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**CRYOPRESERVATION AND  
CHEMOTAXONOMY IN  
*SACCHAROMYCES*  
MEYEN EX REESS**

**GONTSE MORAKILE**

**CRYOPRESERVATION AND CHEMOTAXONOMY IN  
*SACCHAROMYCES MEYEN EX REESS***

by

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Submitted in fulfilment of the requirements for the degree

**MAGISTER SCIENTIAE**

in the

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# Chapter 1

## Introduction

### 1.1 Motivation

The ability to preserve microorganisms can be considered a major biological achievement. Of special importance is an understanding of the principles of culture preservation with minimal occurrence of contamination, genetic and viability change. In biotechnological processes such as brewing, proper maintenance of brewing strains is crucial, since contamination, viability loss or genetic drift following inappropriate preservation protocols can lead to serious production problems. At present, cryopreservation is considered the most successful preservation method for yeasts, yielding high survival levels and good phenotypic stability. As a result, one of the aims of this study was the application and evaluation of a cryopreservation protocol used in the maintenance of a *Saccharomyces cerevisiae* strain presently utilised by a major brewing company in South Africa.

In order to ensure that only pure and stable yeasts with high viability are used after revival from the maintenance protocol, it is essential that appropriate, rapid and inexpensive quality control methods are implemented. Since elaborate and time consuming tests [such as estimation of mutants and bacteria using Wallerstein Laboratory Nutrient Medium (WLN), estimation of respiratory deficient (RDs) yeasts using Wort Agar overlaid with Triphenyl-Tetrazolium-Chloride, detection of the wild yeasts using the Swartz-

Differential Medium (SDM) protocol, 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)] are used today in the brewing industry, another aim of this study was the evaluation of chemotaxonomic characters such as sterols and polar lipids in, a first step determination of the contamination of preserved yeasts with closely related species.

## **1.2 Maintenance of microorganisms**

The ability to preserve microorganisms for a length of time is considered to be of major importance. This endeavour resulted in active research towards understanding the basic principles of culture preservation with minimal contamination, genetic or viability change. The need to maintain cultures is based on the fact that laboratories in teaching institutions require culture collections for demonstrating for example typical reactions, while reference strains are required by pathological laboratories for routine testing and research. Furthermore, a culture bank from which cultures can be drawn for industrial metabolite screening purposes, for taxonomical comparative studies and for initiation of biotechnological processes such as brewing is also frequently needed (Kirsop, 1991).

The need for high quality working cultures necessitated the need for reliable maintenance systems. This is of special importance to the fermentation industry where any change in inoculum performance as a result of poor

viability or genetic drift due to inappropriate preservation can lead to production problems (Hough *et al.*, 1982).

### 1.2.1 Factors influencing maintenance (Snell, 1991):

#### 1.2.1.1 Viability

Viability refers to the capacity to maintain life. Since this may deteriorate during preservation, it is important that the chosen method should minimise the loss of viability and allow survival of the cells once preserved.

#### 1.2.1.2 Population change through selection

Population change through selection of resistant strains may lead to survival of cells with altered phenotypic characteristics when compared to the original preserved culture. The choice of the preservation method should be in such a way that a maximum of viable cells resembling the original population are retained.

#### 1.2.1.3 Genetic change

Genetically engineered cultures for both scientific and industrial use are regarded as fragile and require extensive care during preservation in order to maintain important characters. Consequently, methods of preservation should minimise mutations or for example the loss of plasmids.

#### 1.2.1.4 Purity

It is important that the preserved cultures remain as pure as possible in order to minimise the chance of contamination, thereby changing the output of the culture.

#### 1.2.1.5 Expense

The high cost of establishing state of the art effective preservation methods may lead to the implementation of insufficient maintenance methods which may lead to unstable cell output.

#### 1.2.1.6 Value of cultures

It is best to use a preservation method that minimises the risk of loss since this may lead to the loss of rare and costly strains.

#### 1.2.1.7 Frequency of use

When preserved cultures are to be revived frequently, it is important that a method is used with a reduced risk of contamination. This is of special importance for cultures used in industrial processes or used as reference strains.

### **1.2.2 Major preservation methods for yeasts:**

#### 1.2.2.1 Subculturing

Subculturing include the transfer of cells from exhausted to fresh media (Kirsop, 1991). Transferring is done so that the cells continue to proliferate.

It is repeated at intervals that ensure preparation of the fresh culture before the old one dies. The time allowed to lapse between the subcultures with a minimal risk of losing the culture depends on the type of microorganism. Cell viability being a function of the metabolic rate, can be extended by either lowering the incubation temperature or by limiting oxygen access. Subculturing may be done in water (Odds, 1976), broth or on agar slants (Kirsop, 1991).

Subculturing has been successfully used for many years and is a widely applicable method for the preservation of yeasts. The method is technically simple, inexpensive in terms of the equipment, but relatively expensive in terms of labour. It is applicable to a wide range of microorganisms and provide easy revival as it only requires subculturing to obtain active cultures. However, the method is monotonous to carry out and mislabelling as well as faulty inoculations may occur leading to serious contamination which may outgrow and kill the original culture. Errors of mislabelling may occur mostly due to fatigue and may be minimised by placing the containers in random order, thus maintaining the concentration of the operator and therefore adopting good quality control procedures (Kirsop, 1984).

The method also poses a hazard for loss of viability and high level of degeneration following prolonged subculturing. The larger the inoculum, the lower the risk of selection but the greater the risk of contamination. The method is unsuitable for culture collections where long term stability of strains is important.

### 1.2.2.2 Drying

Drying or desiccation consists essentially of removing water and prevention of rehydration. The technique is mostly applicable to fungi because of their resistance to drying. Methods used include drying on silica gel (Woods, 1976), paper or gelatin discs (Bassel, 1977), sand, soil, or plugs of starch or peptone (Kirsop, 1988). A selection of bacteria and yeasts has been successfully preserved by drying. Species may fail to survive following two years storage on silica gel using milk suspension. The technique, like subculturing, is not expensive. It is particularly suited for small laboratories with limited resources and those that are situated in the region of high ambient temperature. Contamination is less likely to be a problem than with subculturing. The surviving cells may exhibit altered physiological or genetic properties (Kirsop, 1988).

### 1.2.2.3 Liquid Phase Drying (L-Drying)

This is a method by which the microorganisms are preserved by removal of water directly from the liquid phase rather than by sublimation from ice as in freeze drying (Kirsop, 1988). The method was described by Annear (1958) and was subsequently adapted for preservation of yeasts (Mikata *et al.*, 1983). Filamentous yeasts as well as those that are osmotolerant or psychrophilic are, however, sensitive to L-drying.

#### 1.2.2.4 Freeze drying

Freeze drying is a process where water is removed by sublimation from the frozen samples. The technique resembles desiccation, however, water is removed by sublimation from frozen material rather than by evaporation, after which dried microorganisms are stored under vacuum or in an inert gas in vials or ampoules (Kirsop, 1988; Snell, 1991). Phosphorous pentoxide or refrigerated condensers are normally used to trap water vapour that is removed. It is possible to carry out freezing procedures separately from drying or carry out the two as part of a continuing process. Commercial machines are available for this purpose and consist of two types: the centrifugal and the shelf type. In the shelf type, freezing takes place as a result of lowering the temperature of the shelves or by pre-freezing in a deep freeze (-20°C). The drying programme may be carried out continuously without the necessity to use a manifold for secondary drying (Kirsop, 1988).

Shelf drying is less popular than centrifugal drying. With the centrifugal procedure a cotton plug is inserted in the ampoules for the protection against cross-contamination as well as prevention of scattering microorganisms into the environment when the ampoules are opened. Furthermore, the glass seal of ampoules is moisture tight, which makes the centrifugal procedure more suitable for long term storage. Serum bottles commonly used in shelf systems are rubber sealed and therefore not air and moisture tight (Alexander *et al.*, 1980). Freeze drying has been widely used in preservation of microorganisms with wide applicability to bacteria, fungi, yeasts and viruses. Although it has been used for preservation of yeast for some time, it has been

noted that the survival levels are low (Kirsop, 1991). The procedure is suitable for batch production and distribution as well as maintenance of viability during storage. The technique requires skilled technical support and capital investment may be high. It is fairly labour intensive but large batches can be prepared in a relatively short time (Kirsop, 1991).

#### 1.2.2.5 Cryopreservation

Cryopreservation is the most widely applied preservation technique with greatest success on algae, bacteria, bacteriophages, fungi, plant and animal cells, protozoa, yeasts, and viruses. These cells are frozen and thawed with high survival levels and good phenotypic stability. Exceptions exist with particular strains of microorganisms, including genetically engineered strains. The process of cooling and warming may kill a high proportion of these cells, causing a population change (Snell, 1991).

Cells in the post-logarithmic state survive better than the younger cells. The cells yield higher viability figures when grown under aerobic conditions on a shaker. Losses that occur during freezing and thawing may be reduced by using a cryoprotectant. Different cryoprotectants already used with success include 5, 10, and 20% glycerol, glycerol plus dimethyl sulphoxide, 10% dimethyl sulphoxide, ethanol, methanol, YM broth and 5 or 10% hydroxyethyl starch. The success of cryoprotectants depends on their molar concentration and the ease at which they penetrate the cells. Furthermore, the losses may be reduced by adjusting the growth conditions as well as cooling and thawing rate. The latter is the most important factor that affects the cell



survival. Generally a faster cooling rate enhances the damage of cell membranes by formation of ice crystals. When the rate of cooling is slow, ice forms outside the cell and increases the concentration of the solutes. This leads to plasmolysis as water moves out of the cell causing shrinkage (Morris, 1981).

Control of the freezing rate can be obtained by placing ampoules in plastic foam boxes. These are then placed in vapour phase liquid nitrogen until freezing has occurred, after which the ampoules are transferred to liquid nitrogen. The temperature of storage can also affect survival. When using liquid nitrogen, cells are either held at  $-196^{\circ}\text{C}$  in the liquid phase or at  $-130^{\circ}\text{C}$  to  $-100^{\circ}\text{C}$  in the vapour phase. Care should be taken to avoid spillage of liquid nitrogen through the caps of polypropylene cryotubes (Kirsop, 1988).

### **1.3 Methods to evaluate preserved yeasts**

In order to evaluate the quality of yeasts after survival from a maintenance protocol, it is important that efficient accurate and easy to use techniques are available. Since this study dealt with the maintenance of brewing yeasts, only methods used in this respect, as described in Analysis Committee of the Institute of Brewing, (1997) were used. These methods include quantification of the following: (1) Variants through the Wallerstein Laboratory Nutrient Medium (WLN)- a protocol for detecting mutants and bacteria, (2) quantifying respiratory deficient (RDs) yeasts using Wort Agar overlaid with Triphenyl-Tetrazolium-Chloride, (3) quantifying wild yeasts with the Swartz-Differential Medium (SDM) protocol, (4) quantifying the non-*Saccharomyces*

species by the Lysine-Medium (LYS) method, (5) quantifying lactose assimilating and lactose fermenting microorganisms using the Lactose-Peptone-Broth (LP) method and (6) quantifying brewery bacteria using the Universal Liquid Medium (ULM) protocol.

## **1.4 Lipids as a chemotaxonomic tool**

Since some of the above conventional techniques are time consuming and sometimes inaccurate, the aim of this study was to evaluate the use of fatty acid (FA) based lipids as well as sterols as a method to monitor preserved yeasts.

### **1.4.1 Background**

Lipids represent an array of compounds which are defined as being sparingly soluble in water and more readily soluble in organic solvents such as chloroform, hydrocarbons, alcohols, ethers and esters (Ratledge, 1988). On the basis of molecular structure, lipids can be divided into two groups. One group is based on long chain FAs (Fig. 1 a,b,c,d) and the other is characterised by compounds derived from the isoprene units and include terpenoid lipids such as sterols (Fig. 2 a,b).

#### **1.4.1.1 FA based lipids**

FA based lipids (Fig. 1) include normal FAs and molecules such as phospholipids (associated with cell membrane), glyco- and sphingolipids (associated with membrane and cell walls), and triacylglycerols found in oil droplets in cells. Fungal cells contain both  $\omega$ 3 and  $\omega$ 6 series of FAs

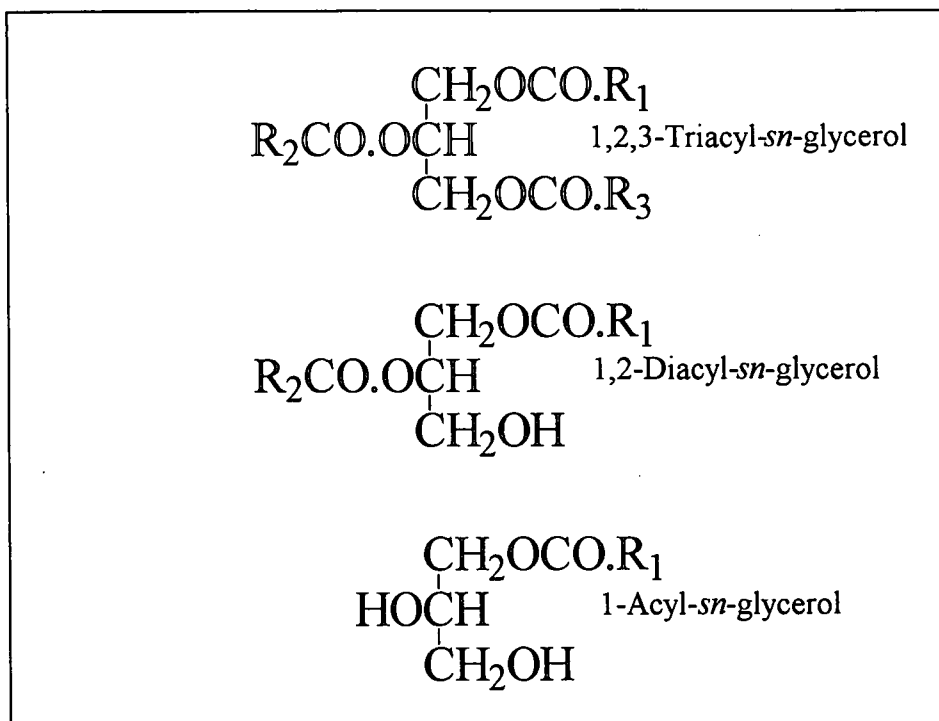


Fig. 1a Neutral lipids, represented by triacyl-, diacyl-, and monoacylglycerol (Ratledge, 1988).  $\text{R}_1\text{CO-}$ ,  $\text{R}_2\text{CO-}$ ,  $\text{R}_3\text{CO-}$  represent fatty acyl groups.

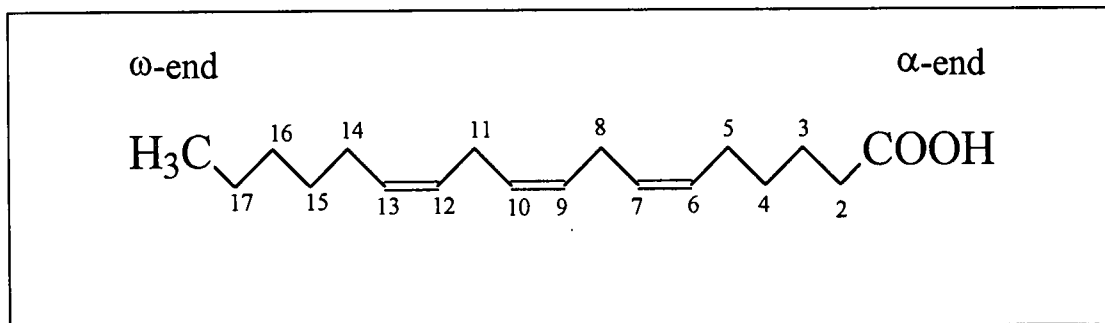


Fig. 1b Gamma-linolenic acid (Cottrell, 1989; Pohl, 1996)

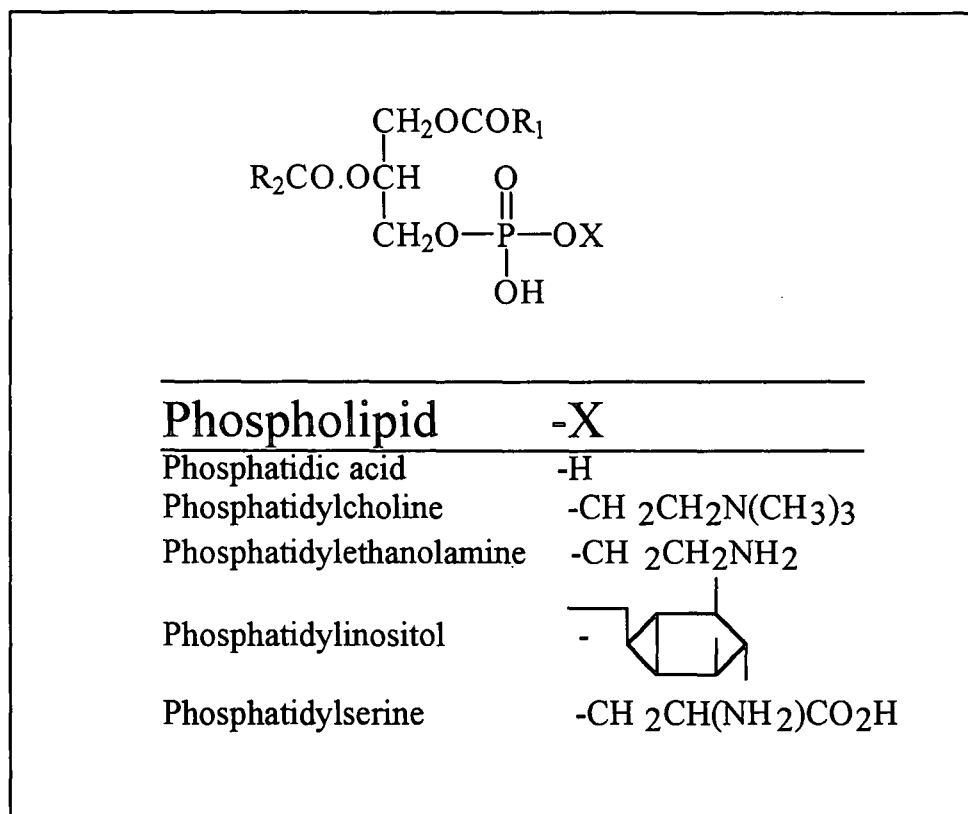


Fig. 1c The general structure and types of phospholipids found in fungi (Ratledge, 1988).  $\text{R}_1\text{CO-}$  and  $\text{R}_2\text{CO-}$ , represent fatty acyl groups; X- represents any of the indicated functional groups

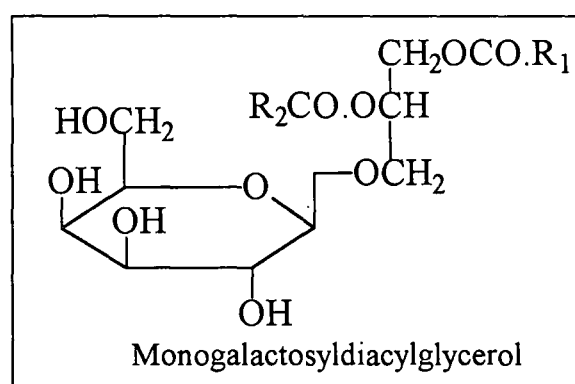


Fig. 1d A typical glycolipid (Ratledge, 1988).  $\text{R}_1\text{CO-}$  and  $\text{R}_2\text{CO-}$  represent fatty acyl groups

(Ratledge, 1988) and include mainly palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2),  $\alpha$ -linolenic (18:3  $\omega$ 3),  $\gamma$ -linolenic (18:3  $\omega$ 6), dihomo gamma linolenic (20:3  $\omega$ 6), arachidonic (20:4  $\omega$ 6) and eicosapentaenoic (20:5  $\omega$ 3) FAs (Kock and Botha, 1998). The most abundant FAs are reported as 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 ( $\omega$ 3) (Table 1). These FAs are, however, very sensitive towards changes in environment such as oxygen availability, temperature, pH, composition of the growth medium as well as growth rate and culture age. It is important that the influencing factors are taken into account before FAs of fungi are compared (Kock and Botha, 1998). FA profiles of fungi have shown to be useful in the demarcation of higher fungal taxa i.e. Protoctista (including the Oomycota, Chytridiomycota and Hyphochytridiomycota)- characterised by the  $\omega$ 6 series of polyunsaturated fatty acids (PUFAs) comprising 18 and 20 carbons; Zygomycota- containing the  $\omega$ 6 series of mainly 18 carbon PUFAs and the Dikaryomycota (i.e. Ascomycotina, Basidiomycotina and Deuteromycotina) that do not produce the  $\omega$ 6 series of PUFAs. Some are capable of producing 18:3 ( $\omega$ 3) and others can produce only up to 18:1 monoenoic FAs (Van der Westhuizen *et al.*, 1987). FAs have also been used successfully in the identification of *Aspergillus*, *Mucor* and *Penicillium* species (Bloemquist *et al.*, 1992) and *Eutipa lata* and *Cryptovalsa cf ampelina* (Ferreira and Augustyn, 1989). Some fungal genera have also been differentiated on the basis of  $\omega$ 3 and  $\omega$ 6 FAs. The zygomycotan fungi *Basidiobolus* and *Cunninghamella* are separated by the presence of 18:3 ( $\omega$ 6) while *Absidia*, *Blakeslea*, *Choanephora*, *Gilbertella*, *Helicostylum*, *Mucor*, *Parasitella*, *Phycomyces*, *Pilaira*, *Piptocephalis*, *Rhizomucor*, *Rhizopus*,

*Saksenaea*, *Syncephalastrum*, *Syncephalis*, *Thamnidium*, and *Zygorhynchus* are distinguished by the presence of 18:3 ( $\omega$ 6) as well as 20:0 and or 20:1 FAs. Also the genera *Conidiobolus*, *Entomophaga*, *Entomophthora* and *Mortierella* were found to be unique and produce 18:3 ( $\omega$ 6) and C20 PUFAs ( $\omega$ 6) (Van der Westhuizen, 1994).

Table 1 The predominant FAs found in fungi (Ratledge, 1988; Jeffery, 1995)

Trivial name (-acid)	*Synonyms	Structures
<b>Saturated fatty acids:</b>		
Palmitic	16:0	$\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$
Stearic	18:0	$\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$
<b>Unsaturated fatty acids:</b>		
Palmitoleic	16:1 (9c)	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Oleic	18:1 (9c)	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Linoleic	18:2 (9c, 12c)	$\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
$\alpha$ -Linolenic acid	18:3 (9c, 12c, 15c)	$\text{CH}_3\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
$\gamma$ -Linolenic	18:3 (6c, 9c, 12c)	$\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=(\text{CH}_2)_5\text{COOH}$

\* FAs are named according to the number of carbon atoms in the acid and the number of the unsaturated centres (e.g. 18:3; = FA consisting of 18 carbon atoms with 3 double bonds. The c denotes a cis double bond and the position is indicated by the number i.e. 9.

An extensive survey regarding FA profiles of the endomycetalean yeasts indicated that the FA compositions of the different yeast families overlap with the *Schizosaccharomycetaceae* occupying an isolated position (Kock and Botha, 1998). This phenotypic characteristic proved to be useful to differentiate between some yeast species of *Rhodospiridium* (Van der Westhuizen *et al.*, 1987), *Schizosaccharomyces* (Yamada and Banno, 1987) and *Nadsonia* (Golubev *et al.*, 1989). Follow-up studies on various yeast taxa showed that many yeast strains show unique FA profiles within species and that especially the presence or absence of unsaturated FAs are useful to separate species (Augustyn *et al.*, 1990). The latter underline the importance of FAs in chemotaxonomy pinpointing the conserved status of the cell membrane which is the site of FA desaturation (Ratledge, 1988). Finally, a quality control method was developed based on FA profiles which successfully monitored fungal biomass in a bioprotein pilot plant (Botha and Kock, 1993).

#### 1.4.1.2 Sterols

Sterols are important compounds in fungi since they associate with FAs by condensing and stabilising the phospholipid bilayers present in cell membranes (Paterson, 1998). A general structure of sterols as well as ergosterol, commonly found in 'true fungi' is shown in Fig. 2. In contrast to the mainly terrestrial true fungi, the Oomycota and Hyphochytridiomycota contain cholesterol and 24-alkylidene sterols (mainly ucosterol). Interestingly, sterols are absent in some Oomycota like *Lagenidium giganteum* and members of the Peronosporales. Cholesterol has also been

found in the Mastigomycotina and Zygomycota while ergosterol has been reported in *Mucor* and other Phycomycetes. According to Wassef (1977) and Paterson (1998), too few studies on sterol composition are available to make reliable conclusions on sterol distribution and value as a taxonomic marker in fungi.

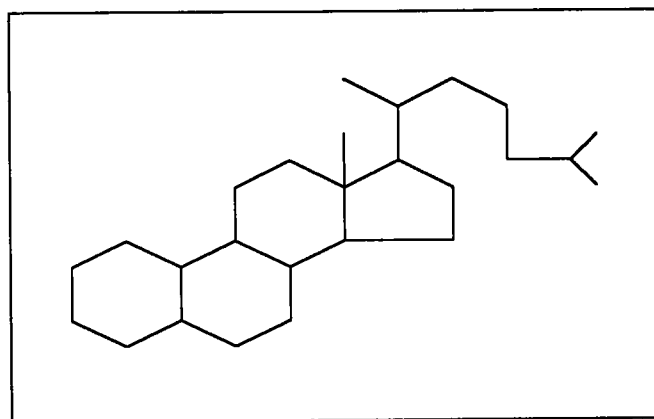


Fig. 2a A generalised structure of sterols (Paterson, 1998)

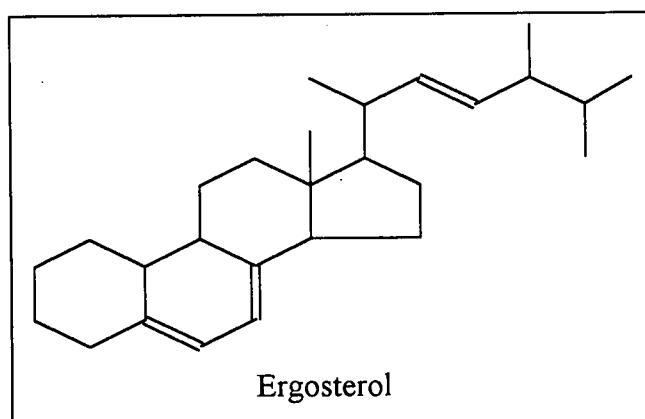


Fig. 2b Chemical structure of ergosterol (Gurr & Harwood, 1991)



It is an acknowledged fact that C28 sterols are present in most fungi. Fungisterol is most common with usually high concentrations of ergosterol. Paterson (1998) reports that most fungi have sterol unsaturation at the  $\Delta^7$  position while lower fungi have C27, C28 and C29 sterols mainly unsaturated at  $\Delta^5$ . Lösel (1988) summarised and tabulated quantitative and qualitative data on sterol presence in fungi. He concluded that refinement on sterol fungal data is necessary in order to obtain greater insight into the significance of this chemotaxonomic method.

Interesting results have recently been obtained (Müller *et al.*, 1994) where sterol and FAs have been included as taxonomic characteristics in 42 strains representing 16 species and 11 genera within the Phycomycetes, Ascomycetes and Basidiomycetes. Improved separation was obtained when FAs profiles as well as sterols were used in combination. The separation of especially the sibling species i.e. S and P types of *Heterobasidium annosum* was possible while ergosterol and ergosta-7,22-dien-3-ol were useful markers.

The value of ergosterol is well established as a measure of fungal growth or content in solid substrates (Gao *et al.*, 1993). It is believed that a large scale study on the sterols of fungi, which employs standard methods, would be interesting (Paterson, 1998). According to this author, the combination of sterols and FAs may have considerable potential in the taxonomy of fungi.

## 1.5 Purpose of the study

Based on this background the purpose of this study encompassed the following:

1.5.1. The application and evaluation of a cryopreservation protocol used in the maintenance of a brewing strain (Chapter 2).

1.5.2 The evaluation of rapid chemotaxonomic markers such as FAs and sterols associated with membranes in determining contamination of preserved yeasts. In order to determine this, the value of these phenotypic characters in differentiating between the species closely related to *Saccharomyces cerevisiae* was determined as a first approach (Chapter 3).

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## Chapter 2

### Maintenance of brewing inocula through cryopreservation

(Parts of this chapter have been submitted for publication in *Journal of Microbiological Methods*. Detailed data are presented in the attached appendix).

#### 2.1 Introduction

Several maintenance methods are available to preserve microorganisms and include subculturing, drying, freeze-drying and freezing (Kirsop, 1991). However, many of these methods may result in poor survival levels and instability of characteristics in yeasts. This is mainly attributed to the relative large size of yeast cells and the absence of resistant spores such as those produced by bacteria and other fungi.

Storage of cultures under liquid nitrogen is the most universally applicable of all preservation methods and fungi, bacteriophages, viruses, algae, protozoa, bacteria, yeasts, animal and plant cells as well as tissue cultures have all been successfully preserved using this method (Snell, 1991). It has been suggested that survival of yeasts following storage in liquid nitrogen can be as high as 100% (Hubalek and Kockova-Kratochvilova, 1978). These authors, furthermore, showed that many strains preserved in liquid nitrogen remain stable (Hubalek and Kockova-Kratochvilova, 1978).

It has been indicated that suitable growth conditions prior to cryopreservation is of major importance in order to obtain good yeast survival rates as well as

stability. Both the age of the culture and the oxygen availability during culturing affect survival levels (Kirsop, 1978). This is of special relevance when cells are preserved in liquid nitrogen. Kirsop (1991) has found that survival following preservation in liquid nitrogen is sometimes higher when cells are cultivated aerobically in shake cultures.

In this study we report on the cryopreservation of a selected *Saccharomyces cerevisiae* strain in ampoules to be used in inoculum preparation in a brewery process. A 4x3x3x2 nested experimental design was performed in order to determine the sources of variation concerning yeast viability, stability and purity over 136 days of storage in liquid nitrogen.

## **2.2 Materials and methods**

### **2.2.1 Strain used**

A selected brewers yeast, *Saccharomyces cerevisiae*, received from a South African brewery, was used throughout this study.

### **2.2.2 Conventional culturing**

This strain was cultivated on YM agar slants at 21°C for 3 days. The cells were then resuspended in 9ml saline solution, after which 1ml was inoculated into a McCartney bottle containing 15ml wort. 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 200ml wort and cultivating this 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 5l inoculum to be used in the brewing process.

### 2.2.3 Cultivation and ampoule preparation

One millilitre of the saline solution containing the culture as described above, was inoculated into 500ml conical side-arm flasks (each containing 50ml YM broth) and incubated at 25°C while shaking at 160 rpm. The cells were grown until logarithmic growth phase. Following this, 7.5ml of sterile 70% (w/w) glycerol was added to each flask as cryoprotectant and thoroughly mixed. Of this mixture, 1.15ml was pipetted aseptically into 1.8ml 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°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°C. Three ampoules per flask were revived after 30 h, 17 days, 87 days and 136 days by immersing each ampoule in water at 35°C until completely thawed. These were then analysed as described below.

### 2.2.4 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 (Analysis Committee of The Institute of Brewing, 1997). 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 by 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 (Analysis Committee of The Institute of Brewing, 1997).

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.

### **2.2.5 Estimation of the variance components**

A 4x3x3x2 nested design (i.e. 4 different liquid nitrogen storage times [St]; 3 separate cultivations [Cult]; 3 ampoules per cultivation flask [Cryo]; 2 analysis per ampoule [Ana]) was performed (Fig. 1) in order to determine the sources of variation in yeast viability, stability and contamination after preservation in liquid nitrogen (Box *et al.*, 1978). Before cryopreservation was attempted, 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 four different storage times (St) i.e. 30 h, 17 days, 87 days and 136 days and processed as described. Therefore, a total of 9 ampoules were revived per culture phase (Cult) at a given revival period which amounts to a total of  $4 \times 9 = 36$  ampoules over the four 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 (Box *et al.*, 1978). 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.

## 2.3 Results and discussion

Conventional inoculum preparation in a major South African brewery includes in short the following steps: (1) the cultivation of the brewer strain on YM agar slants by subculturing from the mother culture maintained on the same medium, (2) resuspension of the culture in saline solution, (3) inoculation of this solution into 15ml wort medium contained in McCartney bottles, (4) cultivation of this at 25°C for 24 h followed by (5) decanting the wort culture aseptically into a round bottom flask containing 200ml wort and cultivating this further for 72 h at 20°C while shaking at 100 rpm until stationary phase is reached and finally (6) adding this to 5l medium to be used as inoculum in the brewing process.

Since the maintenance of yeast cultures on agar slants may result in poor survival levels and instability of characteristics in yeasts (Kirsop, 1991), it was decided to evaluate cryopreservation as a replacement of this section of the inoculum preparation. Consequently, it was decided to prepare ampoules containing yeast culture and cryoprotectant for maintenance under liquid nitrogen and to evaluate the influence of this protocol on yeast viability, stability and purity upon ampoule revival and subsequent cultivations.

### 2.3.1 Influence of cryopreservation protocol on brewery yeast

According to the results obtained, it is clear that a significant decrease in the percentage respiratory deficient yeasts (RDs) as well as variants occurred in the revived ampoules during the cryopreservation phase (Figs. 2, 3). Before cryopreservation the RDs and variants were 3.1% and 8.4% respectively.

These values decreased to 1.3% and 4.8% respectively after 30 h of cryopreservation after which the values remained more or less the same. This phenomenon may be due to a selection against yeast variants and other mutants when stored in liquid nitrogen. A yeast count ranging from 17 to 39 x 10<sup>6</sup> cells. ml<sup>-1</sup> was obtained (viability > 95%) in revived ampoules over 136 days of storage under liquid nitrogen.

When yeasts from slants and yeasts subjected to cryopreservation were cultivated in wort contained in round bottom flasks, no significant changes in the percentage RDs, variants or maximum growth rate ( $\mu_{\max}$ ) could be detected (Figs. 4, 5, 6). In addition no contamination by bacteria and other yeasts was recorded. From these data it is clear that cryopreservation and storage in liquid nitrogen for up to 136 days had no significant impact on the different responses analysed. A total ranging from 200 to 336 x 10<sup>6</sup> cells. ml<sup>-1</sup> (viability > 95%) after 72 h of growth was obtained in round bottom flask cultures produced from the different ampoules. Since these cultures are used for further inocula upscaling in the brewing process under study, it is important that this variation in cell concentration is evaluated in order to determine the viability of this cryopreservation process.

### 2.3.2 Estimation of variances

Storage time under liquid nitrogen (St), culturing in YM broth (Cult), cryopreservation methodology (Cryo) and the analytical tests (Ana) were selected as the major factors contributing to process variation. Consequently, the results obtained from the Nested Experimental Design were subjected to relevant statistical analyses (Box *et al.*, 1978). The results are shown in Table 1.

The results on variants and RDs suggest that the largest source of variation in both cases was the error arising from the analytical tests. Cryopreservation also influenced the variation in the number of RDs obtained, though to a lesser extent. Considering  $\mu_{\max}$ , the largest source of variation was the error arising from the cryopreservation protocol. It is important to realise that the analytical test carried out to measure cell concentration may have contributed significantly to this value. This could not be determined since this has not been separated from the cryopreservation protocol. It is important to note that storage time under liquid nitrogen had no effect on either the variants, RDs or  $\mu_{\max}$ .

From this study it is now possible to construct statistical quality control charts (Duncan, 1974) that can be used to determine if ampoules containing yeasts, manufactured according to our cryopreservation protocol, can be used as inocula in brewing processes. The degree of variation pertaining to the different responses studied, can also be taken into account when deciding to implement this protocol in practice.

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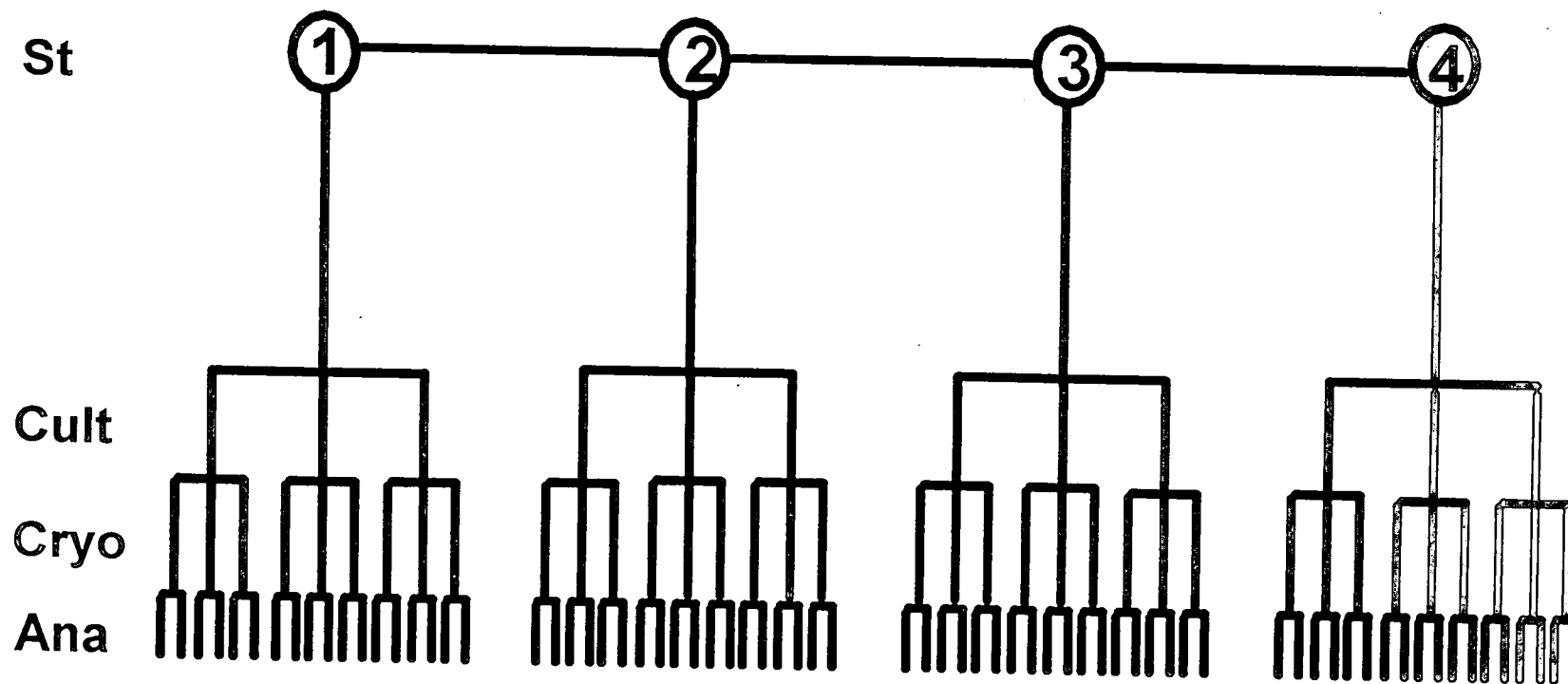


Fig. 1. A 4x3x3x2 hierarchical (nested) design. St: storage time; 1: 30 h; 2: 17 days; 3: 87 days : 4: 136 days; Cult: culturing in YM broth; Cryo: cryopreservation; Ana: analytical tests.

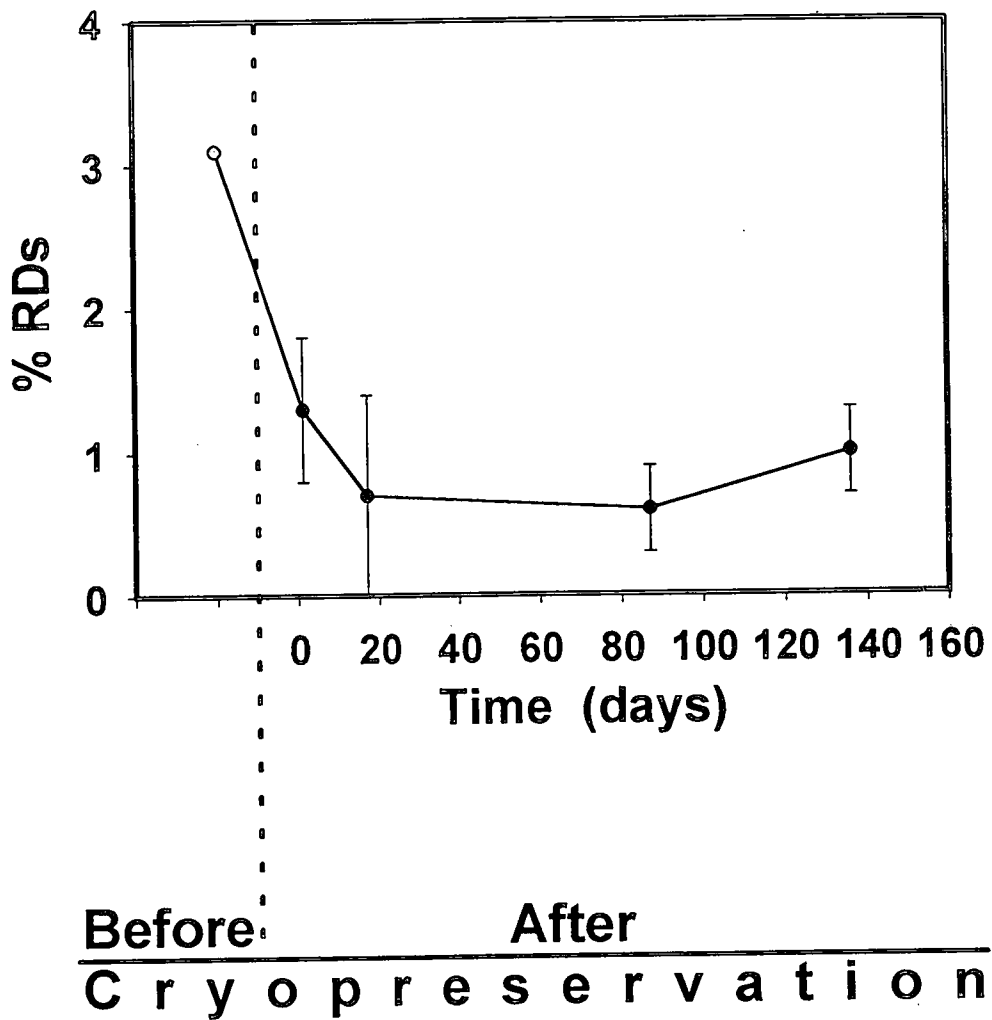


Fig. 2. A comparison between the percentage respiratory deficient yeasts (RDs) present in culture stored on YM agar slant and in cryopreserved ampoules at different liquid nitrogen storage times. The percentage RDs was determined directly upon revival of ampoules.

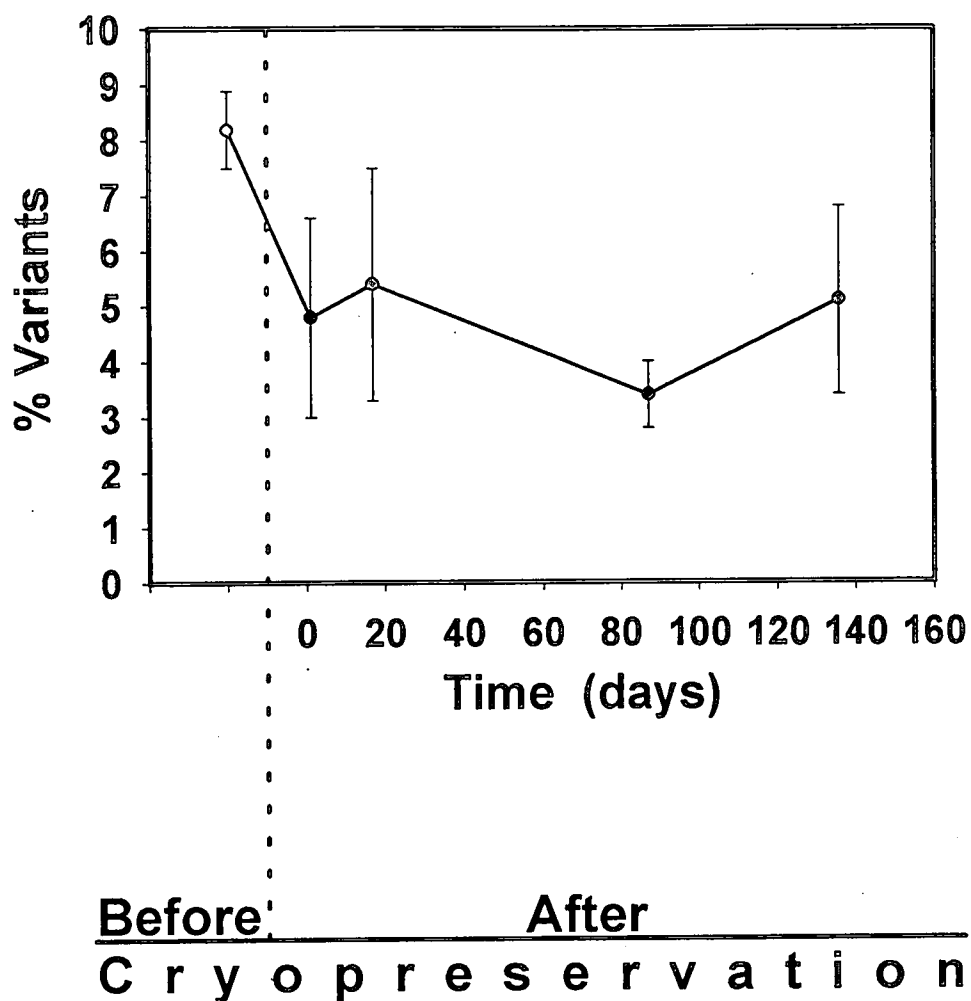


Fig. 3. A comparison between the percentage variants present in culture stored on YM agar slant and in cryopreserved ampoules at different liquid nitrogen storage times. The percentage variants was determined directly upon revival of ampoules.

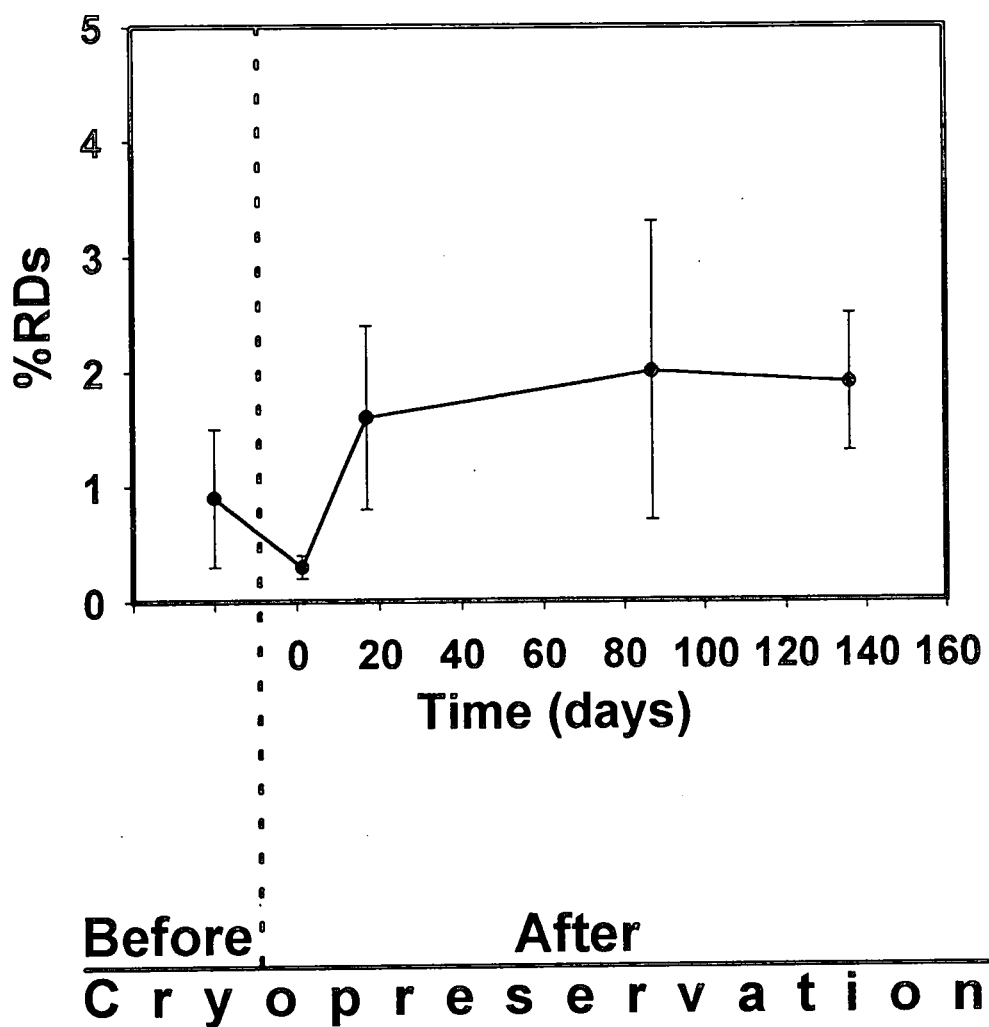


Fig. 4. A comparison between the percentage respiratory deficient yeasts (RDs) present in culture stored on YM agar slant and in cryopreserved ampoules at different liquid nitrogen storage times. The percentage RDs was determined after the yeasts subjected to cryopreservation were cultivated in wort contained in round bottom flasks.

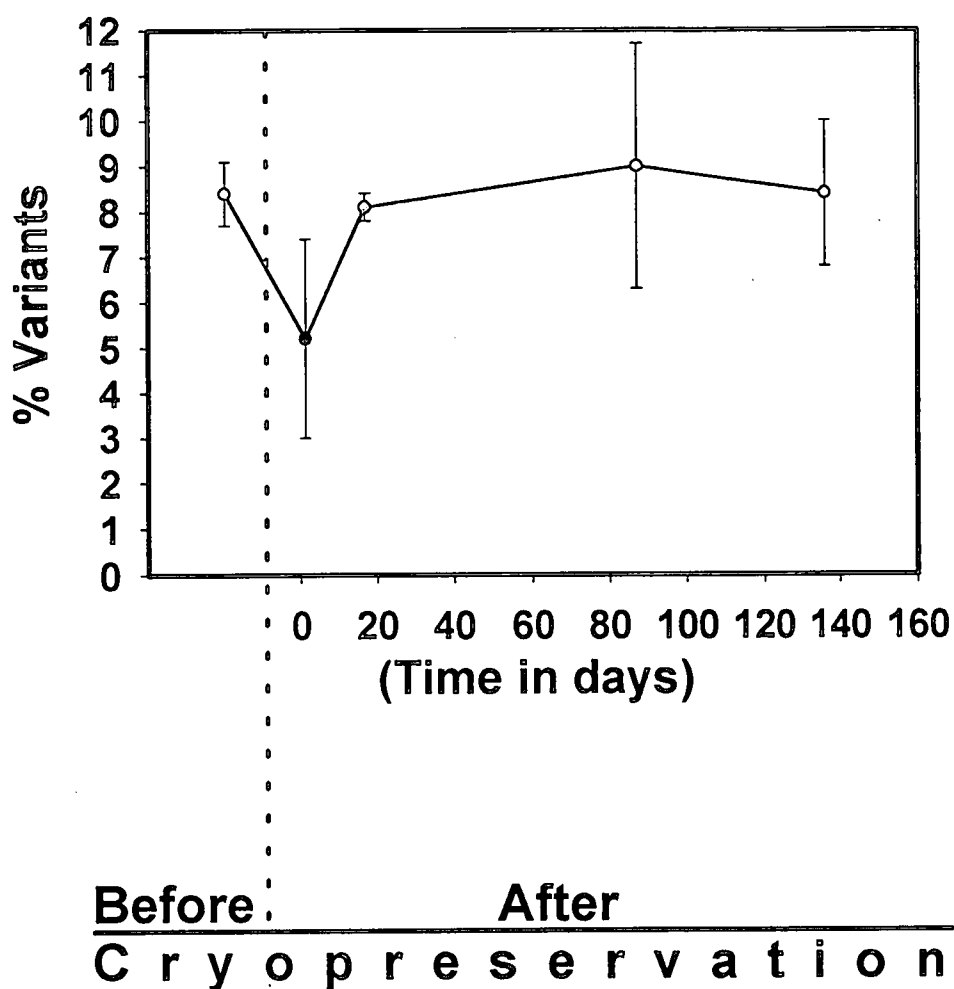


Fig. 5. A comparison between the percentage variants present in cultures stored on YM agar slant and in cryopreserved ampoules at different liquid nitrogen storage times. The percentage variants was determined after the yeasts subjected to cryopreservation were cultivated in wort contained in round bottom flasks.

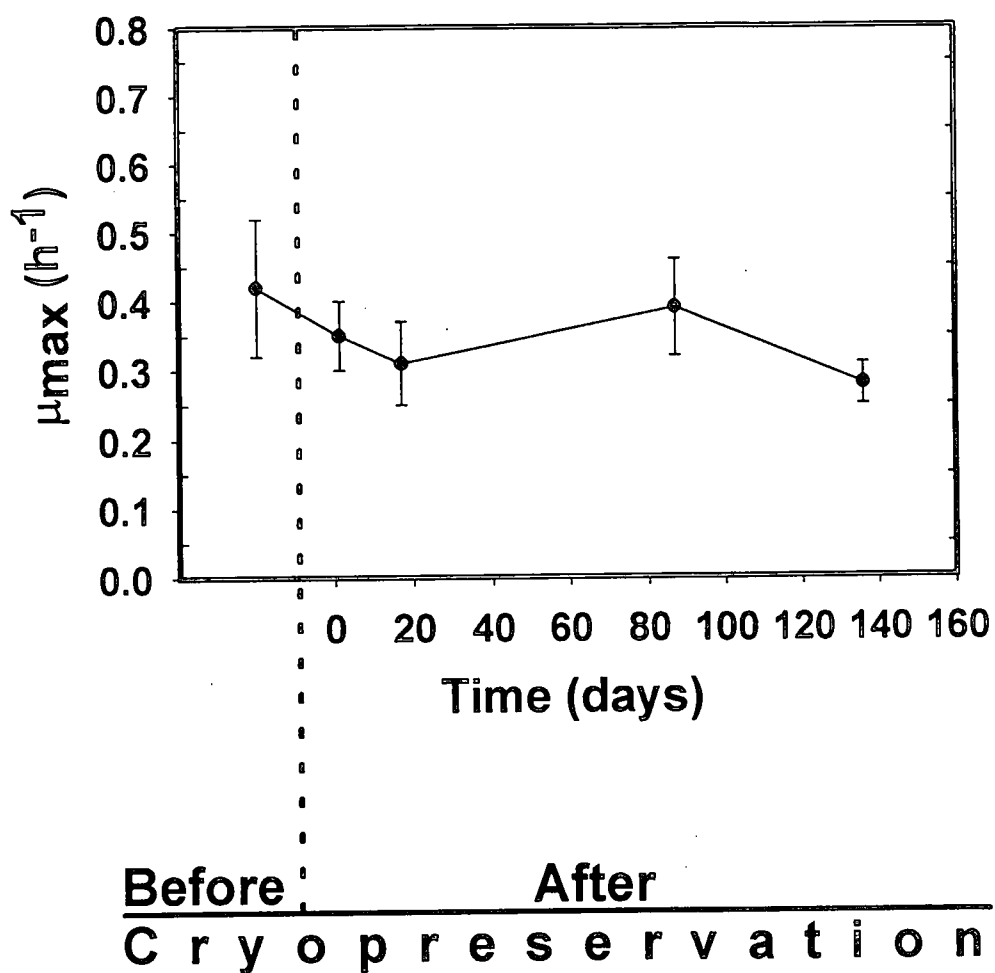


Fig. 6. A comparison of the maximum growth rate ( $\mu_{\max}$ ) of the yeasts stored on YM agar slant and in cryopreserved ampoules at different liquid nitrogen storage times.  $\mu_{\max}$  was determined by a direct cell count using haemocytometer after the yeasts subjected to cryopreservation were cultivated in wort contained in round bottom flasks.

Table 1 Influence of different variance components on yeast maintenance variation.

VARIANCE COMPONENTS	VARIANTS	RDs	$\mu_{\max}$ .
$V_{\text{ana}}$	10.8	1.2	-
$V_{\text{cryo}}$	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>	7.5	1.4	0.33
<b>Grand Standard Dev.</b>	3.3	1.3	0.08

$V_{\text{ana}}$ . Variance component (analytical test) estimate.

$V_{\text{cryo}}$ . Variance component (cryopreservation methodology) estimate.

$V_{\text{cult}}$ . Variance component (culturing in YM) estimate.

$V_{\text{st}}$ . Variance component (storage time under liquid nitrogen) estimate.

**APPENDIX**

**CHAPTER 2**



**NOVEMBER 1997**

**CRYO-TIME: 30 h**

## EXPERIMENT 1

### Cryopreservation of standardised yeast inoculum

#### Variability in cryopreservation methodology

In this first experiment (Exp. 1), the effect of cryopreservation after 30h on brewery yeast viability was investigated. According to the results, no contamination on ULM, LP, LYS and SDM media could be detected at any stage of analysis after cryopreservation. Thirty hours of cryopreservation had no significant influence on growth curves as well as yeast viability in all repetitions performed (see: Growth Studies and Yeast Viability: Exp. 1.1 and 1.2. tables and figures).

# Nested Experimental Design

Variables

Responses  
viability  
stability  
contamination

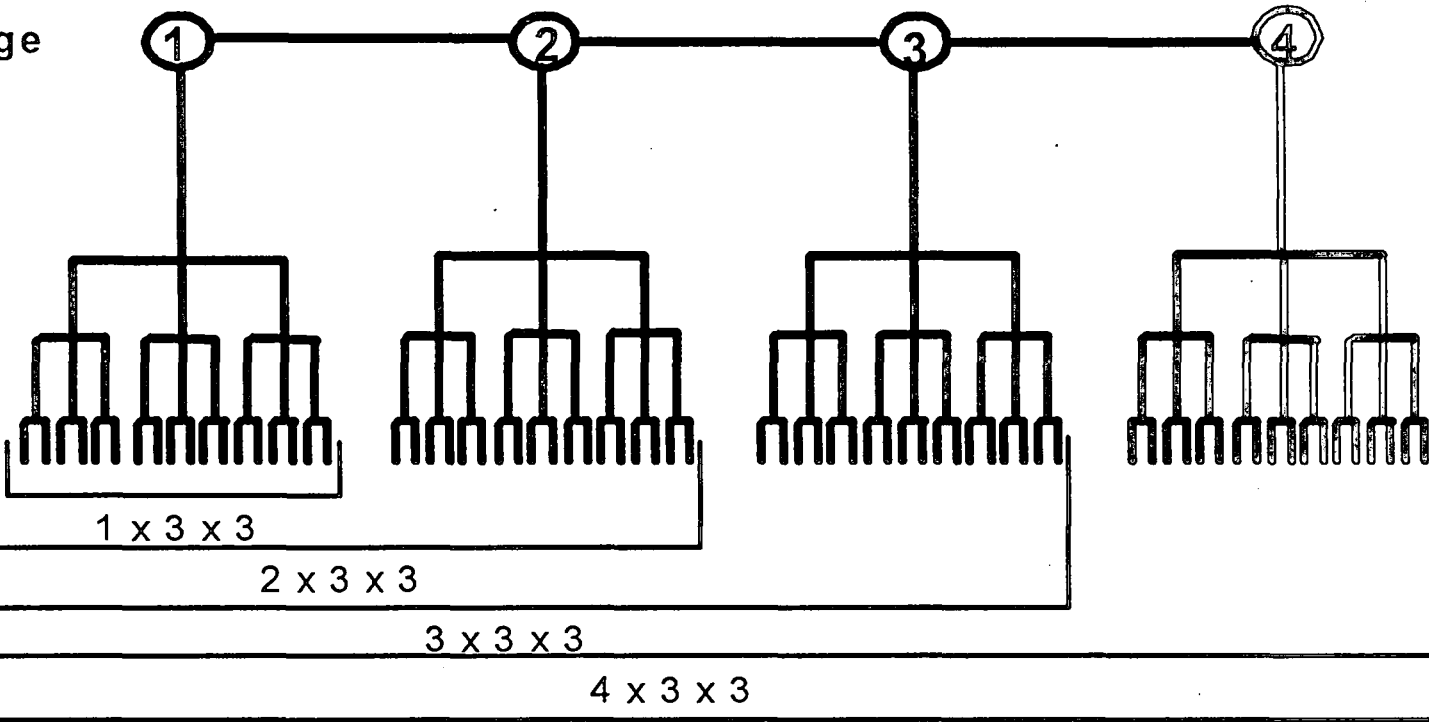
Storage  
time



Cult.

Cryo.

Anal.  
test



1 x 3 x 3

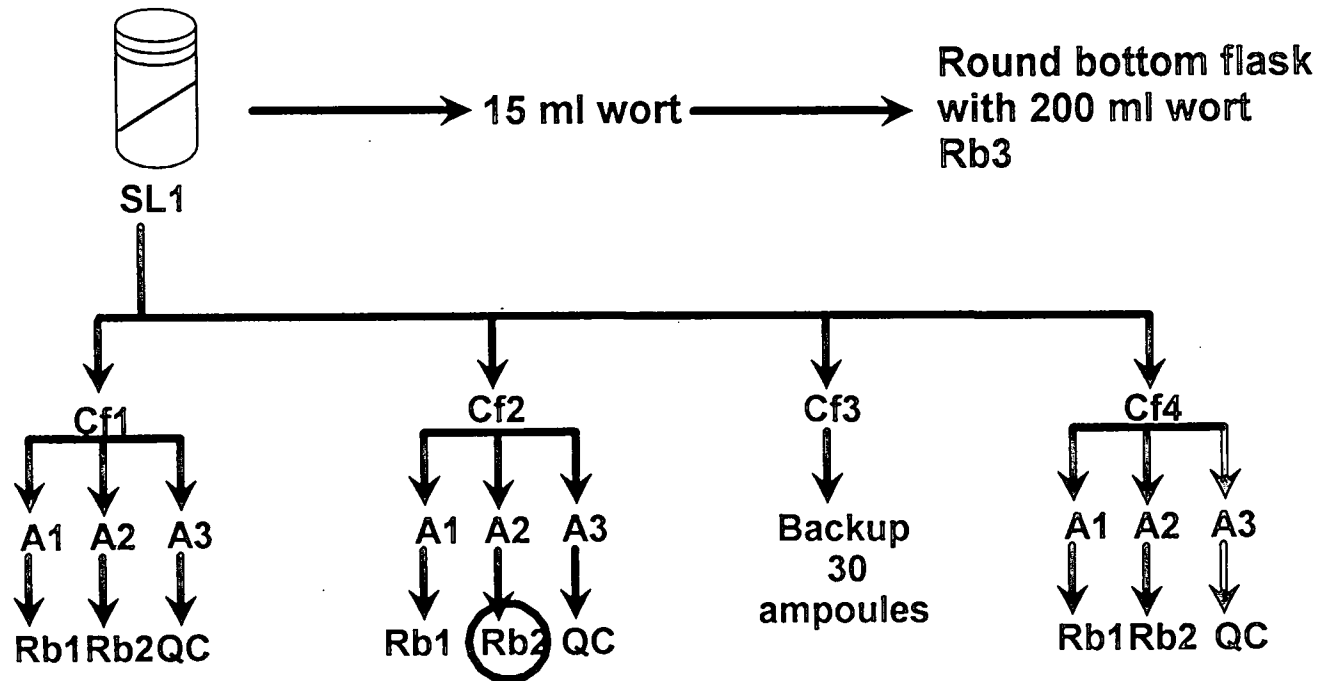
2 x 3 x 3

3 x 3 x 3

4 x 3 x 3

n x 3 x 3

**Experimental design: Experiment 1**



Address example: SL1Cf2A2Rb2

SL: slant; Rb: round bottom flask; Cf: conical flask; A: ampoule; QC: Quality control

## RESULTS OF EXPERIMENT 1: GROWTH MEDIA

WA + TTC = Wort Agar with Triphenyl Tetrazonium Chloride overlay for testing of respiratory deficiency [RD] in yeasts

### Experiment 1.1 Before Cryopreservation

Sample	Address	Dilution	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc. cells.ml <sup>-1</sup>	RD col. in 0.2ml	% RD
Directly from slant	SL1	10 <sup>-3</sup>	5	Tntc	nd	nd	nd
		10 <sup>-4</sup>	5	159	8.75×10 <sup>6</sup>	5	3.1
Round bottom flask, wort, 72h	SL1Rb3	10 <sup>-3</sup>	5	1000	5.00×10 <sup>6</sup>	5	0.5
		10 <sup>-4</sup>	5	149	7.45×10 <sup>6</sup>	2	1.3
Conical flasks, MYGP, 10h, in log. phase	SL1Cf1	10 <sup>-3</sup>	5	582	2.91×10 <sup>6</sup>	6	1.0
		10 <sup>-4</sup>	5	056	2.80×10 <sup>6</sup>	1	1.8
	SL1Cf2	10 <sup>-3</sup>	5	462	2.31×10 <sup>6</sup>	3	0.6
		10 <sup>-4</sup>	5	047	2.35×10 <sup>6</sup>	2	4.3
	SL1Cf3	10 <sup>-3</sup>	5	492	2.46×10 <sup>6</sup>	4	0.8
		10 <sup>-4</sup>	5	055	2.75×10 <sup>6</sup>	0	0.0
	SL1Cf4	10 <sup>-3</sup>	5	464	2.32×10 <sup>6</sup>	4	0.9
		10 <sup>-4</sup>	5	069	3.45×10 <sup>6</sup>	0	0.0

### Experiment 1.2 After 30h cryopreservation

Directly after revival	SL1Cf1A1	10 <sup>-3</sup>	5	Tntc	nd	nd	nd
		10 <sup>-4</sup>	5	222	1.11×10 <sup>7</sup>	4	1.8
	SL1Cf2A1	10 <sup>-3</sup>	5	092	4.60×10 <sup>5</sup>	2	2.2
		10 <sup>-4</sup>	5	013	6.50×10 <sup>5</sup>	0	0.0
	SL1Cf4A1	10 <sup>-3</sup>	5	Tntc	nd	nd	nd
		10 <sup>-4</sup>	5	323	1.62×10 <sup>7</sup>	3	0.9
Round bottom flasks, wort, 72h	SL1Cf1A2Rb1	10 <sup>-3</sup>	5	282	1.41×10 <sup>6</sup>	1	0.4
		10 <sup>-4</sup>	5	030	1.50×10 <sup>6</sup>	0	0.0
	SL1Cf1A3Rb2	10 <sup>-3</sup>	5	243	1.22×10 <sup>6</sup>	2	0.8
		10 <sup>-4</sup>	5	058	2.90×10 <sup>6</sup>	0	0.0
	SL1Cf2A2Rb1	10 <sup>-3</sup>	5	466	2.33×10 <sup>6</sup>	1	0.2
		10 <sup>-4</sup>	5	072	3.60×10 <sup>6</sup>	0	0.0
	SL1Cf2A3Rb2	10 <sup>-3</sup>	5	359	1.80×10 <sup>6</sup>	2	0.6
		10 <sup>-4</sup>	5	060	3.00×10 <sup>6</sup>	0	0.0
	SL1Cf4A2Rb1	10 <sup>-3</sup>	5	741	3.71×10 <sup>6</sup>	0	0.0
		10 <sup>-4</sup>	5	138	6.90×10 <sup>6</sup>	1	0.7
	SL1Cf4A3Rb2	10 <sup>-3</sup>	5	405	2.03×10 <sup>6</sup>	2	0.5
		10 <sup>-4</sup>	5	057	2.85×10 <sup>6</sup>	0	0.0

**WLN = Wallerstein Nutrient Medium for differentiation of wild yeasts [variants], culture yeasts and bacteria**

**Experiment 1.1 Before cryopreservation**

Sample	Address	Dilution	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc. (cells.ml <sup>-1</sup> )	Variant col. in 0.2 ml	% variants
Directly from slant	SL1	10 <sup>-3</sup>	5	Tntc	nd	nd	nd
		10 <sup>-4</sup>	5	179	8.95 × 10 <sup>6</sup>	15	8.4
Round bottom flask, wort, 72h	SL1Rb3	10 <sup>-3</sup>	5	794	3.97 × 10 <sup>6</sup>	71	8.9
		10 <sup>-4</sup>	5	101	5.05 × 10 <sup>6</sup>	08	7.9
Conical flasks, MYGP, 10h, in log. phase	SL1Cf1	10 <sup>-3</sup>	5	333	1.67 × 10 <sup>6</sup>	24	7.2
		10 <sup>-4</sup>	5	063	3.15 × 10 <sup>6</sup>	04	6.3
	SL1Cf2	10 <sup>-3</sup>	5	265	1.33 × 10 <sup>6</sup>	15	5.7
		10 <sup>-4</sup>	5	052	2.60 × 10 <sup>6</sup>	03	5.8
	SL1Cf3	10 <sup>-3</sup>	5	444	2.22 × 10 <sup>6</sup>	32	7.2
		10 <sup>-4</sup>	5	062	3.10 × 10 <sup>6</sup>	04	6.5
	SL1Cf4	10 <sup>-3</sup>	5	569	2.85 × 10 <sup>6</sup>	22	3.9
		10 <sup>-4</sup>	5	064	3.20 × 10 <sup>6</sup>	09	14.1

**Experiment 1.2 After 30h cryopreservation**

Directly after revival	SL1Cf1A1	10 <sup>-3</sup>	5	Tntc	nd	nd	nd
		10 <sup>-4</sup>	5	175	8.75 × 10 <sup>6</sup>	11	6.3
	SL1Cf2A1	10 <sup>-3</sup>	5	117	5.85 × 10 <sup>5</sup>	07	6.0
		10 <sup>-4</sup>	5	010	5.00 × 10 <sup>5</sup>	00	0.0
	SL1Cf4A1	10 <sup>-3</sup>	5	Tntc	nd	nd	nd
		10 <sup>-4</sup>	5	297	1.49 × 10 <sup>7</sup>	15	5.1
Round bottom. flasks, wort, 72h	SL1Cf1A2Rb1	10 <sup>-3</sup>	5	235	1.18 × 10 <sup>6</sup>	16	6.8
		10 <sup>-4</sup>	5	038	1.90 × 10 <sup>6</sup>	01	2.6
	SL1Cf1A3Rb2	10 <sup>-3</sup>	5	251	1.26 × 10 <sup>6</sup>	08	3.2
		10 <sup>-4</sup>	5	025	1.25 × 10 <sup>6</sup>	00	0.0
	SL1Cf2A2Rb1	10 <sup>-3</sup>	5	329	1.65 × 10 <sup>6</sup>	13	4.0
		10 <sup>-4</sup>	5	053	2.65 × 10 <sup>6</sup>	03	5.7
	SL1Cf2A3Rb2	10 <sup>-3</sup>	5	438	2.19 × 10 <sup>6</sup>	29	6.6
		10 <sup>-4</sup>	5	050	2.50 × 10 <sup>6</sup>	05	10.0
	SL1Cf4A2Rb1	10 <sup>-3</sup>	5	755	3.78 × 10 <sup>6</sup>	28	3.7
		10 <sup>-4</sup>	5	119	5.95 × 10 <sup>6</sup>	08	6.7
	SL1Cf4A3Rb2	10 <sup>-3</sup>	5	462	2.31 × 10 <sup>6</sup>	13	2.8
		10 <sup>-4</sup>	5	060	3.00 × 10 <sup>6</sup>	06	10.0

## SDM = Schwartz Differential Medium for differentiation of brewing yeasts from wild yeasts

### Experiment 1.1 Before cryopreservation

Sample	Address	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc.	No. of wild yeast colonies in 0.2ml	% Wild yeast
Directly from slant	SL1	5	-	-	-	-
Round bottom flask wort, 72h	Rb3	5	-	-	-	-
Conical flasks, MYGP, 10h, in log. phase	SL1Cf1	5	-	-	-	-
	SL1Cf2	5	-	-	-	-
	SL1Cf3	5	-	-	-	-
	SL1Cf4	5	-	-	-	-

### Experiment 1.2 After 30h cryopreservation

Directly after revival	SL1Cf1A1	5	-	-	-	-
	SL1Cf1A1	5	-	-	-	-
	SL1Cf1A1	5	-	-	-	-
Round bottom flasks, wort, 72h	SL1Cf1A2Rb1	5	-	-	-	-
	SL1Cf1A3Rb2	5	-	-	-	-
	SL1Cf2A2Rb1	5	-	-	-	-
	SL1Cf2A3Rb2	5	-	-	-	-
	SL1Cf4A2Rb1	5	-	-	-	-
	SL1Cf4A3Rb2	5	-	-	-	-

**LYS = Lysine Medium for differentiation of *Saccharomyces* sp. from non-*Saccharomyces* sp.**

### Experiment 1.1 Before cryopreservation

Sample	Address	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc.	No. of non- <i>Sacch</i> colonies in 0.2 ml	Growth of non- <i>Sacch.</i>
Directly from slant	SL1	5	-	-	-	-
Round bottom flask wort, 72h	SL1Rb3	5	-	-	-	-
Conical flasks, MYGP, 10h, in log. phase	SL1Cf1	5	-	-	-	-
	SL1Cf2	5	-	-	-	-
	SL1Cf3	5	-	-	-	-
	SL1Cf4	5	-	-	-	-

### Experiment 1.2 After 30h cryopreservation

Directly after revival	SL1Cf1A1	5	nd	nd	nd	-
	SL1Cf2A1	5	nd	nd	nd	-
	SL1Cf4A1	5	nd	nd	nd	-
Round bottom flasks, wort, 72h	SL1Cf1A2Rb1	5	nd	nd	nd	-
	SL1Cf1A3Rb2	5	nd	nd	nd	-
	SL1Cf2A2Rb1	5	nd	nd	nd	-
	SL1Cf2A3Rb2	5	nd	nd	nd	-
	SL1Cf4A2Rb1	5	nd	nd	nd	-
	SL1Cf4A3Rb2	5	nd	nd	nd	-



LP = Lactose Peptone Broth for growth of lactose fermenting and lactose assimilating organisms but not strains of *Saccharomyces cerevisiae*

### Experiment 1.1 Before cryopreservation

Sample	Address	Incub. period (days) 30°C	Growth in 0.5 ml
Directly from slant	SL1	4	-
Round bottom flask, wort, 72h	SLRb3	4	-
Conical flasks , MYGP 10h in log. phase	SL1Cf1	4	-
	SL1Cf2	4	-
	SL1Cf3	4	-
	SL1Cf4	4	-

### Experiment 1.2 After 30h cryopreservation

Directly after revival	SL1Cf1A1	4	-
	SL1Cf2A1	4	-
	SL1Cf4A1	4	-
Round bottom flasks wort, 72h	SL1Cf1A2Rb1	4	-
	SL1Cf1A3Rb2	4	-
	SL1Cf2A2Rb1	4	-
	SL1Cf2A3Rb2	4	-
	SL1Cf4A2Rb1	4	-
	SL1Cf4A3Rb2	4	-

**ULM = Universal Liquid Medium for cultivation of brewery  
bacteria**

**Experiment 1.1 Before cryopreservation**

Sample	Address	Incub. period (d) 30°C	Growth in 0.5 ml
Directly from slant	SL1	4	-
Round bottom flask, wort, 72h	Rb3	4	-
Conical flasks, MYGP, 10h, in log. phase	SL1Cf1	4	-
	SL1Cf2	4	-
	SL1Cf3	4	-
	SL1Cf4	4	-

**Experiment 1.2 After 30h cryopreservation**

Directly after revival	SL1Cf1A1	4	-
	SL1Cf2A1	4	-
	SL1Cf4A1	4	-
Round bottom flasks, wort, 72h	SL1Cf1A2Rb1	4	-
	SL1Cf1A3Rb2	4	-
	SL1Cf2A2Rb1	4	-
	SL1Cf2A3Rb2	4	-
	SL1Cf4A2Rb1	4	-
	SL1Cf4A3Rb2	4	-

## RESULTS OF EXPERIMENT 1: GROWTH STUDIES AND YEAST VIABILITY

### Experiment 1.1 Before cryopreservation

Direct from slant (in 9 ml saline) SL1

Total cell concentration:  $93.57 \times 10^6$  cells.ml<sup>-1</sup>

Yeast viability: 98.03 % viable cells

Round bottom flask (slant inoculation) SL1Rb3

Table 1.1.1 Total cell concentration and yeast viability before cryopreservation

Time (h)	Total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> )	Yeast viability (% viable cells)
0	2.54	97.65
2	2.60	85.06
4	3.36	88.10
6	5.76	97.57
8	21.30	99.06
10	38.22	87.50
12	45.40	90.61
14	62.05	92.51
16	72.00	90.28
18	109.50	91.16
20	133.50	93.37
22	185.63	97.31
24	230.00	96.80
26	243.88	97.96
28	262.17	98.71
30	244.75	96.59
32	224.58	95.32
43	261.25	nd
72	268.00	92.31

### Experiment 1.2 After 30h cryopreservation

Table 1.2.1 Total cell concentration and yeast viability after 30h of cryopreservation [SL1Cf1A1; SL1Cf2A1; SL1Cf4A1] directly from ampoule

Ampoule	Total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> )	Yeast viability (% viable cells)
SL1Cf1A1	12.50	94.65
SL1Cf2A1	14.50	99.18
SL1Cf4A1	22.67	95.10

### Experiment 1.2 After 30h cryopreservation

Table 1.2.2 Total cell concentration and yeast viability after 30 h of cryopreservation

[SL1Cf1A2Rb1 and SL1Cf1A3Rb2]

Time (h)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf1A2Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf1A3Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf1A2Rb1	Yeast viability (% viable cells) SL1Cf1A3Rb2	Mean yeast viability
0	1.69	2.07	1.88 (0.19)	99.00	99.50	99.25 (0.25)
2	2.30	2.12	2.21 (0.09)	nd	nd	nd
4	5.11	4.59	4.85 (0.26)	78.38	79.22	78.80 (0.42)
10	28.36	31.76	30.06 (1.70)	91.00	89.93	90.47 (0.54)
12	43.71	37.50	40.61 (3.11)	nd	nd	nd
24	214.50	207.17	210.84 (3.66)	96.00	95.00	95.50 (0.50)
27	250.25	270.67	260.46 (10.21)	nd	nd	nd
30	193.60	183.33	188.47 (5.14)	nd	nd	nd
32	229.17	200.00	214.59 (14.59)	97.50	94.73	96.12 (1.39)
72	226.00	208.00	217.00 (9.00)	98.01	97.34	97.68 (0.34)

Table 1.2.3 Total cell concentration and yeast viability after 30 h of cryopreservation

[SL1Cf2A2Rb1 and SL1Cf2A3Rb2].

Time (h)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf2A2Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf2A3Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf2A2Rb1	Yeast viability (% viable cells) SL1Cf2A3Rb2	Mean yeast viability
0	2.08	1.51	1.80 (0.29)	99.50	99.50	99.50 (0.00)
2	2.16	1.51	1.84 (0.33)	nd	nd	nd
4	3.84	3.82	3.83 (0.01)	46.75	60.42	53.59 (6.84)
9	27.63	32.67	30.15 (2.52)	92.86	88.49	90.68 (2.19)
11	34.00	49.29	41.64 (7.64)	nd	nd	nd
24	308.00	198.92	253.46 (54.54)	96.88	98.28	97.58 (0.70)
26	341.33	238.00	289.67 (51.67)	nd	nd	nd
29	251.00	nd	nd	nd	nd	nd
31	239.56	nd	nd	99.50	nd	nd
72	238.67	nd	nd	99.71	nd	nd

Table 1.2.4 Total cell concentration and yeast viability after 30 h of cryopreservation [SL1Cf4A2Rb1 and SL1Cf4A3Rb2]

Time (h)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf4A2Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf4A3Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf4A2Rb1	Yeast viability (% viable cells) SL1Cf4A3Rb2	Mean yeast viability
0	1.84	2.54	2.19 (0.35)	99.50	99.50	99.50 (0.00)
2	2.70	3.01	2.86 (0.16)	nd	nd	nd
4	3.04	4.74	3.89 (0.85)	36.36	59.02	47.69 (11.33)
9	27.00	28.25	27.63 (0.63)	92.86	88.49	90.68 (2.19)
11	42.88	39.73	41.30 (1.58)	nd	nd	nd
24	192.50	290.13	241.31 (48.8)	97.95	98.00	97.98 (0.03)
26	251.00	283.20	267.10 (16.10)	nd	nd	nd
29	302.00	260.00	281.00 (21.00)	nd	nd	nd
31	291.00	285.00	288.00 (3.00)	96.73	95.07	95.90 (0.83)
72	336.00	268.00	302.00 (34.00)	99.51	95.99	97.75 (1.76)

Table 1.2.5 Mean and standard deviation of the total cell concentration after 30h of cryopreservation [SL1Cf1A2Rb1, SL1Cf1A3Rb2; SL1Cf2A2Rb1, SL1Cf2A3Rb2, SL1Cf4A2Rb1 and SL1Cf4A3Rb2]

Time (hours)	Mean of total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Standard deviation (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )
0	1.94	0.33
2	2.30	0.52
4	4.19	0.76
9	28.89	2.57
10	30.06	1.70
11	41.48	6.38
12	40.61	3.11
24	235.20	50.34
26	278.38	46.07
27	260.46	10.21
29	271.00	27.22
30	188.47	5.14
31	271.85	28.13
32	214.59	14.59
72	255.33	50.12

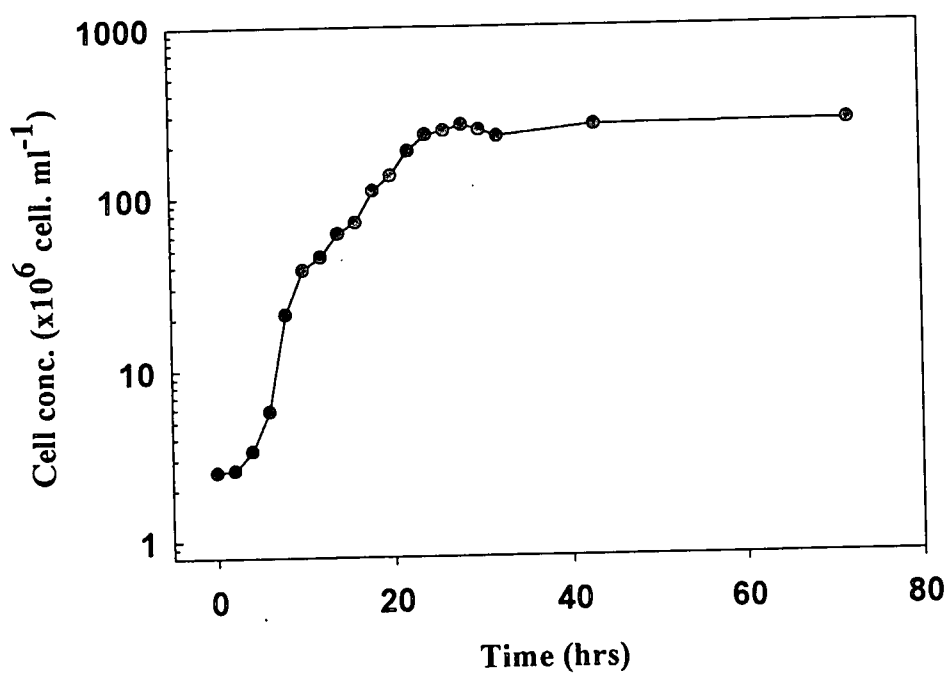


Fig. 1.1 Growth curve of the yeast before cryopreservation (SL1Rb3)

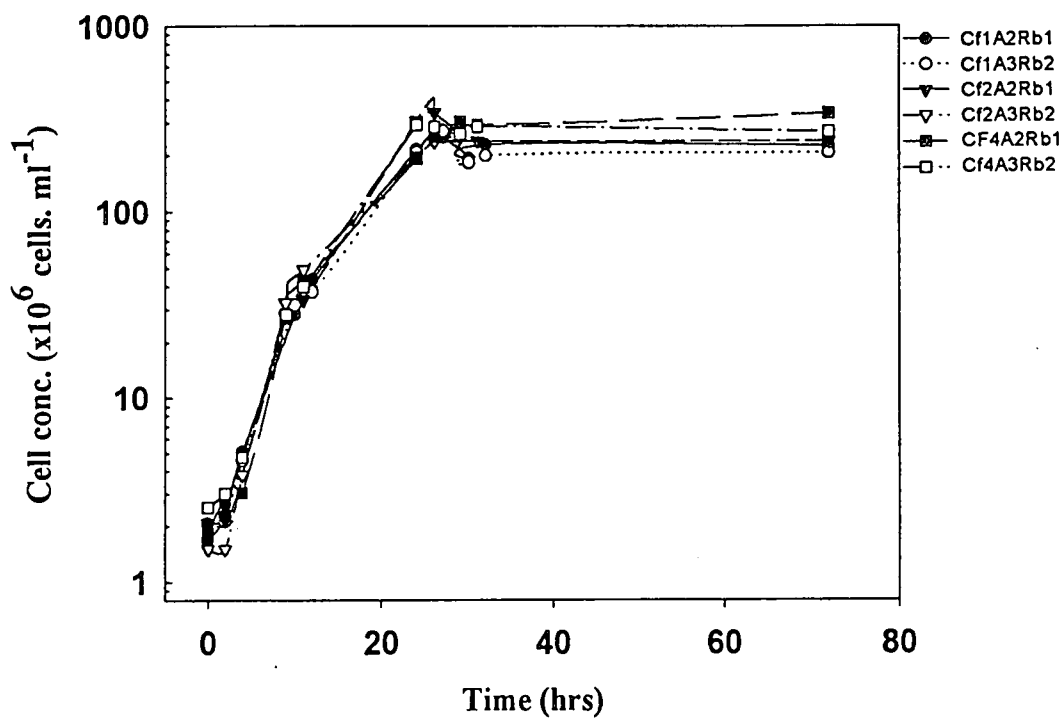


Fig. 1.2 Growth curves of yeasts from ampoules (SL1Cf1A2Rb1, SL1Cf1A2Rb2, SL1Cf2A2Rb1, SL1Cf2A3Rb2, SL1Cf4A2Rb1, S1Cf4A3Rb2) after 30h cryopreservation

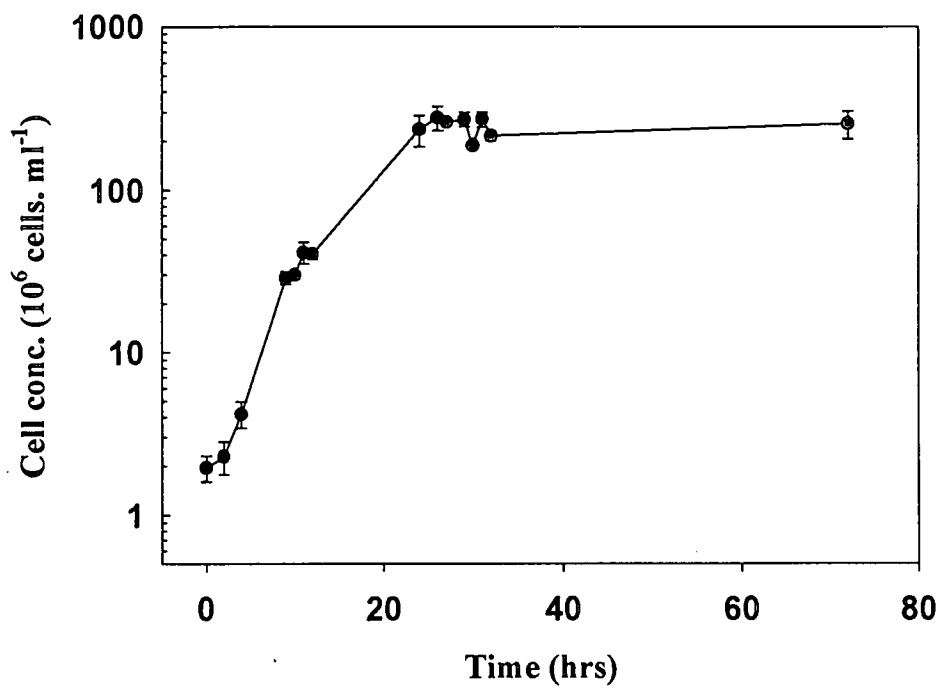


Fig. 1.3 Mean growth curve after 30h cryopreservation



**NOVEMBER 1997**

**CRYO-TIME: 17 days**

## EXPERIMENT 2

### Cryopreservation of standardised yeast inoculum

#### Variability in cryopreservation methodology

In this second experiment (Exp. 2), the effect of cryopreservation after 17 days on brewery yeast viability was investigated. According to the results, no contamination on ULM, LP and SDM media could be detected at any stage of analysis after cryopreservation. On lysine medium, very small colonies were observed on all plates after revival when grown on wort probably due to carry over of nitrogen from wort medium. Seventeen days of cryopreservation had no significant influence on growth curves as well as yeast viability in all repetitions performed (see Growth Studies and Yeast Viability: Exp. 2.1 and 2.2 tables and figures).

# Nested Experimental Design

Responses  
viability  
stability  
contamination

Variables

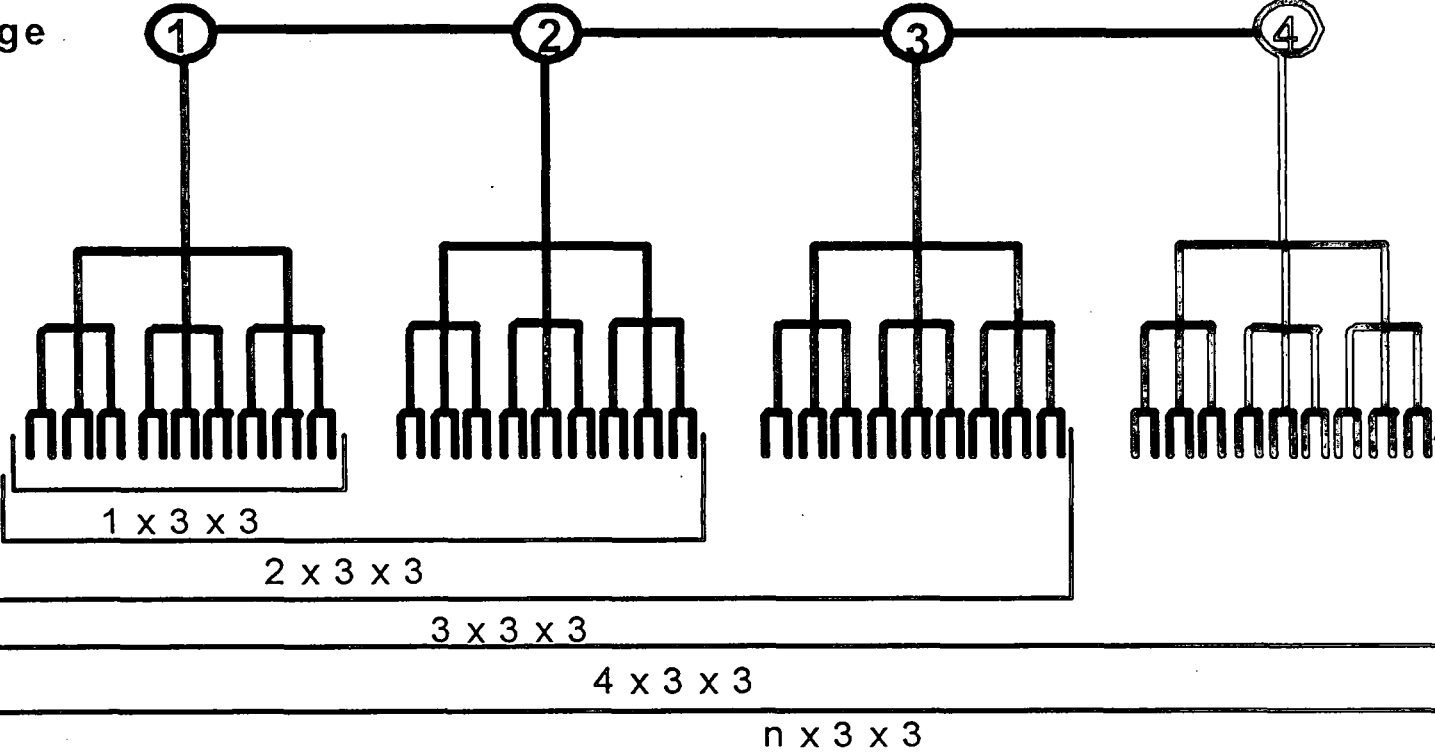
Storage  
time



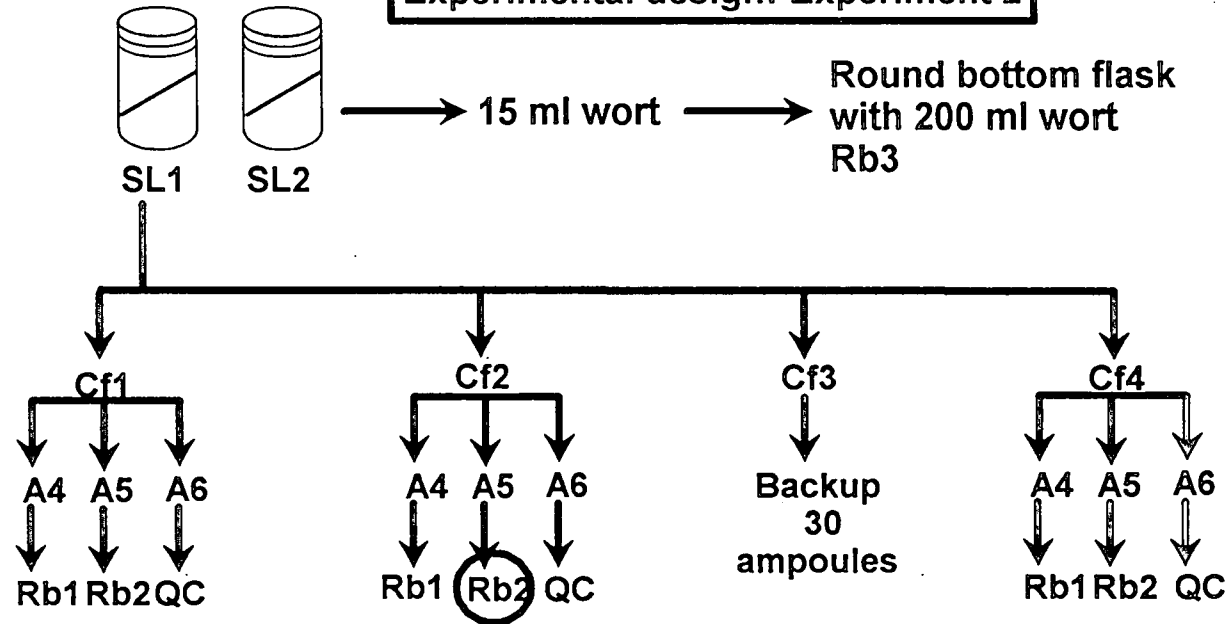
Cult.

Cryo.

Anal.  
test



**Experimental design: Experiment 2**



Address example: SL1Cf2A5Rb2

SL: slant; Rb: round bottom flask; Cf: conical flask; A: ampoule;

Quality control

## RESULTS OF EXPERIMENT 2: GROWTH MEDIA

WA + TTC = Wort Agar with Triphenyl Tetrazonium Chloride overlay for testing of respiratory deficiency [RD] in yeasts

### Experiment 2. After 17 days cryopreservation

Sample	Address	Dilution	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc. (cells.ml <sup>-1</sup> )	RD col. in 0.2ml	% RD
Directly after revival	SL1Cf1A4	10 <sup>-3</sup>	5	341	1.7×10 <sup>6</sup>	2	0.6
		10 <sup>-4</sup>	5	026	1.3×10 <sup>6</sup>	0	0.0
	SL1Cf2A4	10 <sup>-3</sup>	5	447	2.2×10 <sup>6</sup>	7	1.6
		10 <sup>-4</sup>	5	146	7.3×10 <sup>6</sup>	2	1.4
	SL1Cf4A4	10 <sup>-3</sup>	5	463	2.3×10 <sup>6</sup>	2	0.4
		10 <sup>-4</sup>	5	117	5.9×10 <sup>6</sup>	0	0.0
Round bottom flasks, wort, 72h	SL1Cf1A5Rb1	10 <sup>-3</sup>	5	487	2.4×10 <sup>6</sup>	8	1.6
		10 <sup>-4</sup>	5	037	1.9×10 <sup>6</sup>	0	0.0
	SL1Cf1A6Rb2	10 <sup>-3</sup>	5	267	1.3×10 <sup>6</sup>	7	2.6
		10 <sup>-4</sup>	5	030	1.5×10 <sup>6</sup>	1	3.3
	SL1Cf2A5Rb1	10 <sup>-3</sup>	5	266	1.3×10 <sup>6</sup>	6	2.3
		10 <sup>-4</sup>	5	028	1.4×10 <sup>6</sup>	0	0.0
	SL1Cf2A6Rb2	10 <sup>-3</sup>	5	310	1.6×10 <sup>6</sup>	8	2.6
		10 <sup>-4</sup>	5	053	2.7×10 <sup>6</sup>	0	0.0
	SL1Cf4A5Rb1	10 <sup>-3</sup>	5	363	1.8×10 <sup>6</sup>	8	2.2
		10 <sup>-4</sup>	5	053	2.7×10 <sup>6</sup>	1	1.9
	SL1Cf4A6Rb2	10 <sup>-3</sup>	5	336	1.7×10 <sup>6</sup>	8	2.4
		10 <sup>-4</sup>	5	042	2.1×10 <sup>6</sup>	0	0.0

WLN = Wallerstein Nutrient Medium for differentiation of wild yeasts [variants], culture yeasts and bacteria

### Experiment 2. After 17days cryopreservation

Sample	Address	Dilution	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc. (cells.ml <sup>-1</sup> )	Variant col. in 0.2 ml	% variants
Directly after revival	SL1Cf1A4	10 <sup>-3</sup>	5	320	1.6×10 <sup>6</sup>	14	4.4
		10 <sup>-4</sup>	5	054	2.7×10 <sup>6</sup>	06	11.1
	SL1Cf2A4	10 <sup>-3</sup>	5	420	2.1×10 <sup>6</sup>	19	4.5
		10 <sup>-4</sup>	5	071	3.6×10 <sup>6</sup>	03	4.2
	SL1Cf4A4	10 <sup>-3</sup>	5	487	2.4×10 <sup>6</sup>	21	4.3
		10 <sup>-4</sup>	5	082	4.1×10 <sup>6</sup>	03	3.7
Round bottom. flasks, wort, 72h	SL1Cf1A5Rb1	10 <sup>-3</sup>	5	337	1.7×10 <sup>6</sup>	39	11.6
		10 <sup>-4</sup>	5	051	2.6×10 <sup>6</sup>	02	3.9
	SL1Cf1A6Rb2	10 <sup>-3</sup>	5	177	8.9×10 <sup>6</sup>	14	7.9
		10 <sup>-4</sup>	5	026	1.3×10 <sup>6</sup>	02	7.7
	SL1Cf2A5Rb1	10 <sup>-3</sup>	5	233	1.2×10 <sup>6</sup>	18	7.7
		10 <sup>-4</sup>	5	022	1.1×10 <sup>6</sup>	02	9.1
	SL1Cf2A6Rb2	10 <sup>-3</sup>	5	242	1.2×10 <sup>6</sup>	22	9.1
		10 <sup>-4</sup>	5	032	1.6×10 <sup>6</sup>	02	6.3
	SL1Cf4A5Rb1	10 <sup>-3</sup>	5	242	1.2×10 <sup>6</sup>	16	6.6
		10 <sup>-4</sup>	5	036	1.8×10 <sup>6</sup>	04	11.1
	SL1Cf4A6Rb2	10 <sup>-3</sup>	5	292	1.5×10 <sup>6</sup>	26	8.9
		10 <sup>-4</sup>	5	048	2.4×10 <sup>6</sup>	03	6.3

**SDM = Schwartz Differential Medium for differentiation of  
brewing yeasts from wild yeasts**

**Experiment 2. After 17 days cryopreservation**

Sample	Address	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc.	No. of wild yeast colonies in 0.2ml	% Wild yeast
Directly after revival	SL1Cf1A1	5	nd	nd	nd	nd
	SL1Cf2A1	5	nd	nd	nd	nd
	SL1Cf4A1	5	nd	nd	nd	nd
Round bottom flasks, wort, 72h	SL1Cf1A2Rb1	5	-	-	-	-
	SL1Cf1A3Rb2	5	-	-	-	-
	SL1Cf2A2Rb1	5	-	-	-	-
	SL1Cf2A3Rb2	5	-	-	-	-
	SL1Cf4A2Rb1	5	-	-	-	-
	SL1Cf4A3Rb2	5	-	-	-	-

**LYS = Lysine Medium for differentiation of *Saccharomyces* sp. from non-*Saccharomyces* sp.**

**Experiment 2. After 17 days cryopreservation**

Sample	Address	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc.	No. of non- <i>Sacch</i> colonies in 0.2 ml	% non- <i>Sacch.</i>
Directly after revival	SL1Cf1A1	5	nd	nd	nd	nd
	SL1Cf2A1	5	nd	nd	nd	nd
	SL1Cf4A1	5	nd	nd	nd	nd
Round bottom flasks, wort, 72h	SL1Cf1A2Rb1	5	-	-	-	-
	SL1Cf1A3Rb2	5	-	-	-	-
	SL1Cf2A2Rb1	5	-	-	-	-
	SL1Cf2A3Rb2	5	-	-	-	-
	SL1Cf4A2Rb1	5	-	-	-	-
	SL1Cf4A3Rb2	5	-	-	-	-



LP = Lactose Peptone Broth for growth of lactose fermenting and lactose assimilating organisms but not strains of *Saccharomyces cerevisiae*

**Experiment 2. After 17 days cryopreservation**

Sample	Address	Incub. period (days) 30°C	Growth in 0.5 ml
Directly after revival	SL1Cf1A1	4	nd
	SL1Cf2A1	4	nd
	SL1Cf4A1	4	nd
Round bottom flasks wort, 72h	SL1Cf1A2Rb1	4	-
	SL1Cf1A3Rb2	4	-
	SL1Cf2A2Rb1	4	-
	SL1Cf2A3Rb2	4	-
	SL1Cf4A2Rb1	4	-
	SL1Cf4A3Rb2	4	-

**ULM = Universal Liquid Medium for cultivation of brewery bacteria**

**Experiment 2. After 17 days cryopreservation**

Sample	Address	Incub. period (days) 30°C	Growth in 0.5 ml
Directly after revival	SL1Cf1A1	4	nd
	SL1Cf2A1	4	nd
	SL1Cf4A1	4	nd
Round bottom flasks, wort, 72h	SL1Cf1A2Rb1	4	-
	SL1Cf1A3Rb2	4	-
	SL1Cf2A2Rb1	4	-
	SL1Cf2A3Rb2	4	-
	SL1Cf4A2Rb1	4	-
	SL1Cf4A3Rb2	4	-

## RESULTS OF EXPERIMENT 2:GROWTH STUDIES AND YEAST VIABILITY

### Experiment 2.1 Before cryopreservation

#### Round bottom flask (slant inoculation) SL2Rb3

Table 2.1.1 Total cell concentration and yeast viability before cryopreservation

Time (h)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells)
0	4.84	97.75
2	5.72	95.06
4	7.61	98.15
6	19.83	98.46
8	39.83	98.13
10	60.60	95.41
24	228.00	95.85
27	336.00	98.15
29	347.00	98.35
52	272.00	98.67
72	328.00	98.79

### Experiment 2.2 After 17 days cryopreservation

Table 2.2.1 Total cell concentration and yeast viability after 87 days of cryopreservation (directly from ampoule).

Ampoule	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells)
SL1Cf1A4	nd	55.17
SL1Cf2A4	nd	70.00
SL1Cf4A4	nd	99.00

Table 2.2.2 Total cell concentration and yeast viability after 17 days of cryopreservation [SL1Cf1A5Rb1 and SL1Cf1A6Rb2]

Time (h)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf1A5Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf1A6Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf1A5Rb1	Yeast viability (% viable cells) SL1Cf1A6Rb2	Mean yeast viability
0	2.75	3.08	2.92 (0.17)	98.07	99.00	98.54 (0.47)
2	3.33	3.64	3.49 (0.16)	nd	nd	nd
4	8.14	6.72	7.43 (0.71)	98.43	99.28	98.86 (0.43)
6	18.75	13.30	16.03 (2.73)	nd	nd	nd
8	23.50	23.40	23.45 (0.05)	99.57	95.23	97.40 (2.17)
10	63.90	43.07	53.49 (10.42)	nd	nd	nd
24	236.00	160.00	198.00 (38.00)	97.25	95.46	96.36 (0.90)
27	405.33	230.00	317.67 (87.67)	nd	nd	nd
29	320.00	305.33	312.67 (7.34)	98.47	96.34	97.41 (1.07)
52	308.00	268.00	288.00 (20.00)	nd	nd	nd
72	349.33	297.33	323.33 (26.00)	98.87	98.67	98.77 (0.10)

Table 2.2.3 Total cell concentration and yeast viability after 17 days of cryopreservation [SL1Cf2A5Rb1 and SL1Cf2A6Rb2]

Time (h)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf2A5Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf2A6Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf2A5Rb1	Yeast viability (% viable cells) SL1Cf2A6Rb2	Mean yeast viability
0	1.24	1.11	1.18 (0.07)	98.23	97.51	97.87 (0.36)
2	4.18	6.81	5.50 (1.32)	nd	nd	nd
4	10.33	9.92	10.13 (0.21)	96.58	91.28	93.93 (2.65)
6	18.42	16.56	17.49 (0.93)	nd	nd	nd
8	33.33	29.20	31.27 (2.07)	98.46	98.59	98.53 (0.07)
10	62.10	58.75	60.43 (1.68)	nd	nd	nd
24	390.67	180.80	285.74 (104.94)	99.28	98.54	98.91 (0.37)
27	393.33	320.00	356.67 (36.67)	nd	nd	nd
29	328.00	309.60	318.80 (9.20)	98.25	97.88	98.07 (0.19)
52	317.33	272.00	294.67 (22.67)	nd	nd	nd
72	207.20	278.00	242.60 (35.40)	98.11	98.51	98.31 (0.20)

Table 2.2.4 Total cell concentration and yeast viability after 17 days of cryopreservation [SL1Cf4A5Rb1 and SL1Cf4A6Rb2]

Time (h)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf4A5Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf4A6Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf4A5Rb1	Yeast viability (% viable cells) SL1Cf4A6Rb2	Mean yeast viability
0	3.57	3.49	3.53 (0.04)	98.23	97.99	98.11 (0.12)
2	3.57	4.33	3.95 (0.38)	nd	nd	nd
4	8.83	7.34	8.10 (0.75)	97.46	99.20	98.33 (0.87)
6	13.25	10.20	11.73 (1.53)	nd	nd	nd
8	51.83	34.13	42.98 (8.85)	98.66	98.34	98.50 (0.16)
10	58.63	43.50	51.07 (7.57)	nd	nd	nd
24	370.67	278.67	324.67 (46.00)	98.45	98.17	98.31 (0.14)
27	363.00	238.40	300.70 (62.30)	nd	nd	nd
29	294.67	408.00	351.34 (56.67)	97.01	95.42	96.22 (0.80)
52	231.00	350.00	290.50 (59.50)	nd	nd	nd
72	289.33	306.67	298.00 (8.67)	98.64	98.71	98.68 (0.04)

Table 2.2.5 Mean and standard deviation of the total cell concentration after 17 days of cryopreservation [SL1Cf1A5Rb1, SL1Cf1A6Rb2, SL1Cf2A5Rb1, SL1Cf2A6Rb2, SL1Cf4A5Rb1 and SL1Cf4A6Rb2]

Time (h)	Mean of total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Standard deviation (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )
0	2.54	1.10
2	4.31	1.29
4	8.46	1.42
6	15.08	3.38
8	32.57	10.50
10	54.99	9.29
24	269.47	95.90
27	325.01	76.31
29	327.60	41.05
52	291.06	42.32
72	287.98	46.53

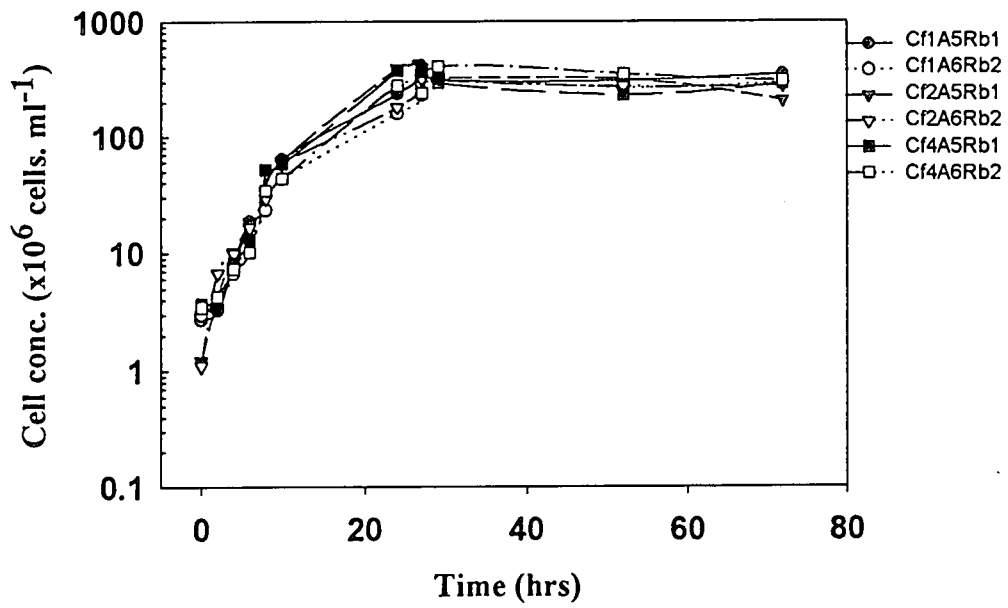


Fig. 2.1 Growth curves of yeasts from ampoules (SL1Cf1A5Rb1, SL1Cf1A6Rb2, SL1Cf2A5Rb1, SL1Cf2A6Rb2, SL1Cf4A5Rb1, SL1Cf4A6Rb2) after 17 days cryopreservation

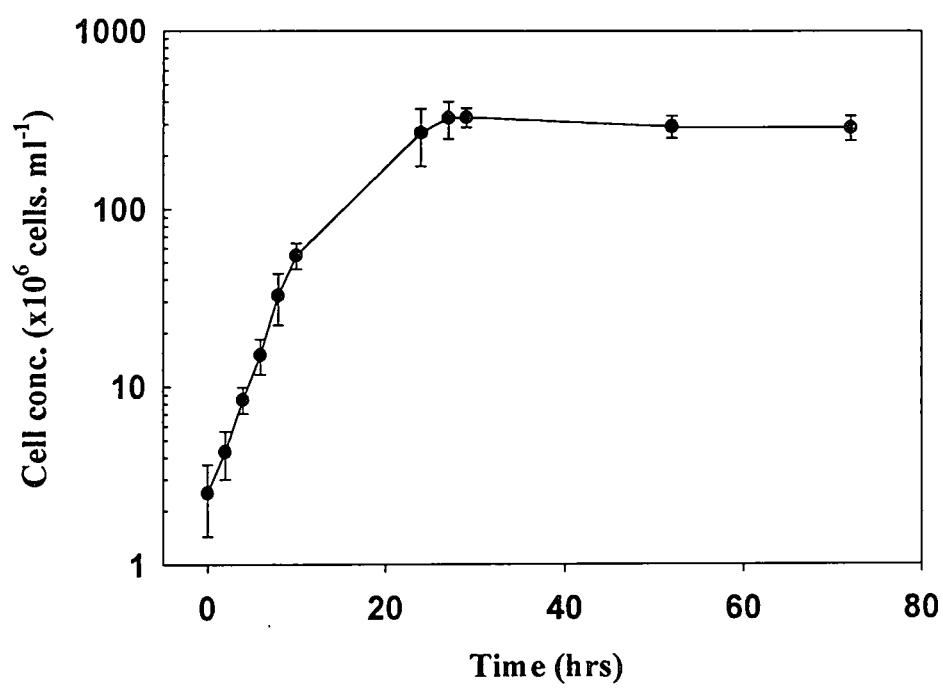


Fig. 2.2 Mean growth curve after 17 days cryopreservation

**JANUARY 1998**

**CRYO-TIME: 87 days**



## EXPERIMENT 3

### Cryopreservation of standardised yeast inoculum

#### Variability in cryopreservation methodology

In this third experiment (Exp. 3), the effect of cryopreservation after 87 days on brewery yeast viability was investigated. According to the results, no contamination on ULM, LP and SDM media could be detected at any stage of analysis after cryopreservation. A new lysine medium was used and no colonies were observed as was reported in experiment 2. Eighty seven days of cryopreservation had no significant influence on growth curves as well as yeast viability in all repetitions performed (see: Growth Studies and Yeast Viability: Exp. 3 tables and figures).

# Nested Experimental Design

Variables

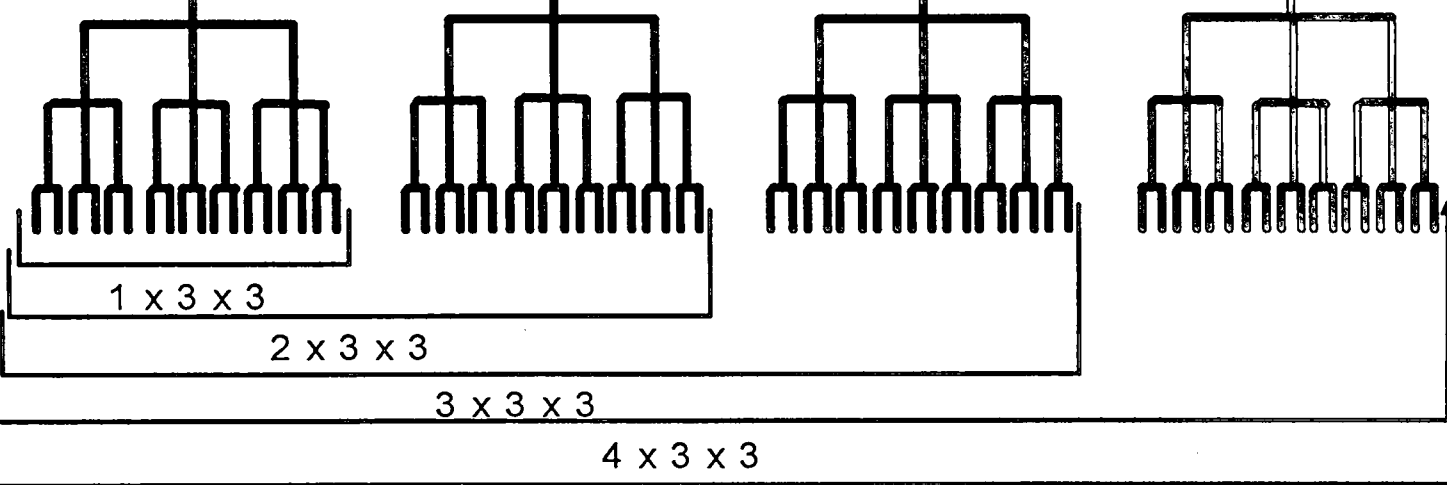
Responses  
viability  
stability  
contamination

Storage  
time

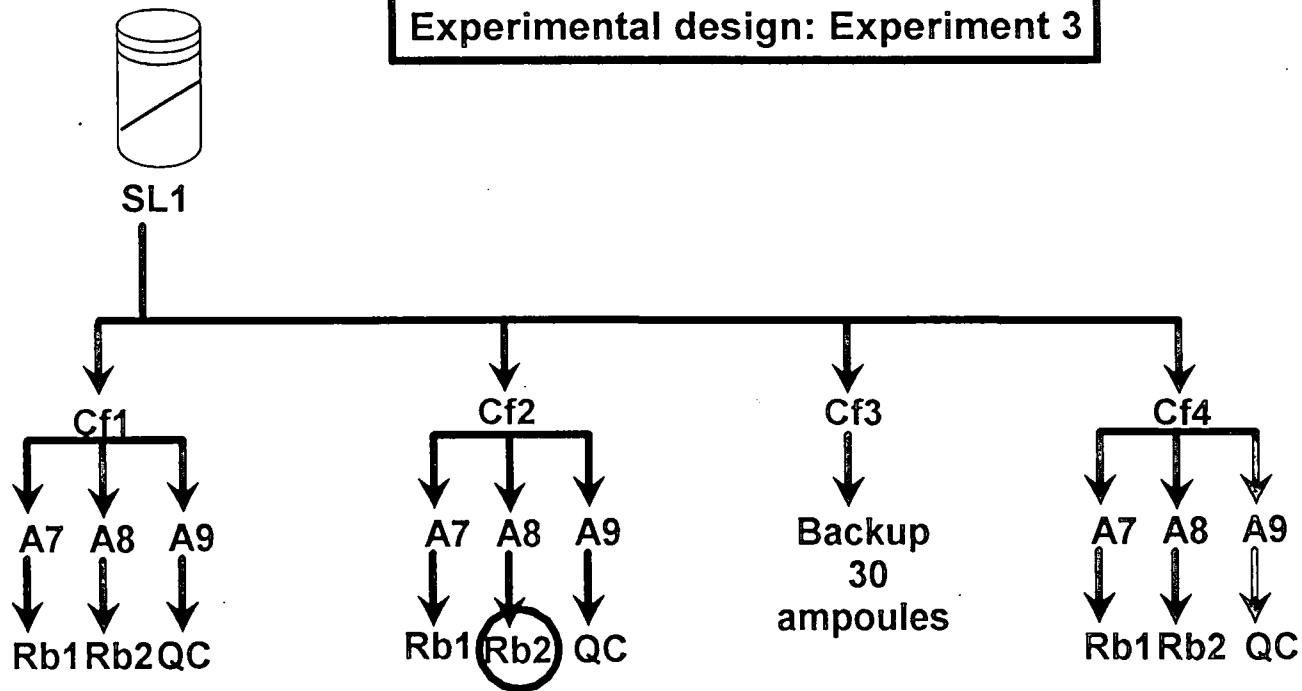
Cult.

Cryo.

Anal.  
test



**Experimental design: Experiment 3**



Address example: SL1Cf2A8Rb2

SL: slant; Rb: round bottom flask; Cf: conical flask; A: ampoule; QC: Quality control

## RESULTS OF EXPERIMENT 3: GROWTH MEDIA

WA + TTC = Wort Agar with Triphenyl Tetrazonium Chloride overlay for testing of respiratory deficiency [RD] in yeasts

### Experiment 3. After 87 days cryopreservation

Sample	Address	Dilution	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc. (cells.ml <sup>-1</sup> )	RD col. in 0.2ml	% RD
Directly after revival	SL1Cf1A9	10 <sup>-3</sup>	5	069	3.5×10 <sup>5</sup>	1	1.4
		10 <sup>-4</sup>	5	011	5.5×10 <sup>5</sup>	0	0.0
	SL1Cf2A9	10 <sup>-3</sup>	5	179	9.0×10 <sup>5</sup>	1	0.6
		10 <sup>-4</sup>	5	081	4.1×10 <sup>6</sup>	1	1.2
	SL1Cf4A9	10 <sup>-3</sup>	5	160	8.0×10 <sup>5</sup>	1	0.6
		10 <sup>-4</sup>	5	010	5.0×10 <sup>5</sup>	0	0.0
Round bottom flasks, wort, 72h	SL1Cf1A7Rb1	10 <sup>-3</sup>	5	494	2.5×10 <sup>6</sup>	8	1.6
		10 <sup>-4</sup>	5	082	4.1×10 <sup>6</sup>	1	1.2
	SL1Cf1A8Rb2	10 <sup>-3</sup>	5	216	1.1×10 <sup>6</sup>	7	3.2
		10 <sup>-4</sup>	5	042	2.1×10 <sup>6</sup>	1	2.4
	SL1Cf2A7Rb1	10 <sup>-3</sup>	5	397	2.0×10 <sup>6</sup>	1	0.3
		10 <sup>-4</sup>	5	054	2.7×10 <sup>6</sup>	1	1.9
	SL1Cf2A8Rb2	10 <sup>-3</sup>	5	nd	nd	nd	nd
		10 <sup>-4</sup>	5	nd	nd	nd	nd
	SL1Cf4A7Rb1	10 <sup>-3</sup>	5	048	2.4×10 <sup>5</sup>	1	2.1
		10 <sup>-4</sup>	5	018	9.0×10 <sup>5</sup>	1	5.6
	SL1Cf4A8Rb2	10 <sup>-3</sup>	5	845	4.2×10 <sup>6</sup>	3	0.4
		10 <sup>-4</sup>	5	152	7.6×10 <sup>6</sup>	2	1.3

WLN = Wallerstein Nutrient Medium for differentiation of wild yeasts [variants], culture yeasts and bacteria

### Experiment 3. After 87 days cryopreservation

Sample	Address	Dilution	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc. (cells.ml <sup>-1</sup> )	Variant col. in 0.2 ml	% variants
Directly after revival	SL1Cf1A9	10 <sup>-3</sup>	5	066	3.3×10 <sup>5</sup>	04	6.1
		10 <sup>-4</sup>	5	004	2.0×10 <sup>5</sup>	00	0.0
	SL1Cf2A9	10 <sup>-3</sup>	5	201	1.0×10 <sup>6</sup>	12	6.0
		10 <sup>-4</sup>	5	022	1.1×10 <sup>6</sup>	00	0.0
	SL1Cf4A9	10 <sup>-3</sup>	5	236	1.2×10 <sup>6</sup>	14	5.9
		10 <sup>-4</sup>	5	049	2.5×10 <sup>6</sup>	01	2.0
Round bottom flasks, wort, 72h	SL1Cf1A7Rb1	10 <sup>-3</sup>	5	504	2.5×10 <sup>6</sup>	60	11.9
		10 <sup>-4</sup>	5	101	5.1×10 <sup>6</sup>	09	08.9
	SL1Cf1A8Rb2	10 <sup>-3</sup>	5	169	8.5×10 <sup>5</sup>	17	10.1
		10 <sup>-4</sup>	5	020	1.0×10 <sup>6</sup>	03	15.0
	SL1Cf2A7Rb1	10 <sup>-3</sup>	5	417	2.1×10 <sup>6</sup>	46	11.0
		10 <sup>-4</sup>	5	056	2.8×10 <sup>6</sup>	02	03.6
	SL1Cf2A8Rb2	10 <sup>-3</sup>	5	nd	nd	nd	nd
		10 <sup>-4</sup>	5	nd	nd	nd	nd
	SL1Cf4A7Rb1	10 <sup>-3</sup>	5	044	2.2×10 <sup>5</sup>	05	11.4
		10 <sup>-4</sup>	5	007	3.5×10 <sup>5</sup>	00	00.0
	SL1Cf4A8Rb2	10 <sup>-3</sup>	5	946	4.7×10 <sup>6</sup>	68	07.2
		10 <sup>-4</sup>	5	105	5.3×10 <sup>6</sup>	11	10.5

**SDM = Schwartz Differential Medium for differentiation of brewing yeasts from wild yeasts.**

**Experiment 3. After 87 days cryopreservation**

Sample	Address	Incub. period (days)] 25°C	Col. in 0.2 ml	Cell conc.	No. of wild yeast colonies in 0.2ml	% Wild yeast
Directly after revival	SL1Cf1A9	5	nd	nd	nd	nd
	SL1Cf2A9	5	nd	nd	nd	nd
	SL1Cf4A9	5	nd	nd	nd	nd
Round bottom flasks, wort, 72h	SL1Cf1A7Rb1	5	-	-	-	-
	SL1Cf1A8Rb2	5	-	-	-	-
	SL1Cf2A7Rb1	5	-	-	-	-
	SL1Cf2A8Rb2	5	nd	nd	nd	nd
	SL1Cf4A7Rb1	5	-	-	-	-
	SL1Cf4A8Rb2	5	-	-	-	-

**LYS = Lysine Medium for differentiation of *Saccharomyces* sp. from non-*Saccharomyces* sp.**

**Experiment 3. After 87 days cryopreservation**

Sample	Address	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc.	No. of non- <i>Sacch</i> colonies in 0.2 ml	% non- <i>Sacch.</i>
Directly after revival	SL1Cf1A9	5	nd	nd	nd	nd
	SL1Cf2A9	5	nd	nd	nd	nd
	SL1Cf4A9	5	nd	nd	nd	nd
Round bottom flasks, wort, 72h	SL1Cf1A7Rb1	5	-	-	-	-
	SL1Cf1A8Rb2	5	-	-	-	-
	SL1Cf2A7Rb1	5	-	-	-	-
	SL1Cf2A8Rb2	5	nd	nd	nd	nd
	SL1Cf4A7Rb1	5	-	-	-	-
	SL1Cf4A8Rb2	5	-	-	-	-

LP = Lactose Peptone Broth for growth of lactose fermenting and lactose assimilating organisms but not strains of *Saccharomyces cerevisiae*

### Experiment 3. After 87 days cryopreservation

Sample	Address	Incub. period (days) 30°C	Growth in 0.5 ml
Directly after revival	SL1Cf1A9	5	nd
	SL1Cf2A9	5	nd
	SL1Cf4A9	5	nd
Round bottom flasks wort, 72h	SL1Cf1A7Rb1	4	-
	SL1Cf1A8Rb2	4	-
	SL1Cf2A7Rb1	4	-
	SL1Cf2A8Rb2	4	nd
	SL1Cf4A7Rb1	4	-
	SL1Cf4A8Rb2	4	-



**ULM = Universal Liquid Medium for cultivation of brewery  
bacteria**

**Experiment 3 After 87 days cryopreservation**

Sample	Address	Incub. period (days) 30°C	Growth in 0.5 ml
Directly after revival	SL1Cf1A9	4	nd
	SL1Cf2A9	4	nd
	SL1Cf4A9	4	nd
Round bottom flasks, wort, 72h	SL1Cf1A7Rb1	4	-
	SL1Cf1A8Rb2	4	-
	SL1Cf2A7Rb1	4	-
	SL1Cf2A8Rb2	4	nd
	SL1Cf4A7Rb1	4	-
	SL1Cf4A8Rb2	4	-

## RESULTS OF EXPERIMENT 3: GROWTH STUDIES AND YEAST VIABILITY

### Experiment 3.1. After 87 days of cryopreservation

Table 3.1.1 Total cell concentration and yeast viability after 87 days of cryopreservation (directly from ampoule)

Ampoule	Total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> )	Yeast viability (% viable cells)
SL1Cf1A9	21.20	nd
SL1Cf1A13	27.64	97.49
SL1Cf2A9	28.80	nd
SL1Cf4A9	38.00	nd

Table 3.1.2 Total cell concentration and yeast viability after 87 days of cryopreservation [Culture flask 1, ampoules 7 & 8: SL1Cf1A7Rb1 and SL1Cf1A8Rb2]

Time (h.)	Total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> ) SL1Cf1A7Rb1	Total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> ) SL1Cf1A8Rb2	Mean total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf1A7Rb1	Yeast viability (% viable cells) SL1Cf1A8Rb2	Mean yeast viability
0	0.46	0.66	0.56 (0.10)	52.94	77.78	65.36 (12.42)
2	0.50	0.67	0.59 (0.09)	57.14	82.86	70.00 (12.86)
4	1.11	0.79	0.95 (0.16)	nd	nd	nd
6	2.39	2.75	2.57 (0.18)	89.79	89.26	89.53 (0.27)
8	6.40	4.14	5.27 (1.13)	nd	nd	nd
20	149.33	107.50	128.42 (20.92)	80.72	98.50	89.61 (8.89)
23	107.50	112.50	110.00 (2.50)	nd	nd	nd
26	142.67	122.29	132.48 (10.19)	97.32	93.71	95.52 (1.81)
48	225.60	182.40	204.00 (21.60)	nd	nd	nd
72	260.00	200.80	230.40 (29.60)	99.79	95.99	97.89 (1.90)

Table 3.1.3 Total cell concentration and yeast viability after 87 days of cryopreservation [Culture flask 2, ampoules 7 & 8: SL1Cf2A7Rb1 and SL1Cf2A8Rb2]

Time(h.)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf2A7Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf2A8Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf2A7Rb1	Yeast viability (% viable cells) SL1Cf2A8Rb2	Mean yeast viability
0	1.97	1.68	1.83 (0.15)	95.33	92.31	93.82 (1.51)
2	4.92	3.48	4.20 (0.72)	99.52	84.55	92.04 (7.49)
4	6.66	5.73	6.20 (0.47)	nd	nd	nd
6	12.60	19.15	15.88 (3.28)	94.94	95.35	95.15 (0.21)
8	29.00	27.90	28.45 (0.55)	nd	nd	nd
20	164.00	268.80	216.40 (52.40)	99.97	99.03	99.50 (0.47)
23	200.80	224.00	212.40 (11.60)	nd	nd	nd
26	201.60	168.00	184.80 (16.80)	93.49	90.97	92.23 (1.26)
48	223.20	nd	223.20	nd	nd	nd
72	230.40	nd	230.40	99.49	nd	99.49

Table 3.1.4 Total cell concentration and yeast viability after 87 days of cryopreservation [Culture flask 4, ampoules 7 & 8: SL1Cf4A7Rb1 and SL1Cf4A8Rb2]

Time (h.)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf4A7Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf4A8Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf4A7Rb1	Yeast viability (% viable cells) SL1Cf4A8Rb2	Mean yeast viability
0	2.71	2.00	2.36 (0.36)	99.50	90.00	94.75 (4.75)
2	3.33	3.41	3.37 (0.04)	90.10	98.01	94.06 (3.96)
4	9.63	9.29	9.46 (0.17)	nd	nd	nd
6	25.40	18.05	21.73 (3.68)	89.17	90.71	89.94 (0.77)
8	40.60	28.90	34.75 (5.85)	nd	nd	nd
20	199.20	197.60	198.40 (0.80)	99.98	99.79	99.89 (0.10)
23	154.67	218.40	186.54 (31.87)	nd	nd	nd
26	168.58	141.33	154.96 (13.63)	95.99	91.93	93.96 (2.03)
48	239.20	217.60	228.40 (10.80)	nd	nd	nd
72	234.40	248.80	241.60 (7.20)	98.24	95.72	96.98 (1.26)

Table 3.1.5 Mean and standard deviation of the total cell concentration after 87 days of cryopreservation. [Culture flasks 1, 2 and 4; ampoules 7 & 8: SL1Cf1A7Rb1, SL1Cf1A8Rb2; SL1Cf2A7Rb1, SL1Cf2A8Rb2, SL1Cf4A7Rb1 and SL1Cf4A8Rb2]

Time (hours)	Mean of total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Standard deviation (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )
0	1.58	0.86
2	2.72	1.76
4	5.53	3.85
6	13.39	9.32
8	22.82	14.39
20	181.07	54.79
23	169.65	52.26
26	157.41	27.93
48	217.60	21.22
72	234.88	22.40

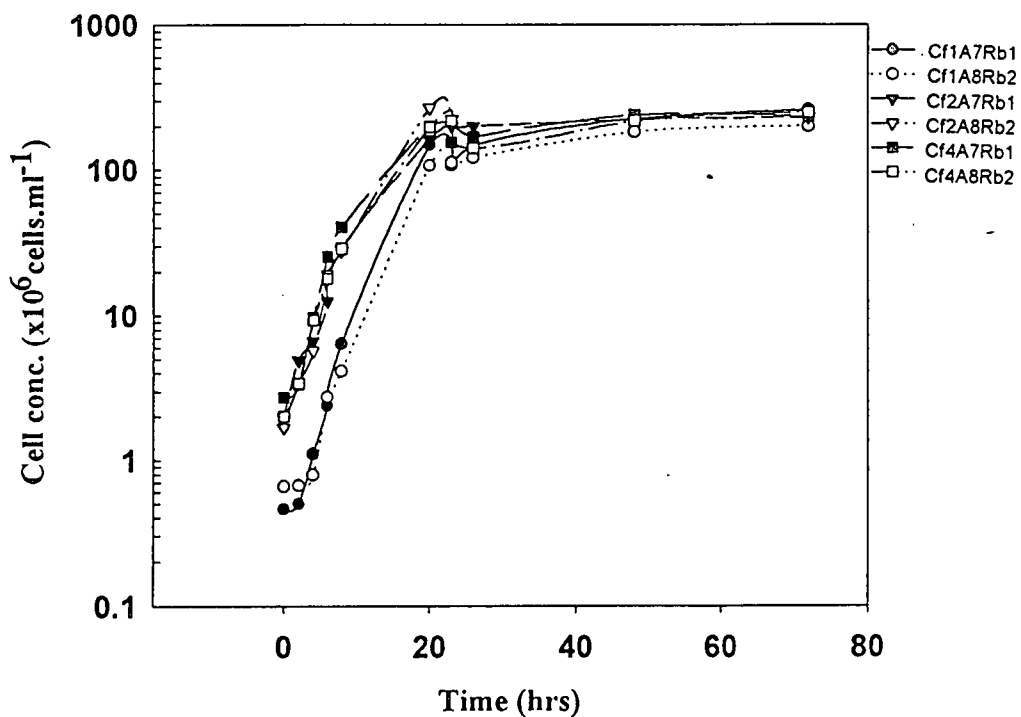


Fig. 3.1 Growth curves of yeasts from ampoules (SL1Cf1A7Rb1, SL1Cf1A8Rb2, SL1Cf2A7Rb1, SL1Cf2A8Rb2, SL1Cf4A7Rb1 and SL1Cf4A8Rb2) after 87 days cryopreservation

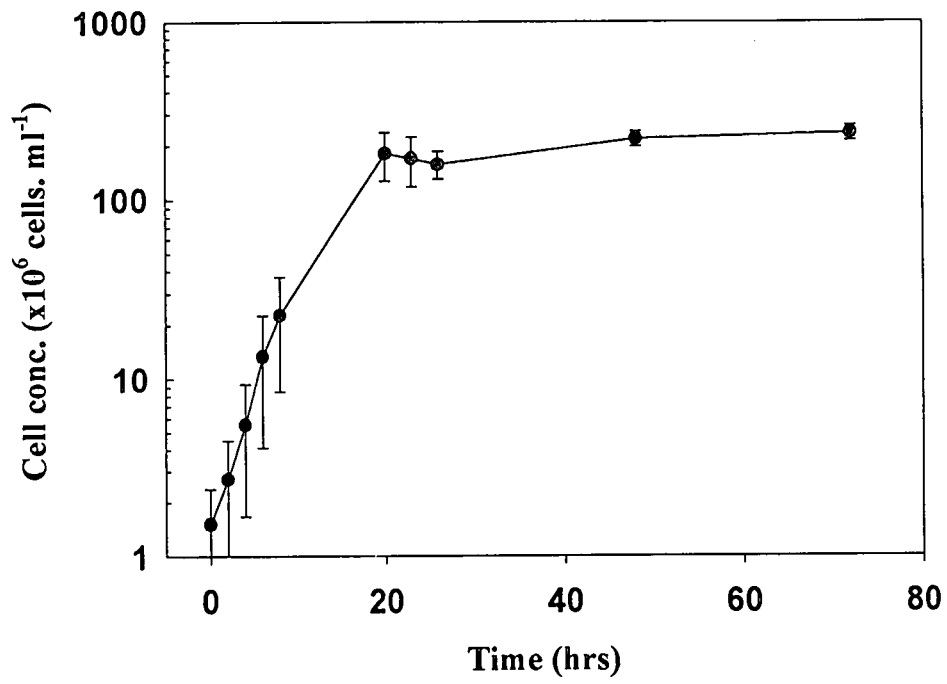


Fig. 3.2 Mean growth curve after 87 days cryopreservation

**MARCH 1998**

**CRYO-TIME: 136 days**

## EXPERIMENT 4

### Cryopreservation of standardised yeast inoculum

#### Variability in cryopreservation methodology

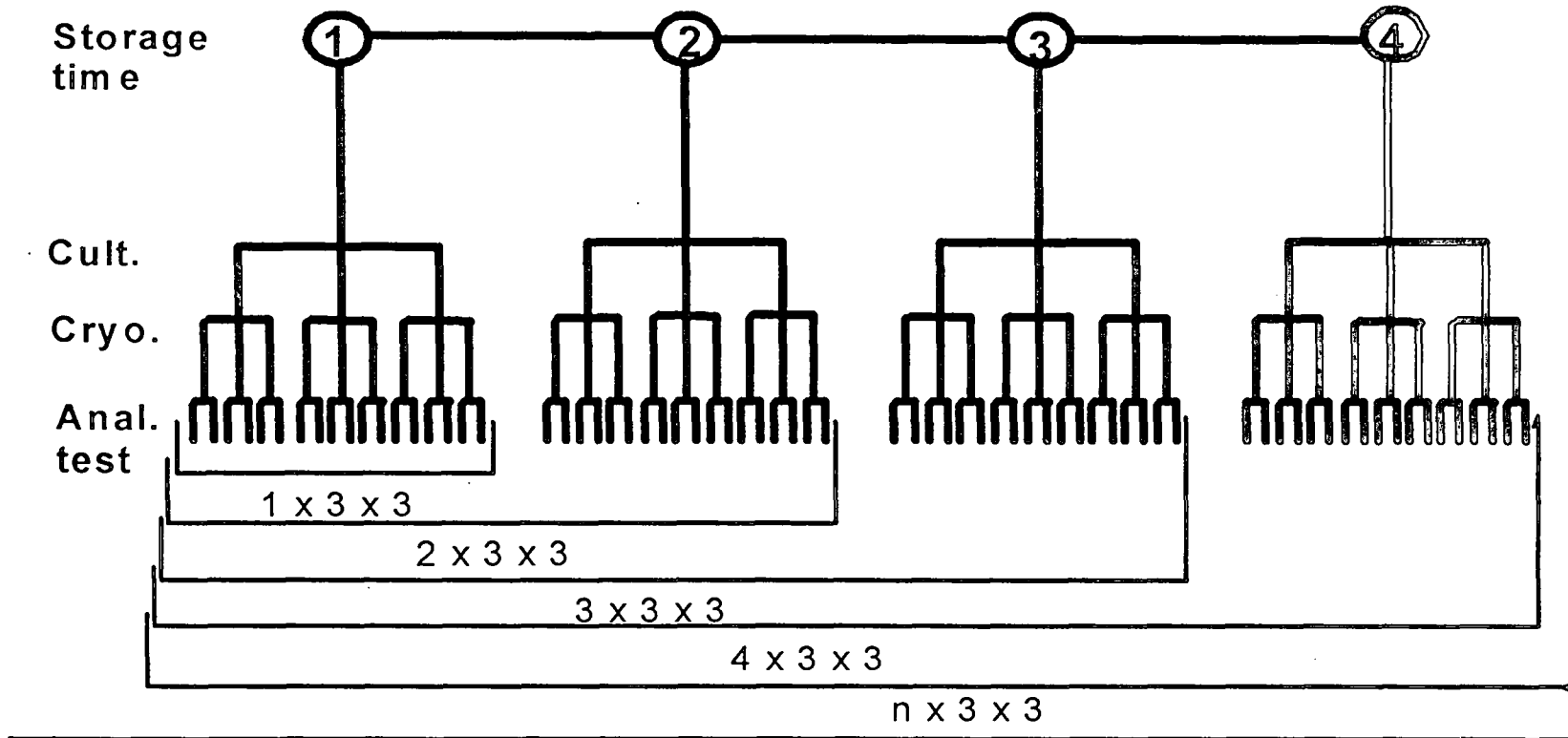
In this fourth experiment (Exp. 4), the effect of cryopreservation after 136 days on brewery yeast viability was investigated. According to the results, no contamination on ULM, LP, LYS and SDM media could be detected at any stage of analysis after cryopreservation. Hundred and thirty-six days of cryopreservation had no significant influence on growth curves as well as yeast viability in all repetitions performed (see: Growth Studies and Yeast Viability: Exp. 4 tables and figures).



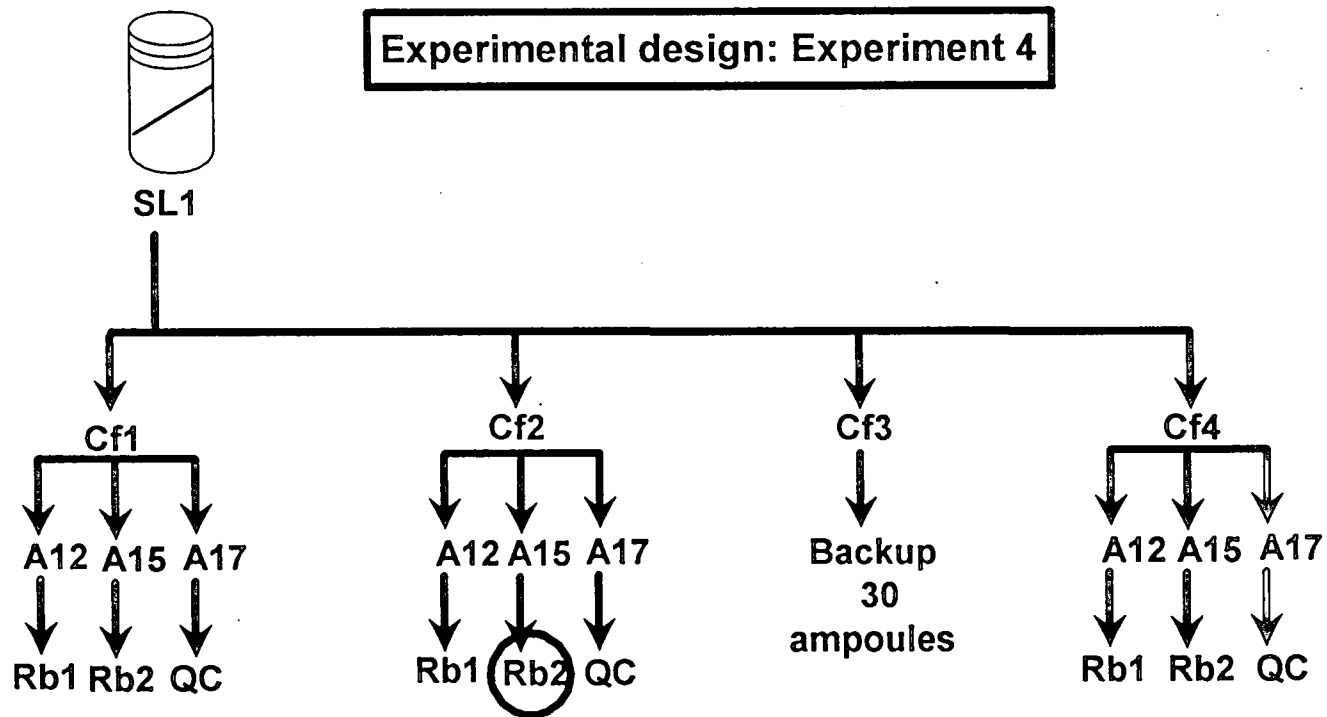
# Nested Experimental Design

Variables

Responses  
viability  
stability  
contamination



# Experimental design: Experiment 4



Address example: SL1Cf2A15Rb2

SL: slant; Rb: round bottom flask; Cf: conical flask; A: ampoule; QC: Quality control

## RESULTS OF EXPERIMENT 4: GROWTH MEDIA

WA + TTC = Wort Agar with Triphenyl Tetrazonium Chloride overlay for testing of respiratory deficiency [RD] in yeasts

### Experiment 4. After 136 days cryopreservation

Sample	Address	Dilution	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc. (cells.ml <sup>-1</sup> )	RD col. in 0.2ml	% RD
Directly after revival	SL1Cf1A17	10 <sup>-3</sup>	5	534	2.67×10 <sup>6</sup>	4	0.7
		10 <sup>-4</sup>	5	162	8.10×10 <sup>6</sup>	2	1.2
	SL1Cf2A17	10 <sup>-3</sup>	5	641	3.21×10 <sup>6</sup>	2	0.3
		10 <sup>-4</sup>	5	209	1.05×10 <sup>7</sup>	2	1.0
	SL1Cf4A17	10 <sup>-3</sup>	5	480	2.40×10 <sup>6</sup>	7	1.5
		10 <sup>-4</sup>	5	217	1.09×10 <sup>7</sup>	2	0.9
Round bottom flasks, wort, 72h	SL1Cf1A12Rb1	10 <sup>-3</sup>	5	567	2.84×10 <sup>6</sup>	04	0.7
		10 <sup>-4</sup>	5	054	2.70×10 <sup>6</sup>	01	1.9
	SL1Cf1A15Rb2	10 <sup>-3</sup>	5	652	3.26×10 <sup>6</sup>	11	1.7
		10 <sup>-4</sup>	5	050	2.50×10 <sup>6</sup>	02	4.0
	SL1Cf2A12Rb1	10 <sup>-3</sup>	5	468	2.34×10 <sup>6</sup>	09	1.9
		10 <sup>-4</sup>	5	065	3.25×10 <sup>6</sup>	01	1.5
	SL1Cf2A15Rb2	10 <sup>-3</sup>	5	283	1.42×10 <sup>6</sup>	05	1.8
		10 <sup>-4</sup>	5	053	2.65×10 <sup>6</sup>	01	1.9
	SL1Cf4A12Rb1	10 <sup>-3</sup>	5	104	5.20×10 <sup>6</sup>	03	2.9
		10 <sup>-4</sup>	5	014	7.00×10 <sup>6</sup>	00	0.0
	SL1Cf4A15Rb2	10 <sup>-3</sup>	5	nd	nd	nd	nd
		10 <sup>-4</sup>	5	nd	nd	nd	nd

**WLN = Wallerstein Nutrient Medium for differentiation of wild yeasts [variants], culture yeasts and bacteria**

**Experiment 4. After 136 days cryopreservation**

Sample	Address	Dilution	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc. (cells.ml <sup>-1</sup> )	Variant col. in 0.2 ml	% variants
Directly after revival	SL1Cf1A17	10 <sup>-3</sup>	5	591	2.96×10 <sup>6</sup>	13	2.2
		10 <sup>-4</sup>	5	118	5.90×10 <sup>6</sup>	05	4.2
	SL1Cf2A17	10 <sup>-3</sup>	5	623	3.12×10 <sup>6</sup>	36	5.8
		10 <sup>-4</sup>	5	137	6.85×10 <sup>6</sup>	08	5.8
	SL1Cf4A17	10 <sup>-3</sup>	5	552	2.76×10 <sup>6</sup>	44	8.0
		10 <sup>-4</sup>	5	086	4.30×10 <sup>6</sup>	04	4.7
Round bottom flasks, wort, 72h	SL1Cf1A12Rb1	10 <sup>-3</sup>	5	330	1.65×10 <sup>6</sup>	27	8.2
		10 <sup>-4</sup>	5	048	2.40×10 <sup>6</sup>	02	4.2
	SL1Cf1A15Rb2	10 <sup>-3</sup>	5	300	1.50×10 <sup>6</sup>	24	8.0
		10 <sup>-4</sup>	5	079	3.95×10 <sup>6</sup>	06	7.6
	SL1Cf2A12Rb1	10 <sup>-3</sup>	5	333	1.67×10 <sup>6</sup>	36	10.8
		10 <sup>-4</sup>	5	076	3.80×10 <sup>6</sup>	07	9.2
	SL1Cf2A15Rb2	10 <sup>-3</sup>	5	226	1.13×10 <sup>6</sup>	28	12.4
		10 <sup>-4</sup>	5	027	1.35×10 <sup>6</sup>	02	7.4
	SL1Cf4A12Rb1	10 <sup>-3</sup>	5	087	4.35×10 <sup>6</sup>	08	9.2
		10 <sup>-4</sup>	5	028	1.40×10 <sup>6</sup>	02	7.1
	SL1Cf4A15Rb2	10 <sup>-3</sup>	5	nd	nd	nd	nd
		10 <sup>-4</sup>	5	nd	nd	nd	nd

**SDM = Schwartz Differential Medium for differentiation of  
brewing yeasts from wild yeasts**

**Experiment 4. After 136 days cryopreservation**

Sample	Address	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc.	No. of wild yeast colonies in 0.2ml	% Wild yeast
Directly after revival	SL1Cf1A17	5	nd	nd	nd	nd
	SL1Cf2A17	5	nd	nd	nd	nd
	SL1Cf4A17	5	nd	nd	nd	nd
Round bottom flasks, wort, 72h	SL1Cf1A12Rb1	5	-	-	-	-
	SL1Cf1A15Rb2	5	-	-	-	-
	SL1Cf2A12Rb1	5	-	-	-	-
	SL1Cf2A15Rb2	5	-	-	-	-
	SL1Cf4A12Rb1	5	-	-	-	-
	SL1Cf4A15Rb2	5	nd	nd	nd	nd

**LYS = Lysine Medium for differentiation of *Saccharomyces* spp. from non-*Saccharomyces* sp.**

**Experiment 4. After 136 days cryopreservation**

Sample	Address	Incub. period (days) 25°C	Col. in 0.2 ml	Cell conc.	No. of non- <i>Sacch.</i> colonies in 0.2 ml	Growth of non- <i>Sacch.</i>
Directly after revival	SL1Cf1A17	5	nd	nd	nd	nd
	SL1Cf2A17	5	nd	nd	nd	nd
	SL1Cf4A17	5	nd	nd	nd	nd
Round bottom flasks, wort, 72h	SL1Cf1A12Rb1	5	-	-	-	-
	SL1Cf1A15Rb2	5	-	-	-	-
	SL1Cf2A12Rb1	5	-	-	-	-
	SL1Cf2A15Rb2	5	-	-	-	-
	SL1Cf4A12Rb1	5	-	-	-	-
	SL1Cf4A15Rb2	5	nd	nd	nd	nd

LP = Lactose Peptone Broth for growth of lactose fermenting and lactose assimilating organisms but not strains of *Saccharomyces cerevisiae*

#### Experiment 4. After 136 days cryopreservation

Sample	Address	Incub. period (days) 30°C	Growth in 0.5 ml
Directly after revival	SL1Cf1A17	2-4	nd
	SL1Cf2A17	2-4	nd
	SL1Cf4A17	2-4	nd
Round bottom flasks, wort, 72h	SL1Cf1A12Rb1	2-4	-
	SL1Cf1A15Rb2	2-4	-
	SL1Cf2A12Rb1	2-4	-
	SL1Cf2A15Rb2	2-4	-
	SL1Cf4A12Rb1	2-4	-
	SL1Cf4A15Rb2	2-4	nd

**ULM = Universal Liquid Medium for cultivation of brewery  
bacteria**

**Experiment 4. After 136 days cryopreservation**

Sample	Address	Incub. period (days) 30°C	Growth in 0.5 ml
Directly after revival	SL1Cf1A17	2-4	nd
	SL1Cf2A17	2-4	nd
	SL1Cf4A17	2-4	nd
Round bottom flasks, wort, 72h	SL1Cf1A12Rb1	2-4	-
	SL1Cf1A15Rb2	2-4	-
	SL1Cf2A12Rb1	2-4	-
	SL1Cf2A15Rb2	2-4	-
	SL1Cf4A12Rb1	2-4	-
	SL1Cf4A15Rb2	2-4	nd



## RESULTS OF EXPERIMENT 4: GROWTH STUDIES AND YEAST VIABILITY

### Experiment 4.1. After 136 days of cryopreservation

Table 4.1.1 Total cell concentration and yeast viability after 136 days of cryopreservation (directly from ampoule)

Ampoule	Total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> )	Yeast viability (% viable cells)
SL1Cf1A14	36.65	98.29
SL1Cf2A14	43.15	98.27
SL1Cf4A14	36.80	95.96

Table 4.1.2 Total cell concentration and yeast viability after 136 days of cryopreservation [Culture flask 1, ampoules 12 & 15: SL1Cf1A12Rb1 and SL1Cf1A15Rb2]

Time(h.)	Total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> ) SL1Cf1A12Rb1	Total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> ) SL1Cf1A15Rb2	Mean total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf1A12Rb1	Yeast viability (% viable cells) SL1Cf1A15Rb2	Mean yeast viability
0	2.40	2.54	2.47 (0.07)	88.89	94.44	91.67 (2.78)
2	2.92	2.64	2.78 (0.14)	nd	nd	nd
4	4.55	4.73	4.64 (0.09)	84.26	99.50	91.88 (7.62)
6	9.25	10.60	9.93 (0.68)	nd	nd	nd
8	17.95	20.00	18.98 (1.03)	94.87	96.01	95.44 (0.57)
23	254.40	196.80	225.60 (28.80)	95.89	98.01	96.95 (1.06)
27	245.60	284.00	264.80 (19.20)	nd	nd	nd
29	246.40	313.60	280.00 (33.60)	94.97	97.03	96.00 (1.03)
45	264.00	312.00	288.00 (24.00)	nd	nd	nd
72	296.80	308.00	302.40 (5.60)	94.98	95.73	95.36 (0.38)

Table 4.1.3 Total cell concentration and yeast viability after 136 days of cryopreservation [Culture flask 2, ampoules 12 & 15: SL1Cf2A12Rb1 and SL1Cf2A15Rb2]

Time(h.)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf2A12Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf2A15Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf2A12Rb1	Yeast viability (% viable cells) SL1Cf2A15Rb2	Mean yeast viability
0	1.28	3.19	2.24 (0.96)	99.50	98.48	98.99 (0.51)
2	1.89	3.72	2.81 (0.92)	nd	nd	nd
4	5.60	7.15	6.38 (0.78)	95.86	99.46	97.66 (1.80)
6	10.15	11.55	10.85 (0.70)	nd	nd	nd
8	15.75	18.75	17.25 (1.50)	96.33	93.99	95.16 (1.17)
23	176.80	241.60	209.20 (32.40)	96.34	94.98	95.66 (0.68)
27	234.44	312.80	273.62 (39.18)	nd	nd	nd
29	240.00	311.20	275.60 (35.60)	92.99	95.37	94.18 (1.19)
45	252.81	302.40	277.61 (24.80)	nd	nd	nd
72	280.00	304.00	292.00 (12.00)	98.01	95.56	96.79 (1.23)

Table 4.1.4 Total cell concentration and yeast viability after 136 days of cryopreservation [Culture flask 4, ampoules 12 & 15: SL1Cf4A12Rb1 and SL1Cf4A15Rb2]

Time(h.)	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf4A12Rb1	Total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> ) SL1Cf4A15Rb2	Mean total cell concentration (x 10 <sup>6</sup> cells.ml <sup>-1</sup> )	Yeast viability (% viable cells) SL1Cf4A12Rb1	Yeast viability (% viable cells) SL1Cf4A15Rb2	Mean yeast viability
0	1.96	2.23	2.10 (0.14)	95.56	98.67	97.12 (1.56)
2	2.44	3.45	2.95 (0.51)	nd	nd	nd
4	5.33	8.50	6.92 (1.59)	99.72	99.48	99.60 (0.12)
6	6.75	10.05	8.40 (1.65)	nd	nd	nd
8	12.50	19.05	15.78 (3.28)	95.98	97.31	96.65 (0.67)
23	176.00	nd	176.00	95.54	nd	95.54
27	212.00	nd	212.00	nd	nd	nd
29	241.60	nd	241.60	98.01	nd	98.01
45	311.20	nd	311.20	nd	nd	nd
72	312.00	nd	312.00	96.78	nd	96.78

Table 4.1.5 Mean and standard deviation of the total cell concentration after 136 days of cryopreservation. [Culture flasks 1, 2 and 4; ampoules 12 & 15: SL1Cf1A12Rb1, SL1Cf1A15Rb2, SL1Cf2A12Rb1, L1Cf2A15Rb2, SL1Cf4A12Rb1, SL1Cf4A15Rb2]

Time (h.)	Mean of total cell concentration ( $\times 10^6$ cells.ml <sup>-1</sup> )	Standard deviation ( $\times 10^6$ cells.ml <sup>-1</sup> )
0	2.27	0.63
2	2.84	0.67
4	5.98	1.54
6	9.73	1.64
8	17.33	2.77
23	209.12	36.74
27	257.77	40.32
29	270.56	38.28
45	288.48	27.99
72	300.16	12.59

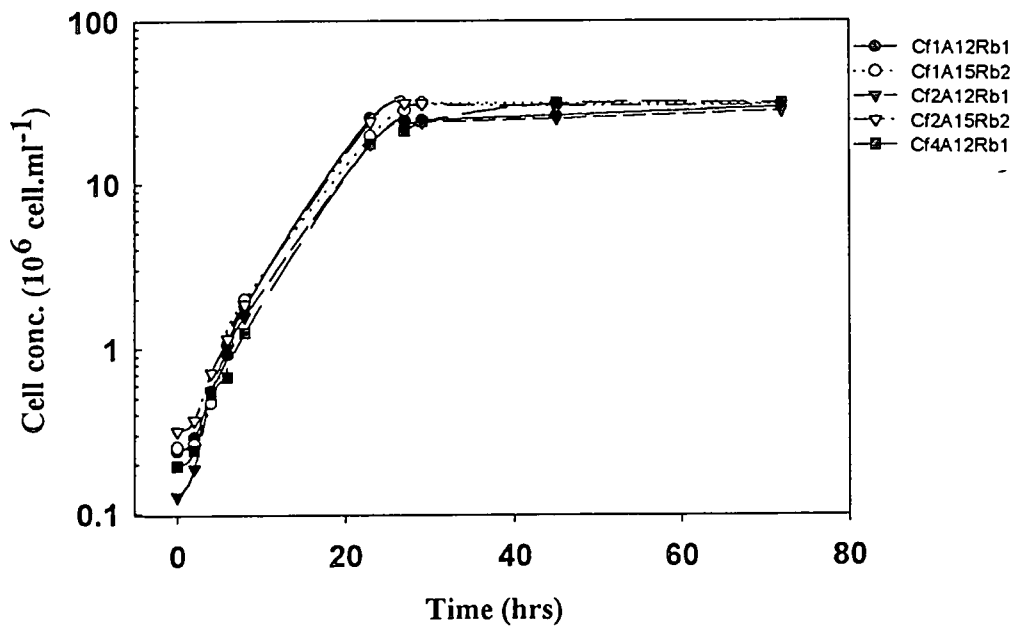


Fig. 4.1 Growth curves of yeasts from ampoules (SL1Cf1A12Rb1, SL1Cf1A15Rb2, SL1Cf2A12Rb1, SL1Cf2A15Rb2, SL1Cf4A12Rb1, SL1Cf4A15Rb2) after 136 days cryopreservation

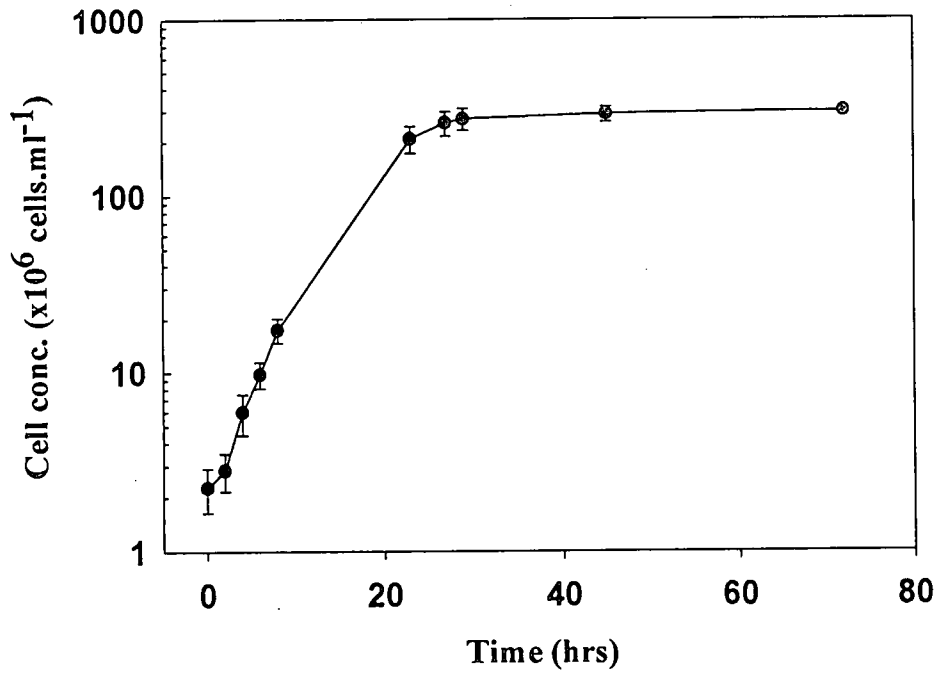


Fig. 4.2 Mean growth curve after 136 days cryopreservation

### List of abbreviations:

- col = colonies  
 d = days  
 h = hours  
 nd = not determined  
 Tntc = Too numerous to count  
 - = No growth  
 A = Ampoule  
 Sl = Slant  
 Cf = Conical flask  
 Rb = Round bottom flask  
 RD = Respiratory deficient

### Media

LP = (Lactose Peptone Broth) Supports growth of lactose fermenting and lactose assimilating organisms but not strains of *Saccharomyces cerevisiae*.

LYS = (Lysine Medium) for differentiation of *Saccharomyces sp.* from non *Saccharomyces sp.*

MYGP = Malt extract, yeast extract, glucose and peptone growth medium

SDM = (Schwartz Differential Medium) for differentiation of brewing yeasts from wild yeasts.

TTC = (Triphenyl Tetrazolium Chloride) for testing respiratory deficiency in yeasts.

ULM = (Universal Liquid Medium) for cultivation of brewery bacteria.

WA = (Wort Agar) for cultivation and enumeration of yeasts.

WLN = (Wallerstein Laboratories Nutrient medium) for differentiation of wild yeast (variants) culture yeast and bacteria.

## Chapter 3

The value of polar lipids and sterols in the identification of species representing the genus *Saccharomyces*

### 3.1 Introduction

The value of cellular fatty acid (FA) profiles in the taxonomy of the yeasts is well established (Lösel, 1988; Augustyn, 1992). The presence or absence of the cellular polyunsaturated fatty acids (PUFAs) linoleic (18:2) and  $\alpha$ -linolenic acid (18:3) have been found to be useful in identifying species of the genus *Saccharomyces* (Botha and Kock, 1993).

Since FA desaturation occurs in the cell membranes (Ratledge, 1988), it was a further aim of this study to evaluate this phenotypic character by analysing FAs associated with different phospholipid fractions of species representing *Saccharomyces* (Vaughan-Martini and Martini, 1998).

It has also been reported that sterols are associated with polar FAs by condensing and stabilising the phospholipid bilayers present in cell membranes (Paterson, 1998). Since sterol composition shows some promise as a taxonomic marker in fungi when used together with the FAs (Müller *et al.*, 1994), the purpose of this study also included an evaluation of sterols in the identification of these yeasts. Rapid identification of these yeasts is of special importance to prevent wine and beer spoilage by yeast



contaminants and to ensure that only pure yeast cultures are used for inoculation procedures.

## 3.2 Materials and methods

### 3.2.1 Yeast used

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

### 3.2.2 Cultivation

Forty strains representing 10 species of the genus *Saccharomyces* were cultivated on YM agar slants (Wickerham, 1951). All strains were inoculated into 11 conical flasks containing 200ml of the following medium ( $\text{g.l}^{-1}$ ); YNB (Difco, USA.), 6.7 and glucose (Merck, Germany), 40.0. Flasks were incubated at  $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , shaken at 160 rpm until stationary phase and then harvested by centrifugation at 8000 rpm for 20 minutes and washed with distilled water. The centrifuged cells were rapidly frozen followed by freeze drying.

### 3.2.3 Lipid analysis

#### 3.2.3.1 Extraction and fractionation

All lipids were extracted from freeze dried cells according to Kendrick and Ratledge (1992) with the aid of chloroform/methanol (2:1, by vol.) as described by Folch *et al.*, (1957). These were then fractionated into neutral-, phospho- and glycolipids by applying organic solvents of different polarities to a column of activated silicic acid (Aldrich, USA.). Extracted lipids and various fractions (neutral-, phospho- and glycolipids)

were dissolved in diethylether and transferred to preweighed vials. The weight of each vial was determined after the samples were dried to a constant weight in a vacuum oven at 50°C over P<sub>2</sub>O<sub>5</sub>.

### 3.2.3.2 Phospholipid separation by thin layer chromatography

Phospholipids were separated on silica gel plates (Merck, Germany) with an aluminium back. These polar lipids were separated using chloroform/methanol/acetic acid/water (65:43:3:1, by vol.) and visualised using iodine vapours. The composition of the phospholipids was determined using standards of phosphatidylcholine (PC), phosphatidylethanolamine (PE) phosphatidylinositol (PI) and phosphatidylserine (PS). All standards were obtained from Sigma (USA.).

In order to obtain phospholipid fractions (i.e. PC, PE, PI and PS), the phospholipid fraction separated by silicic acid column chromatography was chromatographed on TLC plates as described. The bands corresponding with the R<sub>f</sub> of the phospholipid standards were scraped off and extracted from the silica with chloroform/methanol (2:1, by vol.). After filtration through glass wool (Saarchem, RSA.), the solvent was removed under a stream of nitrogen gas and each fraction dried and weighed as described. All of the phospholipid fraction obtained from one cultivation flask was chromatographed on one TLC plate (approximately 1mg).

### 3.2.3.3 FA analysis

The FA composition associated with the various phospholipid fractions (i.e. PC, PE, PI and PS) was determined after trans-esterification by the

addition of trimethyl sulphonium hydroxide according to the method of Butte (1983). FA methyl esters were analysed by using a Hewlett Packard 5890 gas chromatograph equipped with a supelcowax 10 capillary column (30m × 0.75mm) with nitrogen as carrier gas set at a flow rate of 5ml.min<sup>-1</sup>. The inlet temperature was set on 180°C with the initial column temperature set at 145°C increasing at 3°C.min<sup>-1</sup> to 225°C, followed by the same temperature increment 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 standards supplied by Sigma (USA.).

### 3.2.4 Sterol analysis

#### 3.2.4.1 Hydrolysis

Freeze dried cells of each strain prepared from yeast biomass cultivated as described before (see 3.2.2) were hydrolysed as described by Rencken *et al.* (1995). In short, KOH dissolved in methanol: ethanol: dH<sub>2</sub>O (700:315:15, by vol.) were added to preweighed freeze dried yeast biomass. A further 2ml ethanol was added and the mixture was heated to 80°C for 90min in sealed screw cap glass tubes (Schott, USA.) while gently shaken and then cooled to room temperature.

#### 3.2.4.2 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. HPLC was carried out on a Spherisorb C18 reverse phase column (25cm × 0.46cm, 5µm diameter) using a mixture of

ethanol:methanol:water, (100:850:50, by vol.) as solvent phase at a flow rate of  $1\text{ml}\cdot\text{min}^{-1}$ . A Lambda-Max (Waters, Milford, MA, USA.) Model 480 LC spectrophotometer was used to measure ergosterol at 282nm and lanosterol, stigmasterol and squalene at 210nm. The concentration quoted for sterols is based on yeast cell dry weight. The internal standard stock solution (made in ethanol) contained  $2.53\mu\text{g}\cdot 10\mu\text{l}^{-1}$  ergosterol,  $2.53\mu\text{g}\cdot 10\mu\text{l}^{-1}$  lanosterol,  $2.50\mu\text{g}\cdot 10\mu\text{l}^{-1}$  stigmasterol and  $2.72\mu\text{g}\cdot 10\mu\text{l}^{-1}$  squalene. Sterol present in sample was 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.

### 3.3 Results and discussion

#### 3.3.1 Lipid composition

Most strains contained less than six percent (w/w) total lipids while *Sacch. castellii* is characterised by the highest percentage lipids i.e. 8.3 to 9.3%. All strains tested are characterised by relative high amounts of neutral lipids (44.4%-92.8%) and by low amounts of glycolipids and phospholipids. These phenotypic characters showed no taxonomic value and can consequently not be used to differentiate between these strains or higher taxa (Table 1).

When phospholipids (PL) were further analysed, it was found that the major classes i.e. PC, PS, PI and PE were present, with general differences being observed between strains. Although it has been reported that PC is the most abundant phospholipid (Ratray, 1988), similar amounts compared to PS, PI and PE were found (Table 1). Although differences on

strain level were found, these characters showed no taxonomic value at higher taxa level. *Sacch. cerevisiae* CBS 2354 is unique since it contained low percentages PI (i.e. 6.7%) and PE (i.e. 6.7%) and the highest PC and PS i.e. 40.0% and 46.6% respectively. *Sacch. pastorianus* CBS 1503 contained the highest percentage PE (i.e. 56.0%).

Most PL fractions are characterised by the presence of 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid) and 18:2 (linoleic acid) while 18:3 (linolenic acid) could only be detected in some strains (Table 2). It was also found that 16:0 and 18:0 were in many cases the main FAs associated with PL fractions. This is contrary to reports on total FA compositions of these yeasts where the monounsaturated C16 and C18 FAs were without exception, found to dominate (Tredoux *et al.*, 1987). This may be due to 'dilution' of the PL fractions with NL containing mainly 16:1 and 18:1.

It is important to note that 18:2 has been found in most PL fractions which is contrary to the findings reported in literature that all *Saccharomyces* species with the exception of *Sacch. kluyveri* produce no 18:2 or 18:3 (Augustyn *et al.*, 1990). These results are based mainly on cellular FA composition and can be explained by the possibility that the predominating NL fractions do not contain 18:2 which causes the 'dilution' of this FA when studying total FA composition. The 18:2 content of the NL fractions should be further studied. The presence of 18:3 in some PL fractions and its absence in the total FA composition (Kock and Botha, 1998) may be due to a similar reason. Consequently, FA profiles of the PL fractions, especially the monoenoic C16 and C18 FAs, are totally different to that

found in the total lipid fraction of yeasts representing this yeast genus (Augustyn *et al.*, 1990). These profiles can also not be used to differentiate between strains and higher taxa of this group of yeasts.

### 3.3.2 Sterol content

On the basis of the results obtained, ergosterol was found to dominate (Table 3). This sterol was particularly dominant in *Sacch. cerevisiae*, *Sacch. paradoxus* and *Sacch. unisporus*, which all produced high concentrations. The presence of ergosterol is critical to *Saccharomyces* since the vitality of these yeasts depends on the presence of both FAs and this sterol which are essential for membrane function and integrity (Rencken *et al.*, 1995).

Concerning stigmasterol, *Sacch. bayanus*, *Sacch. cerevisiae*, *Sacch. paradoxus* and *Sacch. pastorianus*, i.e. all members of *Saccharomyces sensu strictu* produced high levels of this sterol compared to other species. *Sacch. castellii* and *Sacch. servazzii* did not produce stigmasterol. The concentration of this sterol was less than  $100\text{mg.g}^{-1}$  in most of the other yeasts tested with the exception of *Sacch. unisporus*.

The pattern of squalene composition almost resembles that of stigmasterol, with *Sacch. bayanus*, *Sacch. cerevisiae*, *Sacch. paradoxus* and *Sacch. pastorianus* having slightly higher concentrations than the rest of the species. This general difference in proportion of ergosterol and squalene, may be explained by the fact that ergosterol is an end product of sterol synthesis in a pathway that uses squalene as a metabolic precursor. Since the cells were grown aerobically, ergosterol dominated. However,

squalene can be formed anaerobically. Under these conditions squalene may constitute up to 60-70% of the total cell sterol (Rattray *et al.*, 1975; Henry, 1982).

Different strains of *Sacch. unisporus* produced varying amounts of lanosterol which were, however, in greater concentrations compared to the rest of the species analysed. The strain of *Sacch. unisporus* CBS 3004 produced particularly high concentration of lanosterol.

### 3.4 Conclusions

With this as a background the following conclusions can be drawn:

1. Total lipid content as well as polar lipid fractions and associated FA composition showed no taxonomic value.
2. Both 18:2 and 18:3 were detected in strains characterised by the absence of these FAs in total FA composition. This may be due to the 'dilution' of these FAs by the dominating NL fraction.
3. Sterol content i.e. stigmasterol and squalene, showed promise in the demarcation of the *Saccharomyces sensu strictu* group.

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Table 1 Total lipid content and percentage neutral- (NL), glyco- (GL) and phospho- (PL) lipid fractions in 10 species of the genus *Saccharomyces*. The percentages of the PL fractions i.e. phosphatidylcholine (PC), phosphatidylethanolamine (PE) phosphatidylinositol (PI) and phosphatidylserine (PS) are also included.

ORGANISM		Total lipids (%w/w)	NL	GL	PL	PC	PS	PI	PE
<i>Sacch. bayanus</i>	CBS 395	2.8	84.4	7.5	8.1	24.5	26.5	24.5	24.5
<i>Sacch. bayanus</i>	CBS 424	1.5	76.7	13.3	10.0	22.2	29.7	25.9	22.2
<i>Sacch. bayanus</i>	CBS 1505	2.0	80.6	11.9	7.5	20.0	21.6	29.2	29.2
<i>Sacch. bayanus</i>	CBS 1546	2.3	75.1	12.7	12.2	19.7	22.5	29.6	28.2
<i>Sacch. bayanus</i>	CBS 1604	2.3	84.3	8.1	7.6	23.5	35.3	23.6	17.6
<i>Sacch. castellii</i>	CBS 4309	9.3	88.1	7.6	4.3	19.1	31.9	27.7	21.3
<i>Sacch. castellii</i>	CBS 4310	8.3	83.9	9.0	7.1	22.2	24.5	22.2	31.1
<i>Sacch. cerevisiae</i>	CBS 1171T	6.3	92.2	4.8	3.0	17.2	30.8	21.2	30.8
<i>Sacch. cerevisiae</i>	CBS 2354	2.1	83.5	12.1	4.4	40.0	46.6	6.7	6.7
<i>Sacch. cerevisiae</i>	CBS 3093	2.7	76.5	12.7	10.8	23.6	31.6	22.4	22.4
<i>Sacch. dairensis</i>	CBS 421T	1.6	59.4	26.2	14.4	13.0	30.4	13.0	43.6
<i>Sacch. dairensis</i>	CBS 2913	2.1	76.6	14.2	9.2	18.8	29.2	22.8	29.2
<i>Sacch. dairensis</i>	CBS 6334	1.7	84.3	10.5	5.2	30.8	27.7	15.3	26.2
<i>Sacch. dairensis</i>	CBS 6904	1.2	56.2	25.4	18.4	30.0	10.0	20.0	40.0
<i>Sacch. exiguus</i>	CBS 379T	4.1	65.0	21.1	13.9	22.9	26.5	28.9	21.7
<i>Sacch. exiguus</i>	CBS 3019	5.3	90.4	3.8	5.8	20.7	19.0	36.2	24.1
<i>Sacch. exiguus</i>	CBS 6440	3.8	85.9	6.1	8.0	18.4	44.7	13.2	23.7
<i>Sacch. exiguus</i>	CBS 8134	2.8	78.1	10.1	11.8	27.1	22.9	25.0	25.0
<i>Sacch. kluyveri</i>	CBS 3082T	3.9	76.3	16.1	7.6	16.3	18.7	51.2	13.8
<i>Sacch. kluyveri</i>	CBS 2861	2.2	79.5	10.5	10.0	32.8	16.4	18.0	32.8
<i>Sacch. kluyveri</i>	CBS 4104	1.2	83.5	9.4	7.1	26.6	26.6	24.1	22.7
<i>Sacch. kluyveri</i>	CBS 5828	2.6	76.7	12.7	10.6	44.4	22.2	22.2	11.2
<i>Sacch. kluyveri</i>	CBS 6545	1.5	77.0	13.1	9.9	19.2	25.0	25.0	30.8
<i>Sacch. kluyveri</i>	CBS 6546	0.5	78.4	15.4	6.2	26.3	24.6	18.0	31.1
<i>Sacch. paradoxus</i>	CBS 406T	5.3	88.9	4.6	6.5	19.5	34.8	28.3	17.4
<i>Sacch. paradoxus</i>	CBS 2980	4.4	88.0	4.3	7.7	19.2	21.2	32.7	26.9
<i>Sacch. paradoxus</i>	CBS 5829	6.2	88.1	3.6	8.3	19.1	27.7	25.5	27.7
<i>Sacch. paradoxus</i>	CBS 7400	3.0	87.9	6.9	5.2	18.3	32.4	24.4	24.9
<i>Sacch. pastorianus</i>	CBS 1397T	3.1	84.7	9.9	5.4	23.7	35.5	20.4	20.4
<i>Sacch. pastorianus</i>	CBS 1260	3.8	86.7	7.2	6.1	17.9	34.3	19.4	28.4
<i>Sacch. pastorianus</i>	CBS 1503	1.7	86.7	6.5	6.8	12.0	16.0	16.0	56.0
<i>Sacch. pastorianus</i>	CBS 1513	4.5	92.8	3.6	3.6	19.2	35.9	11.6	33.3
<i>Sacch. pastorianus</i>	CBS 1538	2.6	78.3	11.2	10.5	21.4	19.0	31.0	28.6

Table 1 Continued

ORGANISM		Total lipids (%w/w)	NL	GL	PL	PC	PS	PI	PE
Sacch. servazzii	CBS 4311T	2.3	44.4	38.8	16.8	17.0	17.0	38.3	27.7
Sacch. servazzii	CBS 7721	2.0	79.3	12.6	8.1	20.0	25.7	14.3	40.0
Sacch. unisporus	CBS 398T	3.2	68.0	22.9	9.1	22.3	31.3	19.5	26.9
Sacch. unisporus	CBS 399	5.9	90.6	4.8	4.6	16.1	26.4	25.3	32.2
Sacch. unisporus	CBS 1575	5.9	92.4	3.6	4.0	26.9	25.6	23.1	24.4
Sacch. unisporus	CBS 3004	5.6	85.6	3.4	11.0	24.6	21.1	21.1	33.2
Sacch. unisporus	CBS 4804	4.6	92.0	4.3	3.7	27.3	23.6	18.2	30.9

CBS: Centraal Bureau voor Schimmelcultures, Delft, The Netherlands.

All experiments were performed at least in duplicate while the standard error was less than 5% in most cases.

Table 2 Percentage fatty acids in phospholipid fractions.

Organism	Phosphatidylcholine						Phosphatidylserine						Phosphatidylinositol						Phosphatidylethanolamine					
	16:0	16:1	18:0	18:1	18:2	18:3	16:0	16:1	18:0	18:1	18:2	18:3	16:0	16:1	18:0	18:1	18:2	18:3	16:0	16:1	18:0	18:1	18:2	18:3
<i>Sacch. bayanus</i> CBS 395	49.8	4.0	24.4	9.8	12.0	0.0	54.8	3.9	28.0	9.7	3.6	0.0	50.8	3.9	26.8	8.9	9.6	0.0	49.2	3.8	27.5	9.1	10.4	0.0
<i>Sacch. bayanus</i> CBS 424	46.1	3.3	28.4	9.5	4.4	8.3	46.0	3.0	35.9	6.6	8.5	0.0	45.9	3.2	28.8	9.2	4.3	8.6	50.2	3.8	27.1	8.7	10.2	0.0
<i>Sacch. bayanus</i> CBS 1505	46.8	7.3	25.8	9.9	10.2	0.0	51.2	8.4	22.8	11.2	6.4	0.0	49.2	10.7	25.8	9.9	4.4	0.0	37.8	21.6	20.6	15.1	4.9	0.0
<i>Sacch. bayanus</i> CBS 1546	40.7	19.0	22.1	14.6	3.6	0.0	33.9	4.2	7.5	42.6	11.8	0.0	27.8	9.4	11.9	32.1	18.8	0.0	35.7	5.6	36.7	20.7	1.3	0.0
<i>Sacch. bayanus</i> CBS 1604	50.0	7.8	24.2	10.4	7.6	0.0	38.5	5.1	26.7	9.2	11.0	9.5	40.5	7.4	26.4	19.6	3.3	2.8	41.9	6.3	26.3	10.6	11.0	3.9
<i>Sacch. castellii</i> CBS 4309	41.8	8.0	23.8	13.0	13.4	0.0	43.6	14.4	25.6	10.0	6.4	0.0	41.9	10.5	23.8	11.5	12.3	0.0	35.0	2.3	16.6	40.5	5.4	0.2
<i>Sacch. castellii</i> CBS 4310	15.6	54.3	5.2	23.5	1.4	0.0	33.3	26.3	11.6	28.1	0.7	0.0	16.8	55.7	2.5	24.3	0.7	0.0	18.0	50.3	3.8	26.5	1.4	0.0
<i>Sacch. cerevisiae</i> CBS 1171T	21.0	4.7	11.9	35.7	26.7	0.0	34.4	8.3	26.4	16.9	14.0	0.0	39.2	3.8	40.3	10.5	6.2	0.0	41.3	16.9	23.7	15.4	2.7	0.0
<i>Sacch. cerevisiae</i> CBS 2354	43.3	5.3	27.4	10.7	7.4	5.9	41.0	7.4	25.6	9.1	13.8	3.1	33.8	3.9	28.9	23.0	8.8	1.6	42.6	7.8	26.9	10.3	7.9	4.5
<i>Sacch. cerevisiae</i> CBS 3093	37.0	2.6	40.3	9.0	11.1	0.0	34.4	13.4	22.4	25.7	4.1	0.0	33.7	12.4	31.6	12.6	7.6	2.1	37.8	10.7	30.9	14.6	4.6	1.4
<i>Sacch. dairensis</i> CBS 421T	29.0	4.4	21.9	25.4	19.3	0.0	35.3	2.2	15.2	41.5	5.3	0.5	32.6	6.4	23.9	21.0	14.4	1.7	10.3	0.4	6.8	25.0	57.5	0.0
<i>Sacch. dairensis</i> CBS 2913	42.3	13.0	22.9	14.7	7.1	0.0	38.6	9.7	20.6	21.4	8.7	1.0	19.4	3.2	11.0	23.5	42.9	0.0	40.8	9.6	25.3	11.8	10.8	1.7
<i>Sacch. dairensis</i> CBS 6334	43.6	3.8	40.9	6.9	3.3	1.5	19.2	17.3	31.2	24.1	8.2	0.0	30.6	16.9	24.3	14.0	14.2	0.0	22.1	22.9	20.0	25.1	9.9	0.0
<i>Sacch. dairensis</i> CBS 6904	44.3	5.7	27.1	10.8	10.2	1.9	46.6	26.0	10.9	8.0	8.5	0.0	45.9	6.9	26.5	10.6	5.5	4.6	23.9	25.3	20.1	16.0	14.7	0.0
<i>Sacch. exiguus</i> CBS 379T	33.9	10.4	20.3	17.0	18.4	0.0	40.3	4.8	17.3	27.3	10.3	0.0	15.3	0.9	9.8	39.4	34.6	0.0	34.1	7.8	20.6	22.1	15.4	0.0
<i>Sacch. exiguus</i> CBS 3019	35.5	4.0	39.4	9.3	8.3	3.5	40.1	4.0	38.3	8.6	8.0	1.0	33.6	23.9	14.1	25.1	3.3	0.0	13.9	50.7	6.8	25.9	2.7	0.0
<i>Sacch. exiguus</i> CBS 6440	20.6	32.6	11.6	28.1	7.1	0.0	34.1	36.7	6.1	22.0	1.1	0.0	23.6	42.0	10.6	18.6	3.8	1.4	26.8	35.3	12.4	21.0	4.5	0.0
<i>Sacch. exiguus</i> CBS 8134	48.1	3.7	25.7	9.8	5.1	7.6	50.8	3.6	26.2	9.4	10.0	0.0	50.9	3.7	27.3	8.9	9.2	0.0	52.1	3.6	26.8	8.2	9.3	0.0
<i>Sacch. kluyveri</i> CBS 3082T	31.7	7.6	22.0	22.7	16.1	0.0	39.9	10.9	19.7	21.0	8.5	0.0	34.5	2.4	15.4	42.0	5.2	0.5	35.9	8.9	18.7	22.4	13.2	0.9
<i>Sacch. kluyveri</i> CBS 2861	42.0	4.4	28.3	8.4	16.9	0.0	48.3	4.0	27.8	8.9	4.4	6.6	45.9	3.5	33.3	7.4	9.9	0.0	47.8	4.1	27.0	9.8	11.3	0.0
<i>Sacch. kluyveri</i> CBS 4104	45.7	12.8	15.0	22.5	4.0	0.0	47.6	9.6	4.5	27.9	9.6	0.8	18.2	35.9	24.5	15.9	1.1	4.4	21.5	31.5	6.2	23.6	15.2	2.0
<i>Sacch. kluyveri</i> CBS 5828	41.7	7.0	30.6	9.2	6.9	4.6	42.8	5.7	31.7	8.3	7.6	3.9	46.1	4.9	31.7	8.6	8.7	0.0	42.1	5.8	27.3	9.8	12.2	2.8
<i>Sacch. kluyveri</i> CBS 6545	44.1	3.1	22.0	12.9	3.6	14.3	48.9	3.3	30.0	8.5	9.3	0.0	48.0	3.5	29.5	8.9	10.1	0.0	48.2	3.5	29.5	9.1	4.5	5.2
<i>Sacch. kluyveri</i> CBS 6546	43.2	6.6	28.8	13.0	8.4	0.0	36.3	29.5	14.2	14.8	5.2	0.0	33.0	24.2	14.3	22.6	5.9	0.0	30.1	23.3	16.2	26.7	3.7	0.0
<i>Sacch. paradoxus</i> CBS 406T	33.6	4.3	34.9	12.2	14.9	0.0	24.7	23.0	15.1	35.6	1.6	0.0	6.3	61.4	1.7	29.7	0.9	0.0	46.4	13.9	22.5	7.3	9.9	0.0
<i>Sacch. paradoxus</i> CBS 2980	28.0	20.6	20.1	22.0	9.3	0.0	19.5	24.0	17.7	37.3	1.5	0.0	8.4	48.6	1.7	39.6	1.7	0.0	51.6	7.6	24.8	12.0	2.8	1.2
<i>Sacch. paradoxus</i> CBS 5829	45.8	3.3	25.6	10.4	5.0	9.9	46.3	3.2	31.4	7.8	4.9	6.4	49.4	4.4	27.6	8.6	10.0	0.0	52.2	3.6	26.2	8.9	9.1	0.0
<i>Sacch. paradoxus</i> CBS 7400	33.9	12.9	22.5	15.4	15.3	0.0	30.4	18.6	17.2	27.2	5.4	1.2	16.3	49.6	8.1	22.4	3.6	0.0	12.8	51.8	4.7	28.9	1.8	0.0

Table 2 Continued.

Organism	Phosphatidylcholine						Phosphatidylserine						Phosphatidylinositol						Phosphatidylethanolamine					
	16:0	16:1	18:0	18:1	18:2	18:3	16:0	16:1	18:0	18:1	18:2	18:3	16:0	16:1	18:0	18:1	18:2	18:3	16:0	16:1	18:0	18:1	18:2	18:3
<i>Sacch. pastorianus</i> CBS 1397T	39.5	10.7	24.9	14.0	6.9	4.0	26.5	31.3	12.7	25.8	2.4	1.3	20.0	43.3	7.6	25.0	2.5	1.6	17.9	43.5	6.6	27.9	3.1	1.0
<i>Sacch. pastorianus</i> CBS 1260	21.9	33.1	16.3	22.2	6.5	0.0	26.2	17.5	20.4	28.9	4.9	2.1	23.8	30.5	15.6	24.0	6.1	0.0	14.6	40.0	6.5	35.2	3.7	0.0
<i>Sacch. pastorianus</i> CBS 1503	40.4	8.1	26.1	10.8	8.0	6.6	37.5	15.2	12.7	25.9	8.7	0.0	40.2	13.2	27.0	12.0	4.6	3.0	29.7	13.6	16.6	23.6	16.5	0.0
<i>Sacch. pastorianus</i> CBS 1513	33.8	17.8	14.3	23.0	11.1	0.0	37.4	13.9	20.2	21.5	7.0	0.0	22.9	40.3	7.3	24.4	5.1	0.0	26.1	31.1	14.0	22.4	6.4	0.0
<i>Sacch. pastorianus</i> CBS 1538	28.0	11.0	17.3	29.0	14.7	0.0	41.9	7.7	7.8	30.3	11.1	1.2	34.3	7.7	28.5	14.6	7.8	7.1	34.7	9.8	27.3	15.6	9.2	3.4
<i>Sacch. servazzii</i> CBS 4311T	33.2	9.6	20.4	13.0	13.6	10.2	32.7	9.7	18.3	15.1	17.1	7.1	34.4	7.0	33.3	9.0	11.7	4.6	40.2	9.2	20.9	17.6	6.9	5.2
<i>Sacch. servazzii</i> CBS 7721	33.1	9.6	34.7	12.8	9.8	0.0	31.4	18.0	27.1	17.5	3.7	2.3	32.3	9.7	26.3	15.8	13.6	2.3	33.4	10.2	32.5	11.3	11.0	1.6
<i>Sacch. unisporus</i> CBS 398T	34.3	13.3	19.5	13.9	8.5	10.5	29.3	4.0	19.3	24.7	19.5	3.1	37.6	8.6	26.5	11.5	9.4	6.4	35.9	10.2	19.8	16.8	10.8	6.5
<i>Sacch. unisporus</i> CBS 399	33.7	6.3	47.5	7.9	4.6	0.0	35.3	24.4	7.0	30.5	2.8	0.0	20.6	47.8	8.2	20.0	3.4	0.0	18.6	42.7	6.0	28.1	4.6	0.0
<i>Sacch. unisporus</i> CBS 1575	27.3	29.2	13.2	25.0	4.8	0.0	29.3	22.7	7.2	39.1	1.7	0.0	10.9	54.5	4.0	27.9	2.1	0.6	11.7	42.5	5.7	37.3	2.8	0.0
<i>Sacch. unisporus</i> CBS 3004	47.2	4.1	27.7	9.5	11.5	0.0	48.8	7.8	24.4	9.1	9.9	0.0	45.8	4.0	33.8	7.8	8.6	0.0	44.8	6.2	27.0	11.7	10.3	0.0
<i>Sacch. unisporus</i> CBS 4804	48.6	4.2	24.2	12.2	4.7	6.1	44.1	4.2	26.5	12.0	6.1	7.1	48.8	4.4	25.9	9.9	11.0	0.0	44.4	3.7	27.4	13.6	5.3	5.6

CBS: Centraal Bureau voor Schimmelcultures, Delft, The Netherlands.

All experiments were performed at least in duplicate. The standard error was less than 15% in most cases.

16:0 = (palmitic acid), 16:1 = (palmitoleic acid), 18:0 = (stearic acid), 18:1 = (oleic acid), 18:2 = (linoleic acid) and 18:3 = (linolenic acid).

Table 3 Sterol content in (mg.g<sup>-1</sup>) of 10 species representing the genus *Saccharomyces*

Organism		Ergosterol	Lanosterol	Stigmasterol	Squalene
Sacch. bayanus	CBS 395	217.5	35.5	628.0	225.0
Sacch. bayanus	CBS 424	137.5	18.0	0.0	31.0
Sacch. bayanus	CBS 1505	210.5	67.0	173.5	89.0
Sacch. bayanus	CBS1546	225.5	56.0	111.5	7.5
Sacch. bayanus	CBS 1604	149.5	26.0	232.5	147.0
Sacch. castellii	CBS 4309	357.0	0.0	0.0	9.0
Sacch. castellii	CBS 4310	235.0	0.0	0.0	0.0
Sacch. cerevisiae	CBS 1171T	452.5	152.5	405.5	159.0
Sacch. cerevisiae	CBS 2354	533.5	22.5	80.5	24.5
Sacch. cerevisiae	CBS 3093	275.5	53.5	375.5	133.0
Sacch. dairensis	CBS 421	158.5	40.5	182.5	32.0
Sacch. dairensis	CBS 2913	314.5	0.0	0.0	0.6
Sacch. dairensis	CBS 6334	153.0	22.0	0.0	0.0
Sacch. dairensis	CBS 6904	117.0	47.0	0.0	0.0
Sacch. exiguus	CBS 379T	422.5	0.0	0.0	32.0
Sacch. exiguus	CBS 3019	93.0	0.0	24.0	4.0
Sacch. exiguus	CBS 6440	368.5	13.0	46.0	8.5
Sacch. exiguus	CBS 8134	208.0	0.0	0.0	1.0
Sacch. kluyveri	CBS 3082T	367.5	42.0	86.5	9.0
Sacch. kluyveri	CBS 2861	248.5	17.5	70.5	9.5
Sacch. kluyveri	CBS 4104	186.5	0.0	42.0	3.0
Sacch. kluyveri	CBS 5828	204.0	16.5	51.5	5.5
Sacch. kluyveri	CBS 6545	104.0	16.0	82.5	5.5
Sacch. kluyveri	CBS 6546	45.5	0.0	0.0	1.5
Sacch. paradoxus	CBS 406	443.0	61.5	551.0	24.5
Sacch. paradoxus	CBS 2980	502.5	43.5	443.5	51.5
Sacch. paradoxus	CBS 5829	475.0	37.0	504.0	19.5
Sacch. paradoxus	CBS 7400	475.0	24.0	182.0	41.0
Sacch. pastorianus	CBS 1397T	610.0	134.0	606.0	102.0
Sacch. pastorianus	CBS 1260	454.0	46.0	346.0	47.0
Sacch. pastorianus	CBS 1503	199.0	76.0	72.0	53.5
Sacch. pastorianus	CBS 1513	610.0	125.0	143.5	69.0
Sacch. pastorianus	CBS 1538	182.5	26.0	133.0	12.5
Sacch. servazzii	CBS 4311T	139.5	237.0	0.0	9.0
Sacch. servazzii	CBS 7721	160.0	30.0	0.0	0.0

Table 3 Continued

Organism		Ergosterol	Lanosterol	Stigmasterol	Squalene
Sacch. unisporus	CBS 398T	454.0	132.5	227.0	2.0
Sacch. unisporus	CBS 399	558.0	50.5	178.0	0.0
Sacch. unisporus	CBS 1575	619.0	303.5	142.5	2.5
Sacch. unisporus	CBS 3004	683.5	487.0	199.5	23.0
Sacch. unisporus	CBS 4804	656.0	293.0	110.5	6.5

CBS: Centraal Bureau voor Schimmelcultures, Delft, The Netherlands.

All experiments were performed at least in duplicate while the standard error was less than 5% in most cases.



## Summary

The ability to preserve microorganisms can be considered a major biological achievement. Of special importance is the understanding of the principles of culture preservation with minimal occurrence of contamination, genetic and viability change. At present, cryopreservation is considered the most successful preservation method for yeasts yielding high survival levels and good phenotypic stability. As a result, one of the aims of this study was the application and evaluation of a cryopreservation protocol used in the maintenance of a *Saccharomyces cerevisiae* strain used by a major brewing company in South Africa. In order to ensure that only pure and stable yeasts with high viability are used after revival from maintenance protocol, it is essential that appropriate, rapid and inexpensive quality control methods are implemented. Elaborate and time consuming tests are used today in the brewing industry and include estimation of mutants and bacteria using Wallerstein Laboratory Nutrient Medium (WLN), estimation of respiratory deficient (RDs) yeasts using wort agar overlaid with Triphenyl-Tetrazolium-Chloride, detection of the wild yeasts using the Swartz-Differential Medium (SDM) protocol, 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). According to the results, a decrease in the percentage RDs as well as Variants (i.e. other mutants of *Saccharomyces cerevisiae*) was evident in brewing inocula when maintained through cryopreservation. When yeasts from slants (not cryopreserved) and yeasts subjected to cryopreservation were

cultivated in wort contained in round bottom flasks, no significant changes in the percentage RDs, variants or maximum growth rate ( $\mu_{\max}$ ) could be detected. A 4x3x3x2 nested experimental design was performed in order to determine the sources of variation in yeast viability, stability and contamination after preservation in liquid nitrogen for 136 days. Consequently, results on Variants and RDs suggest that the largest source of variation in the cryopreservation maintenance process was the error arising from the analytical tests. Cryopreservation also influenced the variation in the number of RDs obtained, though to a lesser extent. No contamination was found to occur during the cryopreservation protocol. From this study it is now possible to construct statistical quality control charts that can be used as an aid in the manufacture of brewing inocula maintained through cryopreservation. Another aim of this study was the evaluation of chemotaxonomic characters such as sterols and polar lipids in, as a first step, determining contamination of preserved yeasts with closely related species. According to the results, total lipid content as well as polar lipid fractions and associated fatty acid (FA) composition showed no obvious taxonomic value as the different *Saccharomyces* species could not be differentiated. Both linoleic acid (18:2) and linolenic acid (18:3) were detected in strains characterised by the absence of these FAs in total FA composition, a situation believed to be due to the 'dilution' of these FAs by the dominating NL fraction. Sterol content showed promise in the demarcation of the *Saccharomyces sensu strictu* group.

## Opsomming

Die vermoë om mikroorganismes te bewaar kan as 'n belangrike biologiese prestasie beskou word. Van spesiale belang is die begrip van die beginsels van kultuurbewaring met minimale kontaminasie, genetiese en lewensvatbaarheidsveranderinge. Kriopreservering word huidiglik as die mees suksesvolle bewaringsmetode vir giste beskou en lewer hoë oorlewingsvlakke en goeie fenotipiese stabiliteit. Een van die doelwitte van hierdie studie was gevolglik die toepassing en evaluering van 'n kriopreserveringsprotokol vir die onderhoud van 'n *Saccharomyces cerevisiae* stam wat deur 'n groot Suid-Afrikaanse brouery gebruik word. Om te verseker dat slegs rein en stabiele kulture met 'n hoë lewensvatbaarheid gebruik word na opwekking vanuit die bewaringsprotokol, is die implementering van geskikte, vinnige en goedkoop kwaliteitsbeheermetodes noodsaaklik. Ingewikkelde en tydrowende toetse word tans in die brou-industrie gebruik insluitende bepaling van mutante en bakterieë met Wallerstein Laboratorium Nutrient Medium (WLN), bepaling van respiratories gebrekkige giste (RGGs) met wort agar bedek met Trifeniel-Tetrazolium-Chloried, bepaling van wilde giste met die Swartz-Differensiële Medium (SDM) protokol, bepaling van nie-*Saccharomyces* spesies met Lisien-Medium (LM), bepaling van laktose assimilerende en laktose fermenterende mikroorganismes met Laktose-Peptoon-Sop (LP) en bepaling van brouerybakterieë met Universele Vloeibare Medium (UVM). Volgens die resultate is 'n afname in die persentasie RGGs en variante (d.i. ander mutante van *Saccharomyces cerevisiae*) sigbaar in brou-inokula wat deur kriopreservering onderhou is. In 'n vergelykende studie waar Giste vanaf

skuinstes (nie gekriopreserveer nie) en giste wat wel gekriopreserveer is, in rondeboomflesse met wort gekweek is, kon geen betekenisvolle verskille in die persentasies RGGs, variante of maksimum groeisnelheid ( $\mu_{maks}$ ) waargeneem word nie. Om die oorsprong van die variasie in lewensvatbaarheid, stabiliteit en kontaminasie van giste na preserving in vloeibare stikstof vir 136 dae te bepaal, is 'n 4 x 3 x 3 x 2 geneste eksperimentele ontwerp uitgevoer. Die resultate aangaande variante en RGGs dui gevolglik daarop dat die grootste bron van variasie in die kriopreserveringsproses die fout afkomstig vanaf die analitiese toetse was. Kriopreservering het die variasie in die hoeveelheid RGGs verkry tot 'n geringe mate beïnvloed. Geen kontaminasie het gedurende die kriopreserveringsprotokol voorgekom nie. Uit hierdie studie is dit nou moontlik om statistiese kwaliteitsbeheerkaarte op te stel wat as hulpmiddels in die vervaardiging en kriopreservering van brou-inokula kan dien. 'n Ander doelwit van hierdie studie is die evaluasie van chemotaksonomiese eienskappe soos sterole en polêre lipiede in die bepaling van kontaminasie van bewaarde giste met naby verwante spesies. Die resultate toon aan dat die totale lipiedinhoud en die polêre lipiedfraksies en geassosieerde vetsure (Vse) oënskynlik geen taksonomiese waarde het nie aangesien die verskillende *Saccharomyces* spesies nie onderskei kon word nie. Sterolinhoud behoort geëvalueer te word as 'n vinnige identifikasiehulpmiddel. Beide linoleïnesuur (18:2) en linoleensuur (18:3) is waargeneem in stamme gekenmerk deur die afwesigheid van hierdie Vse in die totale vetsuursamestelling. Dit kan toegeskryf word aan die verdunning van hierdie Vse deur die dominerende neutrale lipiedfraksie. Sterolinhoud blyk belowend te wees in die afbakening van die *Saccharomyces sensu strictu* groep.