methods in detecting contamination by *e.g.* mutants. Mutation of brewing yeasts occurs spontaneously as a result of replication errors<sup>12</sup>. Mutants found in brewing are classified as variants and respiratory deficient mutants (RDs)<sup>15</sup>. Both mutant types are detrimental to beer quality *i.e.* causing stuck fermentation and produce off-flavours<sup>6,11</sup>. Consequently many breweries have strict specifications to combat this type of contamination<sup>5</sup>. In this study FA and sterol analyses were used to differentiate brewing yeast from closely related species. These techniques were also evaluated for sensitivity to detect contamination with mutants of brewing yeasts.

## Materials and Methods

### *Differentiation based on fatty acid and sterol analyses*

#### *Yeasts used*

The yeasts used in this study are listed in Table 1.

#### *Preparation of brewing yeast mutant*

Variants from the brewing strain UOFS Y-0494 were isolated on Wallerstein Laboratory Nutrient Medium (WLN) (Biolab, USA) plates after five days of incubation at 25°C. Mutant colonies are distinguished from normal brewing yeast on WLN since their colony morphology is markedly different (wrinkled, colony size smaller, edges not entire) and their colour is intense blue<sup>2</sup>.

#### *Cultivation*

Thirteen species of the genus *Saccharomyces* including two brewing yeasts and a selected stable variant were cultivated on YM agar slants<sup>17</sup>. All strains were then inoculated into 1 litre conical flasks containing 400 ml of the following medium (g/l); YNB (Difco, USA.), 6.7 and glucose (Merck, Germany), 40.0. Flasks were incubated at 30 °C ± 1 °C, shaken at 160 rpm until stationary phase and then harvested by centrifugation at 8000 rpm for 20 min and washed with distilled water. The centrifuged cells were rapidly frozen followed by freeze drying.

#### *Lipid extraction*

All lipids were extracted from freeze dried cells with the aid of chloroform/methanol (2:1, by vol.)<sup>7</sup>, dried to constant weight and then weighed.

### *Fatty acid analysis*

The FA composition was determined after trans-esterification by the addition of trimethyl sulphonium hydroxide<sup>4</sup>. Fatty acid methyl esters were analysed by using a Hewlett Packard 5890 gas chromatograph equipped with a Supelcowax 10 capillary column (30 m × 0.53 mm) with nitrogen as carrier gas set at a flow rate of 4 ml/min. The inlet temperature was set at 180 °C with the initial column temperature set at 120 °C increasing at 3 °C/min to 225 °C. Following a 10 min isothermal period, temperature was increased at the same rate to a final temperature of 240 °C. The FA peaks were detected using a flame ionisation detector set at 300 °C. Peaks were identified using authentic standards supplied by Sigma (USA). All experiments were performed in triplicate.

### *Sterol analysis*

*Hydrolysis:* Freeze dried cells of each strain were hydrolysed as described by Rencken *et al.*<sup>16</sup>. In short, KOH dissolved in methanol: ethanol: dH<sub>2</sub>O (700:315:15, by vol.) were added to pre-weighed freeze dried yeast biomass. A further 2 ml ethanol was added and the mixture was heated to 80 °C for 90 min in sealed screw cap glass tubes (Schott, USA) while shaken gently and then cooled to room temperature.

*Extraction:* Sterols were extracted with pentane and stored overnight at 4 °C. After evaporation using gaseous nitrogen, ethanol was added to the extract which was also left overnight at 4 °C. The suspension was filtered and injected into a HPLC (Beckman System Gold, 2347). Separations were carried out on a Spherisorb C18 reverse phase column (25 cm × 0.46 cm, 5 µm diameter) using a mixture of ethanol:methanol:water, (100:850:50, by vol.) as solvent phase at a flow rate of 1 ml/min. A Lambda-Max (Waters, Milford, MA, USA) Model 480 LC spectrophotometer was used to measure ergosterol at 282 nm and lanosterol, stigmasterol and squalene at 210 nm. Sterols present in each sample analysed were calculated as the ratio of the sterol

peak area to the internal standard peak area in the sample chromatogram and in the chromatogram of a standard solution. All experiments were performed in triplicate.

#### *Reagents used*

All reagents used were of high purity and obtained from reputable dealers.

### ***Sensitivity of conventional identification tests***

#### *Yeasts used*

The brewing yeast strain UOFS Y-0494 held in the University of the Free State Culture Collection (University of the Free State, Bloemfontein, South Africa) and the derived mutant isolated as described (see *Preparation of brewing yeast mutant*), were investigated.

#### *Culturing*

The brewing yeast UOFS Y-0494 and respective variant were grown in 1000 ml and 400ml YM<sup>17</sup> medium contained in 5 litres and 1 litre Erlenmeyer flasks respectively, at 25 °C for 12 h while shaking at 160 rpm. After harvesting as described before, mixtures were established by adding the selected stable variant to the brewing yeast to yield different concentrations, ranging from 0 % to 100 % (w/w) (theoretical values- see Table 4).

#### *Analysis*

A one millilitre aliquot was then drawn from each mixture and analysed for contaminants according to brewery protocol<sup>2</sup> using WLN plates. All experiments were conducted in triplicate.

#### *Reagents used*

All reagents used were of high purity and obtained from reputable dealers.

## Results and Discussion

According to the results in Table 2, brewing and related yeasts are characterised by the presence of mainly 16:0, 16:1, 18:0 and 18:1 while traces of 18:2 could be detected in the brewing strains and mutant. On the basis of this it is possible to differentiate the brewing strains and mutant from all related yeasts except *Sacch. kluyveri*. The latter could clearly be distinguished from the rest of the species tested due to the presence of 18:2 and 18:3 FAs. Consequently, 18:2 and 18:3 may be used as an identification marker for the brewing yeasts tested. It is however important to note that no significant difference between the brewing strain (UOFS Y-0494) and derived mutant could be found. Other mutants tested in the same way gave similar results (results not shown). This renders FA profiles not sensitive enough to detect mutants formed during the brewing process.

Most organisms contained relatively high amounts of ergosterol, while varying amounts of lanosterol, stigmasterol and squalene were detected (Table 3). *Saccharomyces castellii* and *Sacch. exiguus* did not produce any lanosterol while *Sacch. castellii*, *Sacch. exiguus* and the brewing strains with mutant could not produce stigmasterol. Although sometimes in low concentration, all yeasts studied could produce squalene. Consequently, the ability to produce lanosterol and stigmasterol can be used as marker to differentiate the closely related yeasts studied from the brewing strains and mutant. *Saccharomyces pastorianus* produced generally higher amounts of sterols compared to the rest of the species (1452 mg/g in total). Again no significant difference could be detected between the brewing strain (UOFS Y-0494) and the derived mutant (Table 3).

The results illustrated in Table 4 showed that the conventional methods<sup>2</sup> as applied in the breweries, were sensitive enough to detect a mutant at different theoretical percentages i.e. 0 % to 100 % (correlation coefficient:  $r =$

0.89045). Further research should be performed on other mutants derived in the same way to evaluate these methods for possible use in the brewing industry.

*To conclude:* Although FA and sterol analyses could be used to differentiate qualitatively between the brewing strains (similar profiles) and closely related yeasts, they could not distinguish the mutant studied from the normal brewing strain from which it was derived. These data show that the conventional identification system used in the brewing industry is superior to the two chemotaxonomic methods tested.

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## List of abbreviations and some terminology used

- FA - Fatty acid, molecule based on a hydrocarbon chain attached to a carboxylate group at one end.
- Fingerprinting - Unique and distinctive characteristic used for identifying organisms.
- Freeze drying - Preservation method based on removal of water by sublimation from the frozen material.
- GC - Gas Chromatography
- HPLC - High Pressure Liquid Chromatography
- RDs - Respiratory Deficient yeasts
- Sterol - Compound derived from an isoprene unit.
- Taxonomy - Biological classification of organisms according to their mutual affinities and similarities.
- TTC - Triphenyl Tetrazolium Chloride
- Variants - Mutants encountered in brewing characterised by irregular colony morphology.

WA	-	Wort Agar
WLN	-	Wallerstein Laboratory Nutrient Medium
YM	-	Yeast extract 3%, Malt extract 3%, Peptone 5%, Glucose 10%
YNB	-	Yeast Nitrogen Base
16:0	-	Palmitic acid
16:1	-	Palmitoleic acid
18:0	-	Stearic acid
18:1	-	Oleic
18:2	-	Linoleic acid
18:3	-	Linolenic acid
Erg	-	Ergosterol
Lan	-	Lanosterol
Squa	-	Squalene
Stig	-	Stigmasterol

Table 1 A list of *Saccharomyces* species and brewing strains used in this study

Organism	Strain no.
<i>Saccharomyces barnettii</i>	CBS 5648T
<i>Saccharomyces bayanus</i>	CBS 0380T
<i>Saccharomyces castellii</i>	CBS 4309T
<i>Saccharomyces cerevisiae</i>	CBS 1171NT
<i>Saccharomyces dairenensis</i>	CBS 0421T
<i>Saccharomyces exiguus</i>	CBS 0379T
<i>Saccharomyces kluyveri</i>	CBS 3082T
<i>Saccharomyces paradoxus</i>	CBS 0406T
<i>Saccharomyces pastorianus</i>	CBS 1397T
<i>Saccharomyces servazzii</i>	CBS 4311T
<i>Saccharomyces spencerorum</i>	CBS 3019T
<i>Saccharomyces transvaalensis</i>	CBS 2186T
<i>Saccharomyces unisporus</i>	CBS 0398T
Brewing strain 1	UOFS Y-0494
Brewing strain 2	UOFS Y-0532

CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; T = Type strain; NT = New Type strain; UOFS = University of the Orange Free State, Bloemfontein, South Africa.

Table 2 The lipid composition of the brewing yeasts and 13 species of the genus *Saccharomyces*.

Organism		Total lipids (% w/w)	Fatty acids (% w/w)					
			16.0	16.1	18.0	18.1	18:2	18:3
<i>Sacch. barnettii</i>	CBS 5648T	2.6	12.5	54.9	4.4	28.2	-	-
<i>Sacch. bayanus</i>	CBS 0380T	4.9	23.5	45.7	6.4	24.4	-	-
<i>Sacch. castellii</i>	CBS 4309T	9.3	16.9	50.3	3.8	29.0	-	-
<i>Sacch. cerevisiae</i>	CBS 1171NT	6.3	13.6	44.1	7.1	35.2	-	-
<i>Sacch. dairenensis</i>	CBS 0421T	1.6	15.5	58.9	3.9	21.7	-	-
<i>Sacch. exiguus</i>	CBS 0379T	4.1	16.8	53.5	5.0	24.7	-	-
<i>Sacch. kluyveri</i>	CBS 3082T	3.9	14.1	40.9	4.7	33.8	4.4	2.1
<i>Sacch. paradoxus</i>	CBS 0406T	5.3	9.9	51.7	5.0	33.4	-	-
<i>Sacch. pastorianus</i>	CBS 1397T	3.1	11.9	54.8	3.3	30.0	-	-
<i>Sacch. servazzii</i>	CBS 4311T	2.3	15.1	56.5	8.6	19.8	-	-
<i>Sacch. spencerorum</i>	CBS 3019T	4.4	16.1	51.7	4.9	27.3	-	-
<i>Sacch. transvaalensis</i>	CBS 2186T	1.0	10.2	59.7	2.6	27.5	-	-
<i>Sacch. unisporus</i>	CBS 0398T	3.2	14.5	61.7	5.9	17.9	-	-
Brewing strain 1	UOFS Y-0494	4.8	9.7	58.0	2.8	28.9	0.6	-
Brewing strain 2	UOFS Y-0532	3.9	7.9	53.2	4.4	34.2	0.3	-
Mutant		5.1	9.7	58.0	2.6	29.1	0.6	

CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; T = Type strain; NT = New Type strain; UOFS = University of the Orange Free State, Bloemfontein, South Africa. Standard error < 10%

Table 3 Sterol composition of the brewing yeasts and 13 species of the genus *Saccharomyces*.

Organism		Ergosterol	Lanosterol	Stigmasterol	Squalene
		mg/g	mg/g	mg/g	mg/g
<i>Sacch. barnettii</i>	CBS 5648T	367.3	45.8	109.0	25.0
<i>Sacch. bayanus</i>	CBS 0380T	217.5	35.0	628.0	225.0
<i>Sacch. castellii</i>	CBS 4309T	357.0	-	-	9.0
<i>Sacch. cerevisiae</i>	CBS 1171NT	452.5	152.5	405.5	159.0
<i>Sacch. dairenensis</i>	CBS 0421T	158.5	40.0	182.5	32.0
<i>Sacch. exiguus</i>	CBS 0379T	422.5	-	-	32.0
<i>Sacch. kluyveri</i>	CBS 3082T	367.5	42.0	86.5	9.0
<i>Sacch. paradoxus</i>	CBS 0406T	443.0	61.5	551.0	24.5
<i>Sacch. pastorianus</i>	CBS 1397T	610.0	134.0	606.0	102.0
<i>Sacch. servazzii</i>	CBS 4311T	239.5	237.0	154.0	9.0
<i>Sacch. spencerorum</i>	CBS 3019T	544.8	98.0	332.0	122.9
<i>Sacch. transvaalensis</i>	CBS 2186T	273.8	109.2	145.5	28.6
<i>Sacch. unisporus</i>	CBS 0398T	454.0	132.5	227.0	2.0
Brewing strain 1	UOFS Y-0494	398.3	37.9	-	14.9
Brewing strain 2	UOFS Y-0532	367.8	46.3	-	72.1
Mutant		342.2	30.6	-	11.9

CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; T = Type strain; NT = New Type strain; UOFS = University of the Orange Free State, Bloemfontein, South Africa. Standard error < 10%

Table 4 Results of the mutant mixed with the normal brewing yeast (UOFS Y-0494) to yield different concentrations of mutants (theoretical values) and the percentage actual mutants as counted from the Wallerstein Laboratory Nutrient medium (WLN) plates.

% Mutants (Theoretical values)	% Mutants (WLN)
0	1.7
2.7	4.4
3.7	5.4
4.7	3.5
5.7	3.9
6.7	6.5
7.7	8.5
8.7	6.2
9.7	11.8
100	94.1

Standard error < 10%

## Summary

Techniques to maintain and preserve microorganisms have become important to ensure availability of microorganisms for application in many institutions and processes. Several methods are available to preserve microorganisms and include drying, lyophilization, cryopreservation and sub-culturing. Many of these methods, may lead to population change through selection, loss of viability as well as poor survival rates when used for maintenance of yeasts. In this study, different preservation methods (cryopreservation at  $-196^{\circ}\text{C}$  in liquid nitrogen, freezing at  $-70^{\circ}\text{C}$  and lyophilization) were compared in the maintenance of brewing inocula over a period of two years. Interestingly, a decrease in the percentage of variants and respiratory deficient yeasts (RDs) was generally found when preserved through cryopreservation ( $-196^{\circ}\text{C}$ ) and freezing ( $-70^{\circ}\text{C}$ ). In contrast, the percentage variants when revived yeasts were grown in wort, increased in yeasts maintained through lyophilization. A high percentage viable cells ( $>95\%$ ) was recorded for yeast cultures maintained at  $-196^{\circ}\text{C}$  and at  $-70^{\circ}\text{C}$  while viability was low ( $<50\%$ ) when maintained through lyophilization. Consequently, the maintenance methods of choice are cryopreservation and freezing. The developed cryopreservation method was successfully implemented in the brewing process that produced the champion lager beer "Castle Lager" at Burton-upon-Trent in the UK (April, 2000). The results of the estimate of the components of variance showed that the largest source of variation in all three of the methods tested, was the error arising from the analytical test. On the basis of these results, it subsequently became another aim of this study to explore alternative analytical tests to differentiate and characterise yeasts in the brewing industry. Consequently, PCR based RFLP, fatty acid and sterol profiles were evaluated. Using PCR based RFLP, a study to differentiate brewing yeasts from related yeasts using amplification and restriction polymorphism of the ITS region was conducted. Differentiation was

dependent on the restriction enzymes used to digest the amplified rDNA. Digestion with Hae 111, Cfo 1, Sau3 A1 and Msp 1 divided representatives of the genus *Saccharomyces* into several unique groups. With Msp 1 the DNA patterns for two brewing strains were similar, but could be differentiated from *Sacch. cerevisiae* and other species tested. It was also possible to distinguish some members of the *Saccharomyces sensu stricto* group i.e. *Sacch. bayanus* and *Sacch. pastorianus* from *Sacch. cerevisiae* and *Sacch. paradoxus* using Hae 111 as well as *Sacch. paradoxus* from the other *sensu stricto* members using Msp 1 digestion. Fatty acid and sterol analyses were evaluated as alternative quality control methods to conventional differentiation and characterisation systems in the brewing industry. The presence of linoleic acid (18:2) in brewing yeast could be used to distinguish these from closely related yeast species. Furthermore, the absence of lanosterol and stigmasterol enabled differentiation of the brewing yeast from the rest of the closely related species tested. However, both fatty acid and sterol methods were not sensitive enough to detect mutants (variants) of brewing yeast. Conventional brewing identification tests proved superior to the above researched methods.

## Opsomming

Tegniese vir die bewaring van mikrobies is belangrik ten einde die beskikbaarheid van mikrobies vir aanwending in verskeie instansies en prosesse te verseker. Verskeie metodes vir die bewaring van mikrobies is beskikbaar en sluit in droging, vriesdroging, vries en die maak van subkulture. As hierdie metodes gebruik word vir die bewaring van giste, mag van hulle lei tot veranderinge in die populasie a.g.v. seleksie, verlies aan lewensvatbaarheid en lae oorlewingsvlakke. In hierdie studie is verskillende bewaringsmetodes (vries by  $-196^{\circ}\text{C}$  in vloeibare stikstof, vries by  $-70^{\circ}\text{C}$  en vriesdroging) vergelyk oor 'n tydperk van twee jaar in die bewaring van brou-inokula. Oor die algemeen is 'n afname in variante en respiratories-gebreklike giste gevind as bewaring d.m.v. vriesing ( $-196^{\circ}\text{C}$  en  $-70^{\circ}\text{C}$ ) gedoen is. In teenstelling, het die persentasie variante toegeneem as gevriesdroogde giste weer in moutekstrak opgegroeï is. 'n Hoë persentasie lewensvatbare selle ( $>95\%$ ) is waargeneem in giskulture bewaar by  $-196^{\circ}\text{C}$  en  $-70^{\circ}\text{C}$ , terwyl lewensvatbaarheid laag was ( $<50\%$ ) in gevriesdroogde kulture. Gevolglik is die beste bewaringsmetodes vriesing. Die ontwikkelde vriesingsmetodes is suksesvol geïmplementeer in die brouproses wat gelei het tot die produksie en kroning van die kampioen lager "Castle Lager" by Burton-upon-Trent in die VK (April, 2000). Die resultate van die berekening van variasiekomponente het getoon dat die grootste bron van variasie in al drie die getoetste metodes, die fout a.g.v. die analitiese metode was. Op grond van hierdie resultate, het dit 'n verdere doel van die studie geword om ondersoek in te stel na alternatiewe analitiese metodes om giste in die brou-industrie te differensieer en te karakteriseer. Gevolglik is PKR gebaseerde RFLP, vetsuur- en sterolprofiel geëvalueer. Met behulp van 'n PKR gebaseerde RFLP, is 'n studie uitgevoer om brouersgiste van verwante giste te onderskei m.b.v. amplifisering en beperkings-polimorfisme van die ITS areas. Onderskeiding was afhanklik van die beperkingsensieme wat gebruik is om

die geamplifiseerde rDNS te verteer. Vertering met Hae 111, Cfo 1, Sau 3A1 en Msp 1 het die verteenwoordigers van die genus *Saccharomyces* verdeel in verskeie unieke groepe. Met Msp 1 was die DNS patrone vir twee brouerstamme dieselfde, maar hulle kon onderskei word van *Sacch. cerevisiae* en ander getoetste spesies. Daar kon ook onderskei word tussen sommige lede van die *Saccharomyces sensu stricto* groep, nl. *Sacch. bayanus* en *Sacch. pastorianus* van *Sacch. cerevisiae* en *Sacch. paradoxus* m.b.v. Hae 111 en tussen *Sacch. paradoxus* en die ander lede van die *sensu stricto* groep m.b.v. Msp 1. Vetsuur- en sterolanalises is geëvalueer as alternatiewe kwaliteitsbeheermetodes vir konvensionele onderskeidings- en karakteriseringsisteme in die brou-industrie. Die teenwoordigheid van linoleïensuur (18:2) in brouersgiste is gebruik om hulle te onderskei van naby verwante spesies. Verder het die afwesigheid van lanosterol en stigmasterol dit moontlik gemaak om te onderskei tussen brouersgiste en die ander getoetste naby verwante spesies. Beide die vetsuur- en sterolmetodes was egter nie sensitief genoeg om mutante (variante) van die brouersgiste waar te neem nie. Konvensionele brouersgis-identifikasietoetse het geblyk beter te wees as bogenoemde metodes.

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