# GROWTH AND SURVIVAL OF *SACCHAROMYCES CEREVISIAE* IN SOIL

By


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## Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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# SUMMARY

*Saccharomyces cerevisiae* is commonly associated with the wine industry. However, this yeast was also isolated from soils not associated with vines. Despite the fact that *S. cerevisiae* is not perceived as an autochthonous soil yeast, its interaction with other soil microbiota suggests the contrary. Aside from a few *in vitro* studies, the fate of *S. cerevisiae* in soil is largely unknown. This may partly be ascribed to the lack of reliable methods to enumerate fermentative yeasts in soil.

Consequently, we evaluated an enumeration procedure for fermentative yeasts in soil, whereby yeast malt extract (YM) agar plates containing selective agents, were incubated in anaerobic jars before the colonies were enumerated. This procedure proved to be selective for fermentative yeasts, such as industrial strains of *S. cerevisiae*. We then commenced studying the growth and survival of *S. cerevisiae* in soil differing in moisture content and nutrient levels, using *S. cerevisiae* strain S92 and the genetically modified strain *S. cerevisiae* ML01, as well as two autochthonous soil yeasts, *Cryptococcus laurentii* and *Cryptococcus podzolicus*. The yeast strains were each inoculated into three series of microcosms containing sterile soil with a moisture content of *ca.* 30% (v/w), a moisture content of *ca.* 15% (v/w), or a moisture content of *ca.* 30% supplemented with nutrients used in agriculture. Growth of each strain was monitored for a period of 48 days and all the yeasts were found to grow or survive under these conditions, up until the end of the incubation period. Generally, the cryptococci reached larger population sizes in the soil than the *Saccharomyces* strains, which may be due to their ability to utilize a wider range of carbon sources and to survive in semi-arid soils. Aside from cell numbers observed in nutrient supplemented soil, in which *S. cerevisiae* ML01 reached higher numbers than S92, there was no significant difference between the growth and survival of the *Saccharomyces* strains. In all the microcosms, metabolic rates, as determined by measuring $CO_2$ emissions from soil, reached a maximum within the first day and then declined over the remainder of the trial, possibly due to depletion of nutrients. Differences in $CO_2$ emissions from the different series of microcosms were attributed to

different metabolic rates and energy expenditure needed to maintain yeast populations under different conditions.

Each of the above-mentioned yeasts was subsequently inoculated in a microcosm prepared from non-sterile soil and monitored using selective enumeration procedures. The *Saccharomyces* strains were enumerated using the above-mentioned soil dilution plates incubated in anaerobic jars. The presence of natural soil biota caused a decrease in viable yeast numbers for all strains and this was ascribed to competition with and predation by other soil borne organisms. Further evidence for competition and/or amensalism impacting on *Saccharomyces* populations in soil was obtained when monitoring co-cultures of *Saccharomyces* with *C. laurentii* 1f and *C. podzolicus* 3f in soil microcosms, revealed a significant reduction in *Saccharomyces* numbers during a 28 day incubation period. However, when the two *Saccharomyces* strains were cultured in soil microcosms inoculated with a protistan predator, populations of both strains increased and remained at these high levels for the duration of the trial. These findings point to a possible symbiosis between *Saccharomyces* and the protista whereby the predators ensure continuous nutrient cycling within the soil microcosms.

In the final part of the study, epifluorescence microscopy revealed that, similar to known soil cryptococci, the two *Saccharomyces* strains were able to form biofilms in oligotrophic conditions. The results of this study showed that in the presence of natural soil microbes, no differences exist between the growth and survival of *S. cerevisiae* S92 and *S. cerevisiae* ML01. Also, the findings point to a natural niche for this species somewhere in the soil habitat.

# OPSOMMING

*Saccharomyces cerevisiae* word algemeen met die wynindustrie geassosieer. Hierdie gis is egter ook uit grond geïsoleer wat nie met wingerd geassosieer word nie. Ten spyte van die feit dat *S. cerevisiae* nie as ‘n outogtoniese grondgis beskou word nie, dui sy interaksie met ander grondmikrobiota op die teendeel. Behalwe vir ‘n paar *in vitro* studies, is die lot van *S. cerevisiae* in grond grootliks onbekend. Dit mag gedeeltelik aan die gebrek aan betroubare metodes om fermenterende giste in grond te tel, toegeskryf word.

Ons het gevolglik ‘n tellingsmetode vir fermenterende giste in grond geëvalueer waarin gis-mout ekstrak (GM) agar plate, bevattende selektiewe agente, in anaërobiese flesse geïnkubeer is voordat die kolonies getel is. Hierdie metode was selektief vir fermenterende giste, soos die industriële stamme van *S. cerevisiae.* Hierna is die groei en oorlewing van *S. cerevisiae* bestudeer in gronde met verskillende vog- en nutriëntvlakke deur gebruik te maak van *S. cerevisiae* stam S92 en die geneties gemodifiseerde stam *S. cerevisiae* ML01, asook twee outogtoniese grondgiste, *Cryptococcus laurentii* en *Cryptococcus podzolicus*. Die gisstamme is elk geïnokuleer in drie reekse van mikrokosmosse bestaande uit steriele grond met ‘n vogvlak van *ca.* 30% (v/w), ‘n vogvlak van *ca.* 15% (v/w), of ‘n vogvlak van *ca.* 30% aangevul met landbounutriënte. Die groei van elke stam is waargeneem vir ‘n tydperk van 48 dae en al die giste het onder hierdie omstandighede tot aan die einde van die inkubasietydperk gegroei of oorleef. Oor die algemeen het die cryptococci groter populasies in die grond gevorm as die *Saccharomyces* stamme, wat toegereken kan word aan hul vermoë om ‘n wyer reeks koolstofbronne te benut en om in droë gronde te oorleef. Behalwe dat *S. cerevisiae* ML01 ‘n hoër aantal selle in nutriënt aangevulde grond behaal het as S92, was daar geen beduidende verskil tussen die groei en oorlewing van die *Saccharomyces* stamme nie. In al hierdie mikrokosmosse het die metaboliese tempo, soos bepaal deur $CO_2$ vrystellings vanuit grond te meet, ‘n maksimum bereik binne die eerste dag en dan het dit afgeneem oor die res van die toetsperiode, waarskynlik as gevolg van die uitputting van die nutriënte. Verskille in die $CO_2$ vrystellings wat vir die verskillende reekse van

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mikrokosmosse aangeteken is, is te wyte aan die verskillende metaboliese tempo's en energiegebruik benodig om gispopulasies onder verskillende omstandighede in stand te hou.

Elk van bogenoemde giste is vervolgens geïnokuleer in 'n mikrokosmos wat voorberei is van nie-steriele grond, en waargeneem deur selektiewe enumerasie prosedures toe te pas. Die *Saccharomyces* stamme is getel deur gebruik te maak van bogenoemde grondverdunningsplate wat in anaërobiese flesse geïnkubeer is. Die teenwoordigheid van natuurlike grondbiota het in alle stamme 'n afname in lewensvatbare gisgetalle veroorsaak en is toegeskryf aan die kompetisie met en predasie deur ander grondorganismes. Verdere bewys van die impak van kompetisie en/of amensalisme op *Saccharomyces* populasies in die grond, is die beduidende afname in *Saccharomyces* getalle tydens 'n 28 dag inkubasie tydperk, waartydens ko-kulture van *Saccharomyces* stamme met *C. laurentii* 1f en *C. podzolicus* 3f in grond mikrokosmosse ondersoek is. Toe die twee *Saccharomyces* stamme egter in grond mikrokosmosse opgekweek is wat met 'n protistiese predator geïnokuleer is, het populasies van albei stamme gegroei en om hierdie hoë vlakke gebly tot aan die einde van die toets. Hierdie bevindings dui 'n moontlike simbiose tussen *Saccharomyces* en die protista aan waardeur die predatore deurlopende nutriëntsiklering binne die grondmikrokosmos verseker.

In die laaste deel van die studie toon epifluoressensie mikroskopie aan dat, net soos bekende grond cryptococci, die twee *Saccharomyces* stamme in staat is om biofilms in oligotrofiese omstandighede te vorm. Die resultaat van die studie toon aan dat in die teenwoordigheid van natuurlike grondmikrobe daar geen verskil tussen die groei en oorlewing van *S. cerevisiae* S92 en *S. cerevisiae* ML01 is nie. Die bevindings dui ook aan dat daar 'n natuurlike nis vir hierdie spesie  iewers in die grondhabitat is.

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# MOTIVATION

*Saccharomyces cerevisiae* is a fermentative yeast commonly associated with the wine industry, and has been isolated from vineyards and vineyard soil (Kurtzman and Fell, 2000; Jolly et al. 2003). However, documented cases exist where *Saccharomyces* has been isolated from other soils (Do Carmo-Sousa 1969). Most recently, *S. cerevisiae* and *Saccharomyces paradoxus* were isolated from oak-associated soils, utilizing enrichment techniques (Sniegowski et al. 2002)

*Saccharomyces cerevisiae* is often perceived as a fermentative yeast associated with habitats rich in carbohydrates, rather than an autochthonous soil yeast, but its interactions with soil biota during *in vitro* studies, suggest the contrary. *S. cerevisiae* has been implicated in a number of predator-prey interactions, serving as nutrient source for predatory soil yeast species (Lachance & Pang 1997**;** Kreger-van Rij & Veenhuis 1973), as well as a food source for the nematode *Panagrellus redivivus* (Ricci et al. 2004; Hechler 1970). Aside from these interactions, ethanol production by *S. cerevisiae* was found to increase the pathogenicity of the soil bacterium *Acinetobacter* towards the predacious nematode *Caenorhabditis elegans* (Smith et al. 2004).

The findings mentioned above indicate that soil may act as habitat for *S. cerevisiae*, but relatively little is known about the interactions of this yeast within soil. Previous studies focused on the fate of microbes of potential use in genetic engineering, including *S. cerevisiae*, in model ecosystems such as sewage and lake water (Liang et al. 1982). A study conducted in 1994 tested one genetically engineered and one wild-type strain of *S. cerevisiae* under simulated environmental conditions (Fujimura 1994). However, the strains were only tested in a natural soil/water suspension, soil/medium suspension and in waste water. The fact that so little is known about the fate of *S. cerevisiae* in soil may quite possibly be due to the absence of techniques to enumerate these yeasts in soil.

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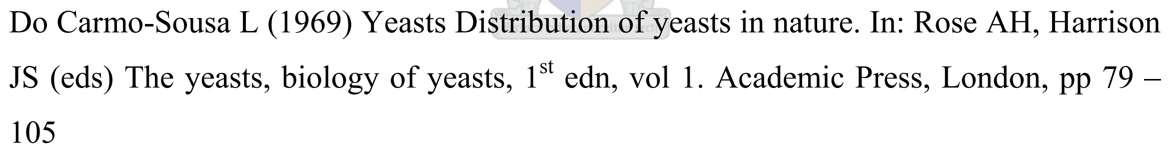
The purpose of this study was to develop a method for the isolation and enumeration of fermentative yeasts in soil and to determine the fate of a wild-type strain of *S. cerevisiae* (S92) and a genetically modified strain (ML01), capable of malolactic fermentation, in soil. The latter strain is a genetically modified commercial yeast to be used in future for winemaking and could potentially be released into the environment. Thus, we set out to develop a technique to selectively enumerate fermentative yeasts, such as *S. cerevisiae,* in soil samples (Chapter 2). Once the enumeration technique was established, the next objective was to monitor the fate of the *S. cerevisiae* S92 and *S. cerevisiae* ML01 in soil microcosms differing in moisture content, nutrient levels and soil biota (Chapter 3). For comparison, two yeasts known to occur in soil (Kurtzman and Fell, 2000), i.e. *Cryptococcus laurentii* and *Cryptococcus podzolicus*, were included in the study. In the final part of the study (Chapter 4), epifluorescence microscopy was used to confirm that, similar to known soil cryptococci, both *S. cerevisiae* S92 and *S. cerevisiae* ML01 were able to produce biofilms under oligotrophic conditions in flow cells.

## References




Do Carmo-Sousa L (1969) Yeasts Distribution of yeasts in nature. In: Rose AH, Harrison JS (eds) The yeasts, biology of yeasts, 1st edn, vol 1. Academic Press, London, pp 79 – 105

Fujimura H, Sakuma Y, Amann E (1994) Survival of genetically-engineered and wild-type strains of the yeast *Saccharomyces cerevisiae* under simulated environmental conditions: a contribution on risk assessment. J Appl Microbiol 77: 689-693

Hechler HC (1970) Reproduction, chromosome number, and postembryonic development of *Panagrellus redivivus* (Nematoda: Cephalobidae). J Nematol 2: 355-361

Jolly NP, Augustyn OPH, Pretorius IS (2003) The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. S Afr J Enol Vitic 24: 35- 42

Kreger-van Rij NJW & Veenhuis M (1973) Electron microscopy of some special cell contacts in yeasts. J Bacteriol 113: 350-356

Kurtzman CP & Fell JW (2000) The yeasts: A taxonomic study, a 4$^{th}$ and enlarged edition. Elsevier, the Netherlands

Lachance MA & Pang WM (1997) Predacious yeasts. Yeast 13: 225-232

Liang LN, Sinclair JL, Mallory LM, Alexander M (1982) Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl Env Mircobiol 44: 708-714

Ricci M, Fifi AP, Ragni A, Schlechtriem C, Focken U (2004) Development of a low-cost technology for mass production of the free-living nematode *Panagrellus redivivus* as an alternative live food for first feeding fish larvae. Appl Microbiol Biot 60: 556-559

Smith MG, Des Etages SG, Snyder M (2004) Microbial synergy via an ethanol-triggered pathway. Mol Cell Biol 24: 3874-3884

Sniegowski PD, Dombrowski PG, Fingerman E (2002) *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. FEMS Yeast Research 1: 299-306

# *TABLE OF CONTENTS*

# *CHAPTER 1*

## *Introduction*


1.1 Soil: A microbial habitat 1

1.2 Interactions of soil microorganisms with their physicochemical environment 3

1.3 Interactions between soil organisms 5

1.4 Yeasts associated with the soil microenvironment 8

1.5 Role of yeasts in soil 10

1.5.1 Mineralization 10

1.5.2 Inorganic nutrient release and soil formation 11

1.6 Yeasts interactions in soil 12

1.7 Yeast interactions in the rhizosphere 12

1.8 Yeast interactions known to occur in bulk soil 16

1.8.1 Predation and yeasts 16

1.9 Genetically modified organisms in the environment 22

1.10 Natural mechanisms of horizontal gene transfer in prokaryotes 23

1.10.1 Transformation 23

1.10.2 Conjugation 24

1.10.3 Transduction 25

1.11 Natural mechanisms of horizontal gene transfer in eukaryotes 26

1.12 Horizontal gene transfer between prokaryotes, eukaryotes and vice versa 27

1.13 The fate of extracellular DNA in the environment 28

1.14 The impact of genetically modified organisms on the native soil biota 29

1.15 References 33

## ***CHAPTER 2***

## *A Method for the Selective Enumeration of Fermentative Yeasts in Soil*

2.1 Introduction 44

2.2 Materials and Methods 45

2.2.1 Yeast strains 45

2.2.2 Survival of yeasts incubated in anaerobic jars 46

2.2.3 Enumeration of yeast strains in soil 46

2.2.4 Enumeration of fermenting wild yeasts in soil samples 47

2.3 Results and Discussion 48

2.3.1 Survival of yeasts incubated in anaerobic jars 48

2.3.2 Enumeration of yeasts in soil 49

2.3.3 Enumeration of fermenting wild yeasts in soil samples 50

2.4 Conclusions 51

2.5 References 54

# ***CHAPTER 3***

## *Growth of* Saccharomyces cerevisiae *in Soil under Different Environmental Conditions*

3.1 Introduction 56

3.2 Materials and methods 58

3.2.1 Yeast strains used 58

3.2.2 Preparation of soil microcosms 58

3.2.3 Preparation of soil microcosms with low moisture content 60

3.2.4 Preparation of microcosms from soil amended with nutrients 60

3.2.5 Preparation of microcosms from non-sterile soil 61

3.2.6 Monitoring yeast metabolic activity 61

3.2.7 Monitoring growth of yeast inoculated into sterile soil 61

3.2.8 Monitoring yeast growth inoculated into non-sterile soil 62

3.2.9 Impact of soil cryptococci on growth of *Saccharomyces* 62

3.2.10 Impact of soil protista on growth of *Saccharomyces* 63

3.2.10.1 Preparation of sterile soil extract 63

3.2.10.2 Isolation of protista 63

3.2.10.3 Preparation of soil microcosms containing protista 63

3.2.11 Analyses of data 64

3.2.11.1 Effect of soil moisture content and nutrients 64

3.2.11.2 Effect of soil biota 65

3.2.11.3 Impact of soil cryptococci on growth of *Saccharomyces* 65

3.2.11.4 Effect of different environmental conditions on growth of *Saccharomyces* 66

3.3 Results and Discussion 66

3.3.1 Effect of soil moisture content and nutrients 66

3.3.2 Effect of soil biota 70

3.3.3 Impact of soil cryptococci on growth of *Saccharomyces* 70

3.3.4 Effect of different environmental conditions on growth of *Saccharomyces* 76

3.4 Conclusions 77

3.5 References 79

## ***CHAPTER 4***

## *Biofilm Formation by* Saccharomyces cerevisiae *under Oligotrophic Conditions*

4.1 Introduction 84

4.2 Materials and methods 84

4.2.1 Yeast strains used 84

4.2.2 Cultivation of biofilm communities 85

4.2.3 Epifluorescent microscopy 86

4.3 Results and discussion 86

4.4 References 88

Pectora roborant cultus recti

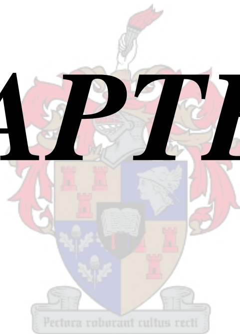
## ***CHAPTER 5***

## *General Conclusions and Hints on Future Research*

5.1 Conclusions and future research 89

5.2 References 91

# *CHAPTER 1*



# *Introduction*

## 1.1 Soil: A microbial habitat

The part of the earth's unconsolidated surface crust, or regolith, which supports plant life, is known as soil (Blain Metting 1993). Soil consists of varying sized organic and inorganic particles, living organisms and their nonliving remains (Stotzky 1972). However, the bulk of the solid phase of soil consists of inorganic materials, with organic matter comprising only 5-20% (Blain Metting 1993). This organic matter consists of cell debris, soluble substances and humus, the latter resulting mainly from lignin degradation and microbial synthesis. It was found that the organic content of soil tends to be higher when low temperatures and waterlogging inhibit the degradation of plant material.

The solid inorganic components of soil originate from the weathering of rock and may vary among different soils (Blain Metting 1993). Thus, the texture of a particular soil depends on the distribution of sand (particle diameter: 0.02-2mm), silt (particle diameter: 0.002-0.02mm) and clay (particle diameter: <0.002mm) occurring in the soil, while classification of soil as sandy, loam or clay, depends on the fraction each component constitutes. The arrangement of these fractions in microscopic and larger aggregates defines the soil fabric and structure. The movement of soil water and exchange of gasses are dependant on the spatial arrangement of pores present in this fabric. Due to the presence of water, gasses and mineral organic particles, each pore potentially serves as a site where microbial activity may occur. However, the microbial species responsible for this activity, show remarkable heterogeneity as a result of the temporal and spatial heterogeneity in the physicochemical composition of soil (Kimura et al. 1998).

Despite this heterogeneity in species composition, many of these microbes need organic carbon, which plays a pivotal role in their metabolism and ultimately originates from autotrophs such as plants (Wardle et al. 2004). Plants therefore impact on soil microbial populations. In turn, the soil microbes indirectly regulate plant growth, by determining

the availability of soil nutrients. This concept of vegetation influencing soil microbial communities and *vice versa*, has been discussed by a number of authors (Saetre & Bååth 2000; Grayston et al. 2004). However, it was found that the organic material originating from plants is often not sufficient to sustain soil microbial growth (Gray & Williams 1971; Poindexter 1981; Williams 1985). Studies performed on the rhizosphere and the associated microbial communities, showed that the energy flow from the roots to the soil often cannot sustain high metabolic activity by these soil microbial communities (Barber & Lynch 1979). Also, water extracts prepared from soil contained less than 2 μg/ml amino acids and 5 μg/ml carbohydrates (Ko and Lockwood 1976). Due to these properties, soil is viewed as an oligotrophic environment (Williams 1985). However, while soil may appear to be a harsh environment low in nutrients, the numbers and diversity of soil borne microbial species alone, dispel the myth of soil being an unfavourable environment for these organisms (Stotzky 1997). It was found that soil microbes are usually restricted to different microcosms, providing the necessary environmental factors required for colonizing, growth and survival of different microbial communities. Such communities may constitute a wide diversity of microbes, some of which may interact with eukaryotes sharing the same habitat. Soil may therefore serve as habitat for archaea, eubacteria, filamentous fungi and yeasts, microalgae, protozoa, nematodes, invertebrates, as well as vertebrates (Blain Metting 1993). Prokaryotes, such as archaea and bacteria, are the most numerous organisms in soil, but their biomass may be similar to that of soil fungi, indicating to an equal importance within the soil microenvironment.

The soil microbes mentioned above, may be classified by the manner in which they obtain energy and carbon (C) to sustain their metabolism and multiply (Blain Metting 1993; Richards 1987). Photoautotrophs obtain energy from sunlight and utilize $CO_2$ as their primary carbon source, while photoheterotrophs derive much of their carbon from organic compounds. Chemoautotrophs oxidize inorganic compounds and use $CO_2$ as their principal carbon source. Chemoheterotrophs derive energy and carbon from organic compounds and include protozoa, fungi and most bacteria. One could go even further and classify organisms that oxidize inorganic materials as lithotrophs and their counterparts

that derive reducing equivalents from organic matter as organotrophs. A simplified classification of soil organisms based on the nature of their energy and principal carbon sources within the foodweb, is depicted in Figure 1 (p 6).

Considering the above, it is obvious that the composition of the microbial population in a particular environment is dependent on available resources (Blain Metting 1993). According to ecological theory, selective pressure dictates the presence of organisms able to survive in dense populations or populations consisting of only a few species. K-selection favours the organism that is able to thrive under a given set of conditions, while r-selection favours the organism which is able to colonize a new environment, or thrive under a sudden flush of nutrients. In reality, organisms may show behavioural characteristics associated with both selection theories, as seasonal changes may cause both K- and r-selection in a microbial population (Andrew & Harris 1986). Many microbial communities may be viewed as genetically promiscuous, due to the presence of plasmids and other mobile genetic elements that are associated with transduction, transformation and conjugation. When a fluctuation in the environment occurs, the microbial community response is an amalgam of the individual programmed and unprogrammed genetic responses. The unprogrammed responses are often due to insertions or deletions, which lead to the expression of a cryptic gene, which in turn may confer a selective advantage to the particular organism. This may explain the occurrence of microbes with broad metabolic potential, or the ability to degrade xenobiotic substrates. An example is *Acinetobacter*, which is able degrade a number of xenobiotic pollutants such as phenol, chlorinated biphenyl and benzoate, making it ideally suited for use in the bioremediation of environmental contaminants (Abdel-El-Haleem 2003).

## 1.2 Interactions of soil microorganisms with their physicochemical environment

The physicochemical environment in which a soil microbe or microbial population is found, is known as a soil microenvironment. Physicochemical properties of this environment may depend on the strength and composition of ions present, pH and the redox potential (Blain Metting 1993). Ionic strength influences the solubility of salts,

electrokinetic potential, activity of soil enzymes, as well as adsorption of cells, viral particles and humic materials (Stotzky & Burns 1982). The soil pH range has been studied extensively and generally varies between 4.0 and 8.5 (Blain Metting 1993). The upper horizons of wetter soils are usually more acidic than the lower horizons, due to leaching of alkaline bases, while soil from arid and semi-arid regions tend to be alkaline. Soil pH influences the availability and toxicity of mineral nutrients. Iron, Mn and Zn become less available at pH levels above 7, while Al, Fe and Mn are toxic at pH values below 5. Phosphor is never readily soluble, but even less so at both low and high pH values (Bohn et al. 1979).

In addition to influencing the availability of nutrients, soil pH also impacts directly on the composition of microbial populations. The microbiota of acidic soils below pH 5.5 is predominantly fungal, whereas bacteria favour environments with a pH ranging from 6 to 8 (Stotzky 1972; Blain Metting 1993). This is a gross generalization however, as pH may vary by 2 or more units over a distance smaller than a microbial cell, due to microbial metabolism or other variables. In addition to changes in pH, the redox potential of a particular environment may vary. This variation was found to be interdependent on factors such as pH, temperature, microbial metabolism, water availability and the composition of the soil atmosphere. Under aerobic conditions microbial activity generates free electrons, which are transferred to free $O_2$ during respiration. However, if the soil environment becomes waterlogged, the $O_2$ will be consumed and the metabolism of facultative and anaerobic organisms will result in the formation of organic acids impacting on soil pH.

From the above it is obvious that the availability of water is an important factor in soil microbial growth and is expressed as the water potential (Blain Metting 1993). The latter indicates the energy expenditure required by microorganisms to assimilate the water present in soil. Water potential depends on the osmotic potential of the water, adsorption to soil particles, capillary effects and gravity. The retention of soil water therefore depends on pore size, aggregate stability and minerals present in the soil. Clay soils retain more water than sandy soils when faced with gravitational pull, but the aforementioned

leads to decreased availability of water to soil microbes. Studies have shown that in contrast to bacteria, yeasts and filamentous fungi are metabolically active at low water potentials.

Water in combination with temperature has also been found to play a cardinal role in the rate of mineral weathering, humus formation and microbial activity (Blain Metting 1993). Tropical soils are approximately 15°C warmer than soils from temperate areas, resulting in higher microbial metabolic rates. Temperature also impacts on the redox potential of the soil fabric, movement of water and diffusion of gasses (Stotzky 1972). It was found that when fluctuations in either water or temperature occur, seasonal variations of less that 7% occur in respiration and root decomposition rates (Smith 1982). However, variations between 70 and 75% were observed when fluctuations in both temperature and water occurred. Changes in the metabolic activity of the soil microbial community brought about by variations in temperature may not be permanent, since experimental evidence showed that although slow freezing of Chernozemic Brown loam to -3°C leads to *ca*. 15% decrease in bacterial and fungal counts, these microbes are able to lay dormant as a result of the formation of resistant spores (Blain Metting 1993). A similar study showed that $CO_2$ evolution changed only slightly under these conditions (Smith 1982), indicating that soil microbial activity is maintained through shifts in community composition towards more cold tolerant types. Evidence suggests that aerobes are more tolerant of freeze-thaw cycles than their anaerobic counterparts (Blain Metting 1993). On the other hand, mesophiles are able to survive high temperatures through sporulization and are more common in soil than thermophilic strains.

### 1.3 Interactions between soil organisms

Any interaction between microbes in a particular population may be viewed as symbioses, whether it is beneficial, detrimental, or neither (Blain Metting 1993). Neutralism occurs when populations have no effect on each other, whatsoever. This is highly improbable in a soil environment, as the soil microbes alter the environment via their metabolic

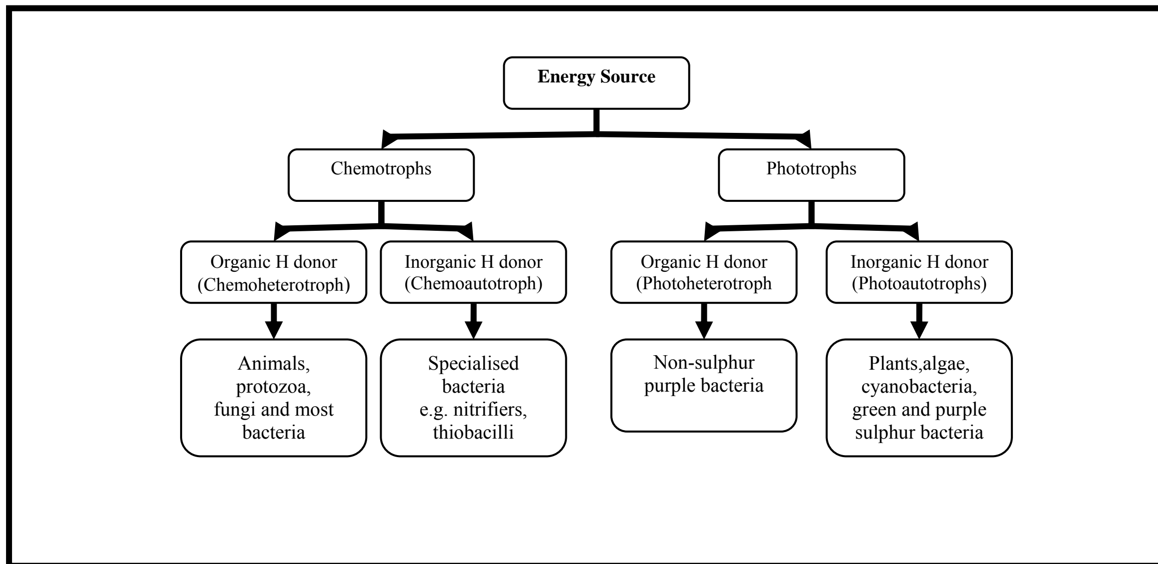



**Figure 1.** Classification of soil organisms based on the nature of their energy and principal carbon sources within the foodweb (adapted from Richards 1987).

processes causing changes, such as in pH, gas ratios, nutrients and growth factors. Commensalism, for example, occurs when one organism is dependent on the production of a growth factor by another. Thus, the auxotroph is dependent on the provider, while the provider neither benefits, nor suffers. This interaction is seen between cellulose degrading fungi and other microbes unable to utilize cellulose (Trevors & Van Elsas 1997). The cellulolytic fungi produce organic acids that serve as substrates for non-cellulolytic microbes. Probably the best known commensalistic relationship is between *Nitrosomonas* and *Nitrobacter* (Gooday 1988). *Nitrosomonas* transforms ammonium to nitrite, which in turn is transformed by *Nitrobacter* to nitrate. The whole process is called nitrification and the ammonium utilized during this process is generated by bacterial breakdown of amino acids (Richards 1987). Another potentially advantageous relationship is protocooperation, where both organisms stand to benefit from their interaction (Blain Metting 1993). Syntrophic associations can be considered to be protocooperative or mutualistic. This association arises when two or more species are required for the successful utilization of a particular resource. In anaerobic habitats this association exists between fermentative organisms capable of degrading complex

compounds and methanogenenic archaea. Methanogens only utilize a limited number of simple substrates, such as acetate, $CO_2$ and $H_2$, produced as the result of metabolism by fermentative anaerobic microbes. With the exception of isopropanol, methanogens are unable to metabolize compounds consisting of more than two carbons. Interestingly, by utilizing $H_2$ the methanogens ensure that the partial pressure of $H_2$ is maintained within a narrow range required by the fermenting microbes, which in turn provide the substrates for the methanogens. Another example of mutualism is lichens, which is the association between specific ascomycetous fungi and a certain green algae or cyanobacteria (Gooday 1988). In such an association the phototroph provides the fungus with carbon compounds and vitamins, while the fungus in turn provides the phototroph with mineral nutrients and water (Richards 1987).

Negative interaction is probably more common than positive interaction, the most important being the competition for limited resources (Stotzky 1972). The competition for carbon and energy sources, mineral nutrients, electron acceptors and space may occur when conditions become limiting. The sudden addition of an easily metabolizable carbon source to a soil microhabitat supporting organisms dependant on complex substrates, may firstly lead to the depletion of $O_2$ and eventually $NO_3^-$. This could cause competition between K-selected species and r-selected species, and ultimately competition among r-selected populations of facultative denitrifiers and strictly aerobic heterotrophs.

Amensalism occurs when a species is favoured by the export of metabolites, which adversely affect other species (Blain Metting 1993). The effect may either be direct, as in the production of antibiotics or inhibitors, or indirect, as when the activity of one species alters the environmental conditions. Whether it is streptomycin, which acts against Gram-positive organisms, or cycloheximide, which acts against eukaryotes, antibiotic production aids in the competitiveness of a particular organism (Gooday 1988). Many protozoa, bacteria and fungi also produce autoinhibitory compounds toxic to closely related taxa, thus “selecting” for more fit genotypes (Blain Metting 1993).

Parasitism and predation are further examples of negative interactions. In the strictest sense of the word, it is the case where one organism directly exploits the other as a food source. Protozoa are common predators in soil and are able to engulf particles or whole organisms via the process of phagocytosis (Richards 1987). Fungi and bacteria are usually not considered to be predators, as the cell wall prevents the uptake of solid particles. However, predacious fungi that trap and digest nematodes do exist. An example of predation among prokaryotes is the interaction between bdellovibrios and their host bacteria (Gooday 1988). These vibroid bacteria act as predators of Gram-negative bacteria, such as pseudomonads and enterobacteria. The *Bdellovibrio* cells collide with their prey and attach to the cell surface. Through enzymatic and physical penetration, the cell is able to lyse a pore through the cell wall of the prey and enter the periplasmic space. Once inside, *Bdellovibrio* grows by consuming nutrients originating from its prey and eventually divides into a number of daughter cells, which are released into the environment.

### 1.4 Yeasts associated with the soil microenvironment

A group of soil microbes of which the interactions has rarely been studied in the past are the yeasts. Although some soil yeasts are able to survive under oligotrophic conditions (Kimura et al. 1998), these fungi are normally isolated from relatively nutrient rich environments, such as the rhizosphere (Kvasnikov et al. 1975), dung and decaying toad stools (Fell & Statzell-Tallman 1998). Due to the aforementioned heterogeneity in the physicochemical composition of soil, yeasts are distributed unevenly in this habitat, nevertheless yeasts have been found in soils differing in texture, pH and chemical composition from diverse locations all over the world (Rose 1969).

Very little is known about the activity of soil yeasts *in situ*, as the majority of research has been performed *in vitro*, often using soil microcosms under controlled conditions (Botha 2005). Due to the absence of countless environmental factors when performing these studies, one is confronted by a fragmented image of what possibly occurs in the natural environment of soil yeasts. Nevertheless, we may gain some insight into soil yeast

biology from the results of these *in vitro* studies and ecological surveys conducted with standard plate count techniques. Using these plate count methods, it was found that the most effective sampling technique for yeasts is to isolate these unicellular fungi from the top 2-10 cm of the soil, where they are most abundant (Di Menna 1957). This is due to the fact that most of the yeasts found in soil are aerobic species and therefore more abundant in the surface layers (Spencer & Spencer 1997). These numbers are consequently expressed as viable units per gram of soil (Rose 1969). While soil may only serve as a temporary haven for certain species, providing protection against desiccation and drought, it serves as an important environmental niche to others. The former are able to survive until they are dispersed by environmental conditions, animals and even growing plants.

While yeasts may not be the most abundant microbes in soil, they are able to multiply in this often highly competitive environment, occurring sometimes at levels as high as $10^4$ and $10^5$ cells/g soil. Soil yeast numbers depend on the available nutrients and metabolizable compounds (Spencer & Spencer 1997). These yeasts often depend on filamentous fungi and bacteria to degrade polymeric recalcitrant compounds to simpler molecules such as the hydrolytic products of lignocellulosic plant materials (Rose & Harrison 1987). However, the species composition of the yeast community in a particular environment is not solely dependant on nutrient availability, but also on the absence of inhibitory agents (Starmer & Phaff 1980). Antibiotic-producing strains of actinomycetes are able to inhibit growth of sensitive yeast strains thereby affecting the composition of a particular soil yeast community.

Although yeasts are generally not as numerous as prokaryotes and moulds in soil, a wide variety of ascomycetous and basidiomycetous yeasts were found in this habitat (Spencer & Spencer 1997). Nutrient rich soils were found to support a wider variety of yeasts species than arid nutrient poor soils. Between 25 and 50% of the yeasts in nutrient rich soils were able to ferment carbohydrates. Yeasts are also more numerous in the soil beneath fruit bearing plants, as the spoiled fruit acts as a nutrient rich yeast inoculum (Phaff et al. 1966). Generally, soil further from the plant contains less yeast species

associated with that particular plant, as observed for oak and pine trees and their associated genera *Saccharomyces* and *Schizosaccharomyces* (Do Carmo-Sousa 1969). Both *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* have recently been isolated from oak-associated soils (Sniegowski et al. 2002).

Species belonging to the genera *Cryptococcus*, *Debaryomyces*, *Lipomyces* and *Schizoblastosporion* were repeatedly isolated from various soils, indicating their preference for this habitat (Phaff & Starmer 1987). Table 1 (pp 13-14) depicts some of the yeast genera associated with soil. It must, however, be noted that the ecological surveys of which the results are listed in Table 1 (pp 13-14), were based on the presence of culturable yeasts able to grow on selected isolation media. Thus, our knowledge of autochthonous soil yeasts is limited to the effectiveness of the isolation media used.

Most soil yeasts possess a wide spectrum of metabolic activities, which enable them to survive in nutrient limited environments. The ability to sporulate (certain *Hansenula* and *Lipomyces* species) may be viewed as an additional survival mechanism, providing protection during dessication and drought (Rose & Harrison 1987). *Lipomyces*, *Cryptococcus* and *Rhodotorula* produce capsules consisting of extracellular polymeric substances (EPS), possibly aiding survival in nutrient limited environments. Certain soil cryptococci are even able to form biofilms, consisting of cells embedded in EPS, when challenged with oligotrophic conditions in a flowcell system (Joubert et al. 2003). The production of EPS and formation of biofilms, is a known mechanism whereby microorganisms sequester and concentrate nutrients in oligotrophic environments (Decho 1990).

## 1.5 Role of yeasts in soil

### 1.5.1 Mineralization

Within the soil ecosystem, the flow of energy usually occurs away from plants, to heterotrophs further along the food chain. These organisms include microbes and

macroscopic fauna (Coleman & Crossley 1996). The primary decomposers, namely bacteria and fungi are able to degrade compounds derived directly or indirectly from plants. While yeasts are considered part of the fungal domain, the majority seem to be saprotrophs, contributing to the mineralization of numerous organic carbon compounds. A number of these yeasts are able to ferment carbohydrates, but the majority respire both carbohydrates and non-fermentable organic compounds (Kurtzman & Fell 2000). The presence of autochthonous soil yeasts (Lachance & Starmer 1998) serves to suggest that yeast play a role in the intricate decomposition processes taking place in soil.

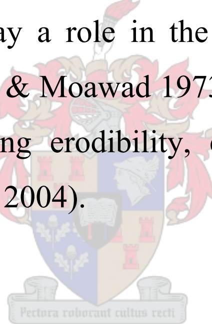
The majority of yeast species most frequently found in soil (Table 1, pp 13-14) are able to aerobically utilize L-arabinose, D-xilose and cellobiose (Kurtzman and Fell 1998). These are the products of the enzymatic degradation of lignocellulosic plant material by bacteria and moulds (Bisaria & Ghose 1981; Tomme et al. 1995). Some of these soil yeasts were also found to assimilate other intermediates of this degradation process, such as ferulic acid, 4-hydroxybenzoic acid and vanillic acid (Middelhoven 1993; Sampaio 1999). Even so, yeasts are not considered to play a major role in the decomposition of organic matter, due to their relatively low numbers compared to other soil microbiota (Phaff & Starmer 1987). However, in desolate habitats like the arctic zones, yeasts may be the dominant culturable soil microbes (Wynn-Williams 1982), hinting at a significant role in the decomposition process.

### 1.5.2 Inorganic nutrient release and soil formation

Some oligotrophic yeasts were found to grow on rocks above ground (Sterflinger & Prillinger 2001; Burford et al. 2003) and ascomycetous and basidiomycetous genera such as *Candida*, *Lipomyces*, *Rhodotorula* and *Trichoderma* were frequently isolated from rock substrates. In addition, euascomycetous taxa are found on an ever wider variety of rock substrates. These fungi include meristematic fungi, known as “black yeasts”. The latter grows yeast-like in culture and consists of the genera *Aureobasidium*, *Exophiala*, *Hormonema*, *Hortaea*, *Lecythophora*, *Phaeotheca*, *Rhinocladia* and *Sarcinomyces*. Their melanized cell walls, chlaymydospore formation and yeast-like morphology with

optimum surface to volume ration, make them suited for epilithic stress conditions, such as UV irradiation, temperature fluctuations, variations in available water and low nutrient conditions. These endo- and epilithic fungi constitute a significant proportion of the microbial communities present on granite, gypsum, limestone, marble, sandstone and siliceous rock types, e.g silica, silicates and aluminosilicates. Microbial communities present on these rocks contribute to the weathering and consequent soil formation, as well as nutrient liberation from this surface (Gadd & Sayer 2000; Burford et al. 2003). Thus, elements present in the rock (phosphorous, sulphur and trace elements) become bio-available. Dissolution is accomplished by the action of $H^+$, organic acids and siderophores.

Another manner in which yeasts impact on biological processes in soil, is through the production of EPS. These extracellular compounds enable soil yeasts from the genera *Cryptococcus* and *Lipomyces* to play a role in the formation of soil aggregates, thus impacting on soil structure (Bab'eva & Moawad 1973; Vishniac 1995). These aggregates stabilize the soil, thereby decreasing erodibility, enhancing porosity, water holding capacity and fertility (Bronick & Lal 2004).




## 1.6 Yeasts interactions in soil

In general, two types of habitats may occur in soil, the first is the bulk of the soil, usually relatively low in available nutrients; the second is the rhizosphere that usually contains more nutrients than the bulk of the soil. Although the type of microbial interactions occurring in these habitats would be similar, the microbial taxa participating in these interactions may differ as a result of differences in the physicochemical composition of the environment.

## 1.7 Yeast interactions in the rhizosphere

The rhizosphere is defined as the few millimeters of soil extending from the plant root into the surrounding soil (Huang & Germida 2002). This could be viewed as the interface

**Table 1**. Yeast species most commonly isolated from soil (Botha 2005).

| *Species according to Kurtzman and Fell (1998).* | *Originally identification during survey* | *Reference* | [a]*Veg* |
|---|---|---|---|
| *Cryptococcus albidus* | *Cryptococcus albidus /diffluens/ terricolus* | Di Menna 1965; Moawad et al. 1986; Bab'eva & Azieva 1980; Polyakova et al. 2001 | F; G; T; V |
| *Cryptococcus curvatus* | *Candida curvata* | Di Menna 1965; Moawad et al. 1986. | F; G; V |
| *Cryptococcus gastricus* | *Cryptococcus gastricus* | Bab'eva & Azieva 1980 | T |
| *Cryptococcus gilvescens* | *Cryptococcus gilvescens* | Polyakova et al. 2001 | T |
| *Cryptococcus humicolus* | *Candida humicola* | Di Menna 1965 | G; V |
| *Cryptococcus laurentii* | *Cryptococcus laurentii* | Babeva & Azieva 1980; Sláviková & Vadkertiová 2000 | F; T |
| *Cryptococcus podzolicus* | *Candida podzolica* | Bab'eva & Reshetova 1975 | V |
| *Cryptococcus terreus* | *Cryptococcus terreus* | Di Menna 1965 | G; V |
| *Filobasidium uniguttulatum* | *Cryptococcus uniguttulatus* | Bab'eva & Azieva 1980 | T |
| *Cystofilobasidium capitatum* | *Cystofilobasidium capitatum* | Sláviková & Vadkertiová 2000 | F |

Pectora roborant cultus recti

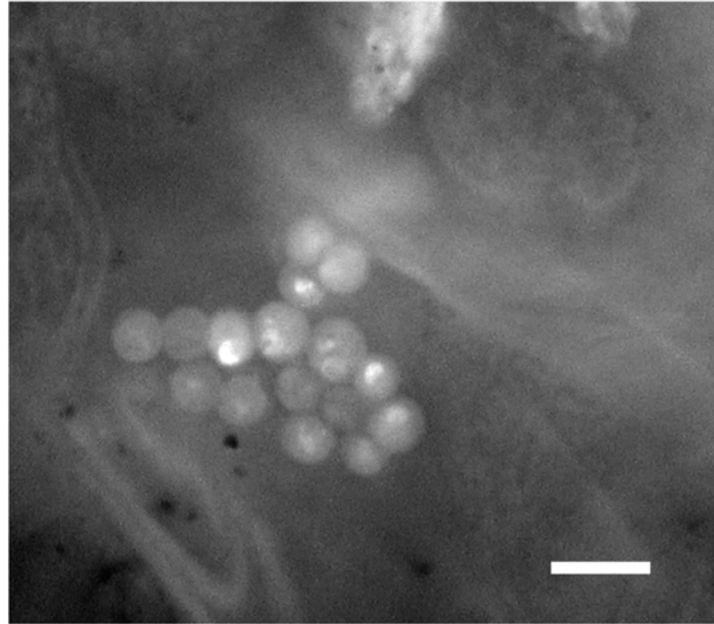
| ***Table 1 continued*** | | | |
|---|---|---|---|
| *Leucosporidium scottii* | *Leucosporidium scottii* | Sláviková & Vadkertiová 2000 | F |
| *Mrakia frigida* | *Candida curiosa/gelida* | Bab'eva & Azieva 1980 | T |
| *Rhodotorula aurantiaca* | *Rhodotorula aurantiaca* | Sláviková & Vadkertiová 2000 | F |
| *Rhodotorula glutinis* | *Rhodotorula glutinis* | Sláviková & Vadkertiová 2000; Moawad et al. 1986. | F; V |
| *Rhodotorula mucilaginosa* | *Rhodotorula mucilaginosa* | Polyakova et al. 2001 | T |
| *Schizoblastosporion starkeyi-henricii* | *Schizoblastosporion starkeyi-henricii* | Di Menna 1965 | G |
| *Sporobolomyces roseus* | *Sporobolomyces roseus* | Polyakova et al. 2001 | T |
| *Trichosporon cutaneum* | *Trichosporon cutaneum* | Di Menna 1965; Sláviková & Vadkertiová 2000 | G; F; V |

[a]General type of vegetation covering the soil that was sampled; F = Forest, G = Grass, T = Tundra, V = various.

through which energy is channeled from the plant to the soil biota. It is estimated that ca. 20 % of the carbon assimilated during photosynthesis is released via the roots into the soil. Due to this efflux of carbon the surrounding soil may differ from the bulk of the soil, with regards to pH, redox potential, as well as composition and concentration of organic compounds. It has been suggested that depending on soil conditions, the rhizosphere may select for specific microbial populations. A number of ascomycetous and basidiomycetous yeasts have been isolated from the rhizosphere (Table 2, pp 18-19). Should the redox potential of the rhizosphere decrease due to waterlogging (Huang & Germida 2002), some of these yeast will still be able to grow as result of fermentative metabolism (Table 2, pp 18-19). As in the case of other soil microbes, larger yeast populations are found in the rhizosphere than in the bulk of the soil not associated with plant roots (Moawad et al. 1986). Figure 2 (p 16) illustrates a yeast colony on the rhizoplane of sorghum. Yeast carbon and nitrogen sources exuded from the roots may contribute to the maintenance of the associated species. These exudates vary between plant species and under different growth conditions (Fan et al. 2001), possibly influencing the composition of the associated microbial populations.

Pectora roborant cultus recti

While the interactions of the yeasts in the rhizosphere may be largely unknown, a few documented cases hint at their role in this microhabitat. Legumes inoculated with *S. cerevisiae* showed increased nodulation and arbuscular mycorrhizal (AM) fungal colonization (Sing et al. 1991). Further, the hyphal growth of the AM fungus *Glomus intraradices* colonizing cucumber roots was found to benefit from the presence of bakers' dry yeast (Ravnskov et al. 1999). Phosphorous uptake by the AM fungi however, remained unaffected. When *Yarrowia lipolytica* and *Glomus deserticola* were used as an inoculum for tomato plants, it was found that the mycorhizal-root colonization was stimulated by the presence of the yeast (Vassilev et al. 2001). Basidiomycetous soil yeasts were also found to stimulate AM fungal growth, with *Rhodotorula mucilaginous* stimulating hyphal lengthening during *in vitro* germination of *Glomus mosseae* and *Gigaspora rosea* (Fracchia et al. 2003). The same effect was observed when exudates from the yeast cultured in liquid medium were added to the spores. These exudates also increased the colonization of the aforementioned AM fungi. *Candida valida*, *Rhodotorula*



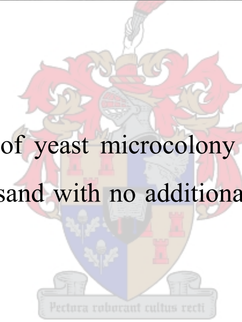

Pectora roborant cultus recti

**Figure 2.** Epi-fluorescence micrograph of yeast microcolony on rhizoplane of two-week- old sorghum seedling growing in medium of washed sand with no additional carbon source. The bar represents 10 μm (Botha 2005).

*glutinis* and *Trichosporon asahii* were also isolated from soil and found to be antagonistic towards the growth of the fungal root pathogen *Rhizoctonia solani* (El-Tarabilly 2004). These yeasts were able to colonize sugar beet roots and protect the seedlings and mature plants from *R. solani* diseases in glasshouse trials. Furthermore, the three yeasts species exerted a synergistic effect on disease suppression and promoted plant growth.

## 1.8 Yeast interactions known to occur in bulk soil

### 1.8.1 Predation and yeasts

Very little is known about the natural predators of yeasts in soil. An extensive search of literature produced only a few documented cases of soil organisms feeding on yeasts.

Nevertheless, evidence was found that the major predators in soil, such as protozoa, nematodes and micro-arthropods (Bardgett & Griffiths 1997), also act as predators of yeasts.

Protozoan activity is limited to the water films in soil pores (Bardgett & Griffiths 1997). While they only comprise a small percentage of the total biomass in soil, these eukaryotes may contribute up to 30% of the total net nitrogen mineralization. Protozoa have an impact on the size and composition of the microbial community and the turnover of nutrients and microbial biomass. The majority of protozoa are thought to feed on bacteria, with the exception of the mycophagous (fungal feeders), predatory (feed on other protozoa) and saprophytic protozoa that are able to absorb soluble compounds. However, very little is known about their interactions with yeasts. *Acanthamoeba* is probably the most common protist found in soil (Sawyer 1989) and has been known to feed on yeast (Allen & Davidowicz 1990). Benting et al. (1979) found that when they incubated a known soil amoeba, *Acanthamoeba polyphaga*, with three strains of *Cryptococcus neoformans*, the amoeba was able to phagocytose and kill these cells. After a period of 9 days, 99% of the *Cryptococcus* cells were phagocytosed. While the predation of yeast in soil may not be as drastic, it is likely that protozoa are able to feed on the yeasts occurring in this habitat. The fact that the majority of yeasts are found in the top 2-10 cm of soil (Di Menna 1957) and protist numbers are more numerous in the upper 10 cm of soil, suggests that interactions between these two groups are inevitable. Like protozoa, nematodes require a water film to be active (Bardgett & Griffiths 1997) and perform the same role in the ecosystem as protozoa, which is nutrient cycling. They are able to feed on animal parasites, bacteria, eukaryotic cells, fauna, fungal hyphae, organic matter and plants. Nematodes present in soil may be grouped according to their mouth parts, which dictate what they are able to feed on. Yeasts have been recorded as food sources (Yeates 1971) for *Alaimus* and *Rhabditis* (previously isolated from soil; Neher et al. 1999; Nielsen 1949), and *Panagrellus* (*Panagrellus redivivus* a known soil nematode; Hechler 1970; Yuen 1968). A well documented case is the interaction between the soil nematode *Caenorhabditis elegans* (Nicholas 1984), *Cryptococcus laurentii* (known soil

**Table 2.** Some yeasts species that were found to occur in the rhizosphere during a number of surveys (Botha 2005).

| *Species according to Kurtzman and Fell (1998a).* | *Originally identification during survey* | *Reference* | [a]*F* |
|---|---|---|---|
| *Bullera* species | *Bullera* species | De Azeredo et al. 1998 | - |
| *Candida azyma* | *Candida azyma* | De Azeredo et al. 1998 | - |
| *Candida krusei* | *Candida krusei* | Kvasnikov et al. 1975 | + |
| *Candida maltosa* | *Candida maltosa* | De Azeredo et al. 1998 | + |
| *Cryptococcus albidus* | *Cryptococcus albidus/ diffluens* | De Azeredo et al. 1998; Moawad et al. 1986; Kvasnikov et al. 1975 | - |
| *Cryptococcus curvatus* | *Candida curvata* | Moawad et al. 1986 | - |
| *Cryptococcus humicolus* | *Candida humicola* | Moawad et al. 1986 | - |
| *Cryptococcus laurentii* | *Cryptococcus laurentii* | De Azeredo et al. 1998; Kvasnikov et al. 1975. | - |
| *Debaryomyces hansenii* | *Debaryomyces hansenii/ kloeckeri* *Torulopsis famata* | De Azeredo et al. 1998; Moawad et al. 1986 | v |
| *Debaryomyces polymorphus* | *Debaryomyces phaffii/ cantarelli* | Kvasnikov et al. 1975. | + |
| *Debaryomyces vanrijiae* | *Debaryomyces vanriji* | Kvasnikov et al. 1975. | v |
| *Fellomyces species* | *Fellomyces species* | De Azeredo et al. 1998 | - |
| *Hanseniaspora uvarum* | *Hanseniaspora apiculata* | Kvasnikov et al. 1975. | + |
| *Leucosporidium scottii* | *Leucosporidium scottii* | De Azeredo et al. 1998 | - |
| *Metschnikowia pulcherrima* | *Metschnikowia pulcherrima* | Kvasnikov et al. 1975. | + |
| *Pichia guilliermondii* | *Pichia guilliermondii* | De Azeredo et al. 1998 | + |
| *Rhodotorula glutinis* | *Rhodotorula glutinis* | De Azeredo et al. 1998; Moawad et al. 1986 | - |
| *Rhodotorula minuta* | *Rhodotorula minuta* | De Azeredo et al. 1998 | - |
| *Rhodotorula mucilaginosa* | *Rhodotorula mucilaginosa* | De Azeredo et al. 1998 | - |

Pectora roborant cultus recti

***Table 2 continued***

| | | | |
|---|---|---|---|
| *Torulaspora delbreuckii* | *Torulaspora delbreuckii* | De Azeredo et al. 1998 | + |
| *Tremella mesenterica* | *Tremella mesenterica* | De Azeredo et al. 1998 | – |
| *Trichosporon cutaneum* | *Trichosporon cutaneum* | Kvasnikov et al. 1975 | – |
| *Saccharomyces cerevisiae* | *Saccharomyces cerevisiae* | De Azeredo et al. 1998 | + |
| *Williopsis californica* | *Hansenula californica* | Kvasnikov et al. 1975 | + |
| *Williopsis saturnus* | *Hansenula saturnus* | Kvasnikov et al. 1975 | + |

[a] Ability of species to ferment carbohydrates according to Kurtzman and Fell (1998); + = positive, - = negative, v = variable

yeast, Kurtzmann and Fell 2000) and *Cryptococcus kuetzingii* (closely related to the soil yeast, *Cryptococcus albidus*; Kurtzmann & Fell 2000; Mylonakis et al. 2002). Under laboratory conditions, *Caenorhabditis elegans* was able to survive on both *Cryptococcus kuetzingii* and *Cryptococcus laurentii* as sole food source, maintaining brood sizes similar to when it is grown on its laboratory food source, *Escherichia coli* OP50. However, when *C. elegans* was cultivated on the opportunistic human pathogen periodically encountered in soil, i.e. *Cryptococcus neoformans*, it was killed by the yeast capsule and a series of gene products associated with mammalian virulence. *Cryptococcus neoformans* caused distention of the nematodes' intestine when ingested, but it is not apparent whether the accumulation of cells led to the death of the nematode. Killing by an acapsular strain of *C. neoformans* has also been observed, where either toxins produced by the yeast, or toxic components of the cell wall were responsible for death. This would lead us to believe that *C. neoformans* virulence may be an environmental adaptation that evolved due to the interaction between this yeast and environmental predators such as free-living nematodes and amoebae.

An article that recently appeared in the Journal of Applied Ichthyology (Ricci et al. 2004), mentions a low cost method for mass production of the free-living nematode,

*Panagrellus redivivus*. It has been reported that *P. redivivus* is able to feed on *S. cerevisiae* (Hechler 1970) and researchers found that they were able to culture *P. redivivus* on a monoxenic *S. cerevisiae* culture. As *S. cerevisiae* has been isolated from soil, it would suggest that *P. redivivus* would be able to feed on *S. cerevisiae* when both organisms are present in soil. Interestingly, it was found that ethanol production by *S. cerevisiae* enhanced the growth of *Acinetobacter*, a bacterium which may be found in the same habitat as the yeast (Smith et al. 2004). The ethanol led to increased pathogenicity of the bacterium towards the predacious nematode *C. elegans*. Through this interaction, *S. cerevisiae* indirectly reduces the number of predators present. Furthermore, researchers found that when culturing the hermaphrodite nematode *Acrobeloides*, with *Pseudomonas cepacia* as its food source, yeast contamination of the bacterial culture is necessary for reproduction of the nematode (Ikonen 2001). They did, however, state that heavily contaminated bacterial cultures or yeast alone, did not support nematode population growth.

Prokaryotes may also prey on yeasts (Goto-Yamamoto et al. 1993). Yeast-lysing bacteria have been isolated from numerous sites in Brazil (soils, flowers, fermented foods and others) and screened for the ability to cause lysis of *S. cerevisiae* K-701. Fifty strains of yeast-lysing bacteria were isolated from 271 samples, all of which were Gram-positive. Forty six of these strains agglutinated with *S. cerevisiae* and caused lysis of viable cells. A well documented case is the lysis of the basidiomycetous yeast, *Rhodotorula glutinis* (known soil yeast, Vital et al. 2002; Yamanaka et al. 1993) by bacteria. In another study, twenty-seven strains of Myxobacteria were isolated from natural environments and tested against a number of yeasts. All the yeasts were lysed by nearly all the myxobacterial strains tested. Among the yeasts screened were *R. glutinis* AJ 5012, *Rhodosporidium toruloides* AJ 5212, *S. cerevisiae* AJ 14396, *Candida albicans* AJ 4297, *C. albidus* AJ 4297, *Geotrichum candidum* AJ 4884 and *Candida japonica* AJ 4692. The aforementioned species have all been isolated from soil (Kurtzmann & Fell 2000). Researchers found that Myxobacteria (soil bacteria, Reichenbach 1999) were able to lyse R. *glutinis*, which is known to be rather resistant to lytic enzymes, by apparently evacuating the cytoplasm and leaving the cell wall relatively intact. It would therefore

appear that the permeability of the cell surface is disrupted in such a way that the intracellular ingredients (like amino acids) are excreted. This allows the Myxobacteria to live on organisms that have a resistant cell wall.

In contrast to the role of yeasts as prey, yeasts may also act as predators themselves (Kreger-van Rij & Veenhuis 1973; Lachance & Pang 1997 ). The ability to penetrate and consume other yeasts has been observed in the type strain of *Arthroascus javanensis* (previously isolated from soil, Kurtzmann & Fell 2000) and three other filamentous yeast species. A number of yeasts, auxotrophic for sulphur-containing amino acids, were tested for predation against potential prey such as *S. cerevisiae* and *Metschnikowia* species. Among the potential predator yeasts tested were *A. javanensis, Pichia membranefaciens* (isolated from vineyard soil, Kurtzmann & Fell 2000) and *Saccharomycopsis fibuligera* (previously isolated from orchard soil**,** Kurtzmann & Fell 2000). Both *A. javanensis* and *S. fibuligera* were found to be predacious (Lachance & Pang 1997), provided that the following three requirements were met: Firstly, only low concentrations of complex nutrients had to be present in the medium. Secondly, excess organic sulphur, excreted by the prototrophic prey and serving as an attractant to predacious strains needed to be present. Thirdly, a solid surface, such as that of agar media, allowing sufficient contact between predator and prey needed to be present. The aforementioned requirements are met in soil, as soil is ultimately an oligotrophic environment, actively growing prey would be present in porous spaces allowing for attraction of the predator and there is a solid surface available on the soil particles. While auxotrophy for organic sulphur may seem detrimental to the yeasts in their natural environment, it may be a survival mechanism, as a number of toxic substances share common permeases with sulphate.

From the above it is obvious that the survival of yeasts and other microbes are the result of interactions between different organisms, as well as interactions of the yeast with its chemical and physical environment. This makes accurate predictions on the fate of particular yeast strains in soil very challenging. In future such predictions will become a necessity, especially since an ever-increasing number of genetically modified organisms

are being evaluated for industrial applications and the fate of such organisms, once released into the environment, is being contemplated.

## 1.9 Genetically modified organisms in the environment

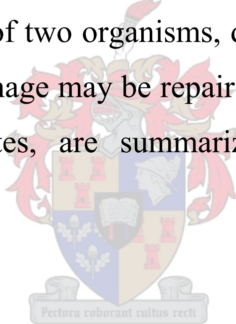
Considerable research has been conducted on the impact of transgenic plants and microbes on the environment, but the impact on the soil biota per se, has received very little attention (O'Callaghan 2001**.** This is understandable, as monitoring this ecological niche poses numerous challenges. Partly due to extreme variation in community structure, but also due to limited techniques in monitoring changes brought about by an external perturbation.

One of the major concerns about introducing new genes into the environment, is whether the introduced gene can cause harm to humans, domestic or wild animals, plants and crops (Bailey et al. 2001). This would depend on the nature of the introduced gene and the possibility that it may spread, although being highly unlikely. A large number of pathogenic bacteria are known and their genome sequences are available. An in depth study of pathogenic bacteria revealed that pathogenesis requires a number of associated genes often found in organizational clusters on plasmids and chromosomes. As pathogenicity is a highly complex trait, the chances of it occurring due to a simple mutation are very slim. *Salmonella typhimurium* requires at least 10-20 genes in order to infect mice, with more than 100 genes expressed during infection. Of all the genes utilized in genetic manipulation, the greatest concern surrounds antibiotic resistance genes. There is due concern, as the emergence of multidrug resistant bacteria have increased worldwide (Dzidic & Bedeković 2003). This is mainly due to microbial characteristics, selective pressure from the use of antibiotics, transfer of resistance genes between microbes and negligence in infection control practices. Microbes posses a wide range of mechanisms to deal with antibiotics, the main mechanisms being the production of inactivation enzymes (Gold & Moellering 1996; Paterson 2001), alteration of cell-wall target sites, alteration of DNA gyrase targets, permeability mutations and active efflux (Bearden and Danziger 2001; Levy 2002), as well as ribosomal modification. Through

recombination the resistance genes can be inserted into expression cassettes and spread through the microbial population via horizontal gene transfer mechanisms such as conjugation, transduction and transformation (Dzidic & Bedeković 2003). Should resistance genes become localized on plasmids, they can not only be transferred to closely related bacteria in the same environment, but even between different bacterial genera.

## 1.10 Natural mechanisms of horizontal gene transfer in prokaryotes

Bacteria do not engage in sexual reproduction, as most species of eukaryotes do (Bailey et al. 2001). Instead they utilize a number of mechanisms to facilitate gene transfer between species and even higher taxonomic groups. It is also believed that gene transfer plays a role in DNA repair among asexual prokaryotes (Frost 1992). By promoting recombination between the genes of two organisms, deleterious mutations that may have occurred due to environmental damage may be repaired. The mechanisms whereby genes are transferred among prokaryotes, are summarized in Table 3 (p 31) and are discussed in more detail below.




### 1.10.1 Transformation

Transformation occurs when a bacterial cell imports DNA from the surrounding environment and incorporates it into its own genetic material (O'Callaghan 2001). Free DNA may originate from dead cells, or may be actively secreted into the environment. The mechanism for DNA uptake varies among bacterial taxa, but generally it involves absorption onto the cell surface, followed by uptake via a protein channel (Bailey et al. 2001). The specificity of this mechanism varies from the uptake of conspecific DNA only, to the uptake of any DNA.  A prerequisite for transformation is that the cell must be in a competent state and be able to take up and incorporate the foreign DNA. Competence was found to be greatest under intermediate nutrient conditions. Species belonging to bacterial genera such as *Acinetobacter*, *Bacillus*, *Micrococcus* and *Pseudomonas* are

known to be naturally competent under appropriate environmental conditions. This behaviour could possibly accelerate the rate of adaptation by these organisms.

The majority of DNA taken up is degraded by restriction nucleases, which is a mechanism used to protect cells against foreign DNA, i.e. viral DNA (Bailey et al. 2001). The host DNA is protected either by methylation or by the absence of restriction enzyme target sites. This mechanism promotes incorporation of DNA originating from closely related species. Should this DNA be taken up by another organism and not be degraded, incorporation into the host genome will depend on sequence homology. However, if the foreign DNA is able to self- replicate, as in the case of plasmids and bacteriophages, it can remain inside the host organism. Thus, the foreign DNA may be potentially advantageous to the recipient cell and my even result in a phenotypical trait. Should the foreign DNA not be incorporated into the host genome, it may serve as a nutrient source for cellular metabolism, or provide nucleotides for DNA synthesis.

### 1.10.2 Conjugation

Conjugation is the transfer of a self-replicating mobile genetic element between bacteria (Frost 1992) and may occur between closely related species, different genera and even between bacteria differing in their Gram staining reactions (O'Callaghan 2001). A bacterial genome usually consists of a single chromosome, but plasmids may also be present (Bailey et al. 2001). The plasmid may impart a selective advantage on the host organism, increasing its fitness in a particular environment. Many cases of antibiotic resistance, resistance against toxins and environmental stresses are associated with plasmid-borne genes. Plasmids not only contain the sequences needed to replicate, but also the genes necessary to facilitate their transfer, via conjugation, from one cell to another.

The conjugation process entails physical contact, followed by the formation of a conjugative pilus (Bailey et al. 2001). Successful transfer of DNA during this process requires a recipient cell able to maintain the plasmid, as well as a metabolically active

host and donor cell. Should a cell contain a plasmid coding for the conjugative pilus and another plasmid unable to do so, the latter could be transferred to a recipient cell through the complementary action of the former. It is possible for plasmids to move from a recipient cell to a donor cell, via a process known as retrotransfer (Sia 1996). Should homology exist between the plasmid and the host cell, recombination may occur, incorporating the plasmid DNA into the host genome.

A Transposon is another type of genetic element which may occur either in the host genome or on a plasmid (Bailey et al. 2001). These mobile genetic elements code for their excision and incorporation elsewhere in the genome. Thus transposons are able to transfer DNA between chromosomes and plasmids.

### 1.10.3 Transduction

Bacteriophages consist of a DNA molecule surrounded by a protein sheath or capsid (Bailey et al. 2001) and have been studied in the soil rhizosphere (Ashelford et al. 2000). Upon contact with a susceptible host, these bacterial viruses transfer their DNA into the bacterial cell. Should the virus enter the lytic cycle, it will rapidly replicate and eventually rupture the host cell, releasing newly synthesized phages into the surrounding environment. Alternatively, the phage DNA may be incorporated into the host DNA where it would replicate as part of the bacterial genome. In the event of the phage carrying a beneficial gene, it could even increase the fitness of the host cell. This kind of replicative process of a phage and is known as lysogeny.

These interactions with the host cell may lead to generalized or specialized transduction (Bailey et al. 2001). The former occurs when host DNA, whether it be chromosome or plasmid fractions, become encapsulated either along with viral DNA, or without viral DNA. Thus, bacterial DNA can be transferred to a new host bacterium and possibly incorporated into its genome. Specialized transduction on the other hand, occurs when viral DNA incorporated into the host genome, enters the lytic phase. During the excision

process, host DNA may be excised along with the viral DNA, packaged into the capsid of the next generation of bacteriophages and transferred to the next host.

It is not known whether barriers to horizontal gene transfer exists in eukaryotes, but such mechanisms do exist in their prokaryotic counterparts (Krishnapillai 1996). A major mechanism is the effect of restriction enzymes on foreign DNA, which inhibits the incorporation of this DNA into the bacterial genome. *Escherichia coli* possess at least 11 naturally occurring restriction systems (Barcus 1995). DNA molecules that are not destroyed are methylated, facilitating recombination into the host genome (Krishnapillai 1996). A second barrier is the DNA mismatch repair system found in *E. coli* and *Salmonella typhimurium* (Matic et al. 1995). This mechanism prevents nonhomologous recombination between *E. coli* and *S. typhimurium*. However the error-prone SOS system stimulates nonhomologous recombination by triggering the RecBC-dependant SOS system.

**1.11 Natural mechanisms of horizontal gene transfer in eukaryotes**

Indirect evidence for horizontal gene transfer, based on non-homologous genetic recombination, has been found among eukaryotes and three main types of transposable genetic elements were suggested to be involved in these processes, i.e. transposons, retrotransposons and retroposons (Krishnapillai 1996).

Transposons contain either perfect or imperfect terminal repeats, which transpose via a DNA intermediate, leading to target site duplications 8bp in size (Krishnapillai 1996). They show similarity to prokaryotic transposons and tend to be highly promiscuous and occur in hosts as diverse as fungi and vertebrates. Retrotransposons possess long terminal repeats and transpose via a RNA intermediate, leading to 5bp duplications. Retroposons generate 12bp duplications and lack the long terminal repeats found in retrotransposons.

Although direct evidence for horizontal gene transfer in eukaryotes is lacking, there is enough circumstantial evidence to support this theory (Krishnapillai 1996). The

distribution of the *Tc1-mariner* family of transposons among arthropods, ciliates, fungi, nematodes, planarians and vertebrates is a perfect example. Even though these organisms possess highly heterologous genomes, the transposase responsible for the transposition of this element is highly conserved in all these cases. Homologues of *Tc1-mariner* have been found in bacteria, making it highly improbable that this transposon originated from a common ancestor. Another example of horizontal gene transfer is the inverted repeat sequences found at the termini of 23 genetic elements present in both animal and plant genomes. Members of both the *Ac*-like (8bp duplications) and 'CACTA' (3bp duplications) families of transposable elements were found to occur in the animal and plant kingdoms. Furthermore, the transposases of four of the 23 elements mentioned above are highly conserved.

### 1.12 Horizontal gene transfer between prokaryotes, eukaryotes and vice versa

Evidence does exist to support the theory of horizontal transfer of DNA between prokaryotes and eukaryotes. As mentioned above, homologues of the *Tc1-mariner* family of transposons have been found both in eukaryotes and prokaryotes (Krishnapillai 1996). Another prime example is the gene for glyceraldehydes-3-phosphate dehydrogenase i.e. *Gapdh* (Smith et al. 1992). *Escherichia coli* possess two versions of this gene. The first was most likely acquired by an ancestor of *E. coli* from a eukaryote, as phylogenetic studies revealed that it shows more similarity to the eukaryotic sequence than to any prokaryotic example. The second is similar to that of the bacterial ancestor. This theory was substantiated when the eukaryotic form of *Gapdh* was found in numerous species of enteric bacteria. It is possible that horizontal gene transfer may have taken place in the gut of a eukaryotic organism, before the divergence of animals and plants.

A similar example is the gene for glutamine synthetase (*GS2*) found in *Bradyrhizobium japonicum*, which shows great structural similarities with the eukaryotic version of the gene, while the alternate form, *GS1*, has only been found in archaea and eubacteria (Smith et al. 1992). As *B. japonicum* is associated with plants, it is plausible that transfer of the gene could have taken place between early plants and ancestors of this bacterium.

There is an even stronger case for horizontal transfer of the gene for glucose phosphate isomerase in *Escherichia coli*, as the sequence of this gene shows 88% homology with that of eukaryotic glucose phosphate isomerase. The possibility therefore exists that somewhere in the distant past some *E. coli* strains lost this gene and reacquired it from a eukaryote.

Phylogenetic studies also revealed evidence for the transfer of DNA from prokaryotes to eukaryotes (Smith et al. 1992). The first example is that of Fe-superoxide dismutase (Fe-SOD) found in the protist *Entamoeba histolytica*. This particular dismutase shows 60% similarity with a number of bacterial dismutases, but only 30% similarity to those found in other eukaryotes. As *E. histolitica* feeds on bacteria, the theory of Fe-SOD being of bacterial origin therefore seems not be too far fetched.

Further evidence for the transfer of genes from eubacteria to eukaryotes was obtained after sequence analysis of the genes for fructose-biphosphate aldolase (Smith et al. 1992). Only four class II aldolases were reported, three from eubacteria and one from the yeast *S. cerevisiae*. The yeast aldolase was found to be more similar to the *E. coli* aldolase than any of the bacterial aldolases are to each other. Interestingly, Heinemann and Sprague (1989) found that a conjugative plasmid of *E. coli* could be transferred to *S. cerevisiae* via a process resembling conjugation, providing more evidence for the direct transfer of genes between these phylogenetic unrelated groups.

**1.13 The fate of extracellular DNA in the environment**

The possibility of transformation by naked DNA is dependent on the half life of the DNA involved as well as the physicochemical properties of the soil (Bailey et al. 2001). The greater majority of soil microbes possesses DNA-degradation mechanisms and may be responsible for the destruction of a significant fraction of extracellular DNA. Despite the activity of these mechanisms, notable quantities of naked DNA can still be extracted from soil and aquatic environments. DNA may be protected by adhesion to soil particles, but also by the formation of complexes with soil minerals such as clay, quartz and humic

acid. However, the latter may prevent the uptake of naked DNA by soil microbes (Aardema et al. 1983; Blum et al. 1997). Table 4 (p 32) lists the estimated half life of DNA in different environmental settings.

### 1.14 The impact of genetically modified organisms on the native soil biota

Once a GMO is released into the environment it may not only be able to multiply, but it may also be able to evolve under selective pressures exerted on it by the soil environment (De Leij et al. 1995). As previously explained, these organisms may be able to exchange DNA with other soil microbes using a number of different processes. Therefore, their genetic information has to be taken into consideration, should they be released into the environment. Aside from the dangers of gene transfer, the possible impact of a GMO on the soil microbial community should also be taken into consideration. Earlier studies performed in experimental environments showed detrimental effects on protozoa (Austin et al. 1990), increased carbon turnover (Wang et al. 1990), displacement of indigenous microbes of the soil rhizosphere and suppression of fungal populations in soil (Short et al. 1990). One such study focused on the impact of a genetically modified *Pseudomonas fluorescens* and a wild-type *P. fluorescens* strain on the soil microbiota in the field. The release of both strains led to significant, yet transient, perturbations of the culturable microorganisms associated with the soil rhizosphere. However, no significant changes were found in the bulk soil away from the rhizosphere. Yeasts and other fast growing organisms that were unable to form resistant structures appeared to be the most sensitive to the release of *P. fluorescens*. Interestingly, the changes brought about by the modified *P. fluorescens* strain did not differ significantly from those brought about by the wild-type strain. No changes were observed in plant growth or health. This is but one case and illustrates the need for field studies before each and every release of a GMO.

Very little is known about the fate of genetically modified strains of the wine yeast *S. cerevisiae* in soil. Since this species is known to occur naturally in soil (Sniegowski et al. 2002), it does interact with the soil biota (see: “Yeasts interactions in soil”); and as genetically modified strains of this species do exist and the future release thereof into the

environment is inevitable, studies on the growth of genetically modified strains of *S. cerevisiae* in soil is expedient. However, the growth and interactions of wild type *S. cerevisiae* within soil has not even been studied. As will be explained in the next chapter, the latter may partly be ascribed to inappropriate methods to monitor viable cells of this species in soil.

**Table 3.** Some characteristics of the recognized mechanisms of horizontal gene transfer in bacteria (Nielsen et al. 1998).

| Characteristic | Mechanism of gene transfer | | |
|---|---|---|---|
| | **Transduction** | **Conjugation** | **Transformation** |
| **Donor organism** | Bacterium infected with a bacteriophage | Bacterium harboring a plasmid or conjugative transposon | Any organism with double- stranded DNA |
| **Requirements to the recipient bacterium**[a] | Attachment sites for binding of bacteriophage (phage receptors) | Able to bind pilli from donor bacterium | Expression of competence for uptake of DNA |
| **Vector** | Bacteriophage | Plasmid or chromosome with an inserted plasmid or a conjugative transposon | Free DNA |
| **Stability of vector DNA (in soil, water, or on plants)** | Long-term stability expected due to protection of DNA in the protein envelopes of bacteriophages | Stability depends on the survival and activity of the donor bacterium | Stability of free DNA is poor due to nucleases, chemical modifications, shearing or binding to solids |
| **Host range dependence** | Presence of phage attachment sites | Binding of pili and plasmid incompatibility[b] | Ability of DNA to integrate into the genome or recircularize into plasmids |
| **Size range of transferred DNA (average size transferred)** | ≤ 100 kb (45 kb) | < 100 bp to $10^6$ kb[c] (size of bacterial plasmids) | 0- > 25 kb (< 100 bp- 10 kb) |
| **Shown to occur in/on:** | Soil, plants and water | Soil, plants and water | Soil, plants and water |

[a]The recipients must be able to stabilize the transferred DNA by either homologous/illegitimate recombination into the genome, or recircularization into plasmids.
[b]Unless the plasmid is integrated into the bacterial genome.
[c]Partial to whole genomes can be transferred if the plasmid is chromosomally integrated in the donor (e.g. high frequency of recombination strains of *E.coli*).

Pectora roborant cultus recti

**Table 4.** Estimated DNA half life in various environments (Bailey et al. 2001).

| Location | Halflife (h) |
|---|---|
| **Aquatic environment** | |
| *Waste water* | 0.017-0.17[a] |
| | 0.23[b] |
| *Freshwater* | |
| Oligotrophic | 4.2[c] |
| Eutrophic | 5.5[b] |
| *Marine water* | |
| Estuarine | 3.4-5.2[c] |
| | 5.5[b] |
| *Ocean surface* | |
| Oligotrophic | 12.8[c] |
| P limited | 4.5[c] |
| Not P limited | 45.0-83.0[c] |
| *Marine sediment* | 235[d] |
| | 140[c] |
| **Terrestrial environment** | |
| *Soil* | |
| Loamy sand soil | 9.1[e] |
| Silty clay soil | 15.1[e] |
| Clay soil | 28.2[e] |

[a]Conversion of supercoiled into relaxed-circular or linear plasmid DNA.
[b]Loss of hybridization signals of plasmid DNA in Southern transfers or blots.
[c]Loss of acid-precipitated material (colourimetric DNA determination of $^{32}$P-labelled plasmid DNA).
[d]In dead cells degradation measured as in footnote c.
[e]Loss of transformation activity of plasmid DNA.

Pectora roborant cultus recti

## 1.15 References

Aardema BW, Lorenz MG, Krumbein WE (1983) Protection of sediment-adsorbed transforming DNA against enzymatic inactivation. Appl Env Mircobiol 46: 417-420

Abdel-El-Haleem D (2003) *Acinetobacter*: Environmental and biotechnological applications. Afr J Biotech 2: 71-74

Allen PG & Davidowicz EA (1990) Phagocytosis in *Acanthamoeba*: 1. A mannose receptor is responsible for the binding and phagocytosis of yeast. J Cell Physiol 145: 508-513

Andrew JH & Harris RF (1986) r- and K-selection and microbial ecology. Adv Microb Ecol 9: 99-147

Ashelford KE, Norris SJ, Fry JC, Bailey MJ, Day MJ (2000) Seasonal populational dynamics and interactions of competing bacteriophages and their host in the rhizosphere. Appl Env Mircobiol 66: 4193-4199

Austin HK, Hartel PG, Coleman DC (1990) Effect of genetically-altered *Pseudomonas solanacearum* on predatory protozoa. Soil Biol Biochem 22: 115-117

Bab'eva IP & Moawad H (1973) Soil yeasts of the genus Lipomyces as soil-conditioning agents. Russian Journal of Soil Science 8: 430 – 432

Bailey MJ, Timms-Wilson TM, Lilley AK, Godfray HCJ (2001) The risks and consequences of gene transfer from genetically-manipulated micro-organisms in the environment. Research report for the Department for Environment, Food and Rural Affairs (DEFRA)

Barber WL & Lynch JM (1979) Microbial growth in the rhizosphere. Soil Biol Biochem 9: 305-308

Barcus V A,' Titheradge AJB, Murray NE (1995) The diversity of alleles at the hsd locus in natural populations of *Escherichia coli*. Genet 140: 1187-1197

Bardgett RD & Griffiths BS (1997) Ecology and biology of soil protozoa, nematodes and microarthropods. *Modern Soil Microbiology* (Van Elsas JD, Trevors JT, Wellington EMH, eds.), Marcell Dekker, Inc., New York, pp. 129-159

Bearden DT & Danziger LH (2001) Mechanism of action of and resistance to quinolones. Pharmacotherapy 21: 224S-232S

Benting LA, Neilson JB, Bulmer GS (1979) *Cryptococcus neoformans*: Gastronomic delight of a soil ameba. Sabouraudia 17: 225-232

Bisaria VS & Ghose TK (1981) Biodegradation of cellulosic materials: substrates, microorganisms, enzymes and products. Enzyme Micro Technol 3: 90-104

Blain Metting F, Jr (1993) Structure and physiological ecology of soil microbial communities. In: Blain Metting F, Jr (ed) Soil microbial ecology. Applications in agricultural and environmental management. Marcel Dekker, Inc. New York, pp 3-14

Blum SAE, Lorenz MG, Wackernagel W (1997) Mechanism of retarded DNA degradation and Prokaryotic origin of DNases in nonsterile soils. System Appl Microbiol 20: 513-521

Bohn HL, McNeal BL, O'Conner GA (1979) Soil chemistry. Wiley-Interscience. New York

Pectora roborant cultus recti

Botha A (2005) Yeasts in soil. In: Ecophysiology of Yeasts, eds. C. A. Rosa & G. Péter. Springer-Verlag, Heidelberg (Accepted for publication)

Bronick CJ & Lal R (2004) Soil structure and management. Geoderma (in press)

Burford EP, Kierans M, Gadd GM (2003) Geomycology: fungi in mineral substrata. Mycologist 17: 98- 107

Coleman DC & Crossley DA (1996) Secondary production: Activities of heterotrophic organisms – microbes. In: Coleman DC & Crossley DA (eds) Fundamentals of soil ecology, Academic Press, London, pp 33 – 195

Decho AW (1990) Microbial exopolymer secretions in ocean environments: Their role(s) in food webs and marine processes. Oceanogr Mar Biol Annu Rev 28: 73 – 153

De Leij FAAM, Sutton EJ, Whipps JM, Fenlon JS, Lynch JM (1995) Impact of field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. Appl Env Mircobiol 61: 3443-3453

Di Menna ME (1957) The isolation of yeast from soil. J Gen Microbiol 17: 678-688

Do Carmo-Sousa L (1969) Yeasts Distribution of yeasts in nature. In: Rose AH & Harrison JS (eds) The yeasts, biology of yeasts, 1st edn, vol 1. Academic Press, London, pp 79 – 105

Dzidic S & Bedeković V (2003) Horizontal gene transfer-emerging multidrug resistance in hospital bacteria. Acta Pharmacol Sin 24: 519-526

El-Tarabily KA (2004) Suppression of Rhizoctonia solani diseases of sugar beet by antagonistic and plant growth-promoting yeasts J Appl Microbiol 96: 69-75

Fan T W-M, Lane AN, Schenker M, Bartley JP, Crowley D, Higashi RM (2001) Comprehensive chemical profiling of gramineous plant root exudates using high-resolution NMR and MS. Phytochemistry 57: 209-221

Fell JW & Statzell-Tallman A (1998) *Cryptococcus* Vuillemin. In: Kurtzman CP & Fell JW (eds) The yeasts, A taxonomic study, 4th edn. Elsevier, Amsterdam, pp 742 – 767

Fracchia S, Godeas A, Scervino JM, Sampedro I, Ocampo JA, García-Romera I (2003) Interaction between the soil yeast *Rhodotorula mucilaginosa* and the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora rosea*. Soil Biol Biochem 35: 701–707

Frost LS (1992) Bacterial conjugation: everybody's doin' it. Can J Microbiol 38: 1091-1096

Gadd GM & Sayer GM (2000) Fungal transformations of metals and metalloids. In: Lovley DR (ed) Environmental microbe-metal interactions, ASM, Washington

Gold HS & Moellering RC Jr (1996) Antimicribial-drug resistance. N Engl J Med 335: 1445-1453

Gooday GW (1988) The potential of the microbial cell and its interaction with other cells. In: Lynch JM & Hobbie JE (eds) Micro-organisms in action: Concepts and applications in microbial ecology. Blackwell Scientific Publications. Oxford, pp 24-32

Goto-Yamamoto N, Sato SI, Miki H, Park YK, Tadenuma M (1993) Taxonomic studies on yeast-lysing bacteria, and a new species *Rarobacter Incanus*. J Gen Appl Microbiol 39: 261-272

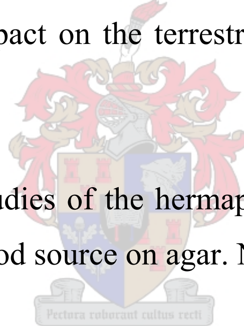
Gray TRG & Williams ST (1971) Soil micro-organisms. Longman, London

Grayston SJ, Campbell CD, Bardgett RD, Mawdsley JL, Clegg CD, Ritz K, Griffiths BS, Rodwell JS, Edwards SJ, Davies WJ, Elston DJ, Millard P (2004) Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Appl Soil Ecol 25: 63-84

Hechler HC (1970) Reproduction, chromosome number and postembryonic development of Panagrellus redivivus (Nematoda: Cephalobidae). J Nematol 2: 355-361

Heinemann JA & Sprague Jr GF (1989) Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nat 340: 205-209

Huang PM & Germida JJ (2002) Chemical and biological processes in the rhizosphere: Metal pollutants. In: Haung PM, Bollag J-M, Senesi N (eds) Interactions between soil particles and microorganisms, Impact on the terrestrial ecosystem. John Wiley & Sons Ltd, Chichester




Ikonen EK (2001) Life history studies of the hermaphrodite nematode *Acrobeloides* sp. with *Pseudomonas cepacia* as a food source on agar. Nematology 8: 759-766

Joubert L-M, Botha A, Wolfaardt G M (2003) Feeding relationships in yeast-ciliate biofilms. In: McBain A, Allison D, Brading M, Rickard A, Verran J, Walker J (eds) BBC 6, Biofilm communities: Order from chaos? Bioline, Cardiff

Kimura Y, Nakano Y, Fujita K, Miyabe S, Imasaka S, Ishikawa Y, Sato M (1998) Isolation and characteristics of yeast able to grow at low concentrations of nutrients. Yeasts 14**:** 233 - 238

Ko WH & Lockwood JL (1976) Soil fungistasis: relation to fungal spore nutrition. Phytopathology 57: 894-901

Kreger-van Rij NJW & Veenhuis M (1973) Electron microscopy of some special cell contacts in yeasts. J Bacteriol 113: 350-356

Krishnapillai V (1996) Horizontal gene transfer. J Genet 75: 219-232

Kurtzman CP & Fell JW (1998) The yeasts: A taxonomic study, 4th edition. Elsevier, Amsterdam

Kurtzman CP & Fell JW (2000) The yeasts: A taxonomic study, a 4th and enlarged edition. Elsevier, the Netherlands

Kvasnikov EI, Nagornaya SS, Shchelokova, IF (1975) Yeast flora of plant rhizosphere and phyllosphere. Microbiologia

Lachance MA & Pang WM (1997) Predacious yeasts. Yeast 13: 225-232

Lachance MA & Starmer WT (1998) Ecology of yeasts. In: Kurtzman CP & Fell JW (eds) The yeasts, a taxonomic study, 4th edn. Elsevier, Amsterdam, pp 21 – 30

Levy SB (2002) Active efflux, a common mechanism for biocide and antibiotic resistance. J Appl Microbiol 92 Suppl: 65S-71S

Matic I, Rayssiguier C, Radman M (1995) Interspecies gene exchange in bacteria: The role of SOS and mismatch repair systems in evolution of species. Cell 80: 507-515

Middelhoven WJ (1993) Catabolism of benzene compounds by ascomycetous and basidiomycetous yeasts and yeastlike fungi. Antonie van Leeuwenhoek 63: 125-144

Moawad H, Salem SH, Badr El-Din SMS, Khater T, Iskandar M (1986) Yeasts in soils of Egipt. Zentralbl Mikrobiol 141: 431-435

Pectora roborant cultus recti

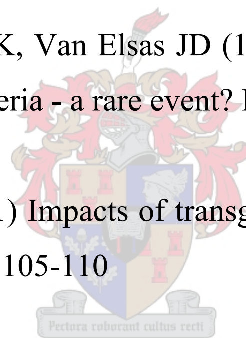
Mylonakis E, Ausubel FM, Perfect JR, Heitman J, Calderwood B (2002) Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. PNAS 99: 15675-15680

Neher DA, Weicht TR, Savin M, Görres JH, Amador JA (1999) Grazing in a porous environment.2. Nematode community structure. Plant and Soil 212: 85-99

Nicholas WL (1984) The biology of free-living nematodes, 2nd edition. Oxford University Press, New York, pp. 168

Nielsen CO (1949) Studies on the soil microfauna 2.The soil inhabiting nematodes. Natura jutl. 2: 1-132

Nielsen KM, Bones AM, Smalla K, Van Elsas JD (1998) Horizontal gene transfer from transgenic plants to terrestrial bacteria - a rare event? FEMS Micro Rev 22: 79-103



O'Callaghan M & Glare TR (2001) Impacts of transgenic plants and microorganisms on soil biota. NZ Plant Protection 54: 105-110

Paterson DL (2001) Extended-spectrum beta-lactamases: The European experience. Curr Opin Infect Dis 14: 697-701

Phaff HF, Miller MW, Mrak EM (1966) Chapter VIII, Ecology. In: Phaff HF, Miller MW, Mrak EM (eds) The life of yeasts. Harvard University Press, Cambridge, pp 93–123

Phaff HJ & Starmer WT (1987) Yeasts associated with plants, insects and soil. In: Rose AH & Harrison JS (eds) The yeasts, biology of yeasts, 2nd edn, vol 1. Academic Press, London, pp 123 - 180

Poindexter JS (1981) Oligotrophy. Fast and famine existence. In: M Alexander (ed) Advances in Microbiol Ecology, vol 5. Plenum Press, New York, pp 63-89

Ravnskov S, Larsen J, Axel Olsson P, Jakobsen I (1999) Effects of various organic compounds on growth and phosphorus uptake of an arbuscular mycorrhizal fungus. New Phytol 141: 517-524

Reichenbach H (1999) The ecology of the myxobacteria. Env Microbiol 1: 15-21

Ricci M, Fifi AP, Ragni A, Schlechtriem C, Focken U (2004) Development of a low-cost technology for mass production of the free-living nematode *Panagrellus redivivus* as an alternative live food for first feeding fish larvae. Appl Microbiol Biot 60: 556-559

Richards BN (1987) The microbiology of terrestrial ecosystems. Longman Group UK limited. Harlow Essex, pp 110-114

Rose AH & Harrison JS (1969) The yeasts. Academic Press, London, pp 82-89

Rose AH & Harrison JS (1987) The yeasts, yeasts and the environment, 2nd edn, vol 2. Academic Press, London

Saetre P & Bååth E (2000) Spatial variation and patterns of soil microbial community structure in a mixed spruce-birch stand. Soil Biol Biochem 32: 909-917

Sampaio JP (1999) Utilization of low molecular weight aromatic compounds by heterobasidiomycetous yeasts: taxonomic implications. Can J Microbiol 45: 491 – 512

Sawyer TK (1989) Free-living pathogenic and nonpathogenic amoebae in Maryland soils. Appl Environ Microbiol 55: 1074-1077

Short KA, Seidler RJ, Olsen RH (1990) Survival and degradative capacity of *Pseudomonas putida* induced or constitutively expressing plasmid-mediated degradation of 2,4-dichlorophenoxyacetate (TFD) in soil. Can J Microbiol 36: 821-826

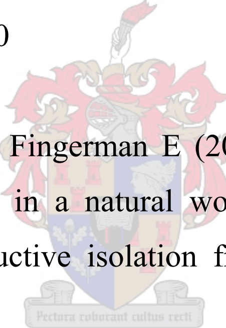
Sia EA, Kuehner DM, Figurski DH (1996) Mechanism of retrotransfer in conjugation: Prior transfer of the conjugative plasmid is required. J Bac 178: 1457-1464

Singh CS, Kapoor A, Wange SS (1991) The enhancement of root colonization of legumes by vesicular-arbuscular mycorrhizal (VAM) fungi through the inoculation of legume seed with commercial yeast (*Saccharomyces cerevisiae*). Plant Soil 131: 129-133

Smith MG, Des Etages SG, Snyder M (2004) Microbial synergy via an ethanol-triggered pathway. Mol Cell Biol 24: 3874-3884

Smith MW, Feng D, Doolittle RF (1992) Evolution by acquisition: The case of horizontal gene transfers. TIBS 17: 489-493

Smith OL (1982) Soil microbiology: A model of decomposition and nutrient cycling. CRC Press, Inc. Florida, pp 161-170




Sniegowski PD, Dombrowski PG, Fingerman E (2002) *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. FEMS Yeast Research 1 299-306

Spencer JFT & Spencer DM (1997) Ecology: Where yeasts live. In: Spencer JFT, Spencer DM (Eds) Yeasts in natural and artificial habitats. Springer, Berlin, pp 33 - 58

Starmer WT & Phaff HJ (1980) Specificity of natural habitats for yeasts and yeast-like organisms. In: Skinner FA, Passmore SM, Davenport RR (eds) Biology and activities of yeasts. Academic Press. London, pp 79-100

Sterflinger K & Prillinger H (2001) Molecular taxonomy and biodiversity of rock fungal communities in an urban environment (Vienna, Austria) Antonie van Leeuwenhoek 80: 275 – 286

Stotzky G (1972) Activity, ecology and population dynamics of microorganisms in soil. CRC Crit Rev Microbiol 2: 59-137

Stotzky G & Burns RG (1982) The soil environment: Clay-humus-microbe interactions. In: Burns RG & Slater JH (eds) Experimental microbial ecology. Blackwell Scientific. Oxford, pp 105-133

Stozky G (1997) Soil as an environment for microbial life. In: Van Elsas JD, Trevors JT, Wellington EMH (eds) Marcel Dekker, Inc. New York, pp 1-19

Tomme P, Warren RAJ, Gilkes NR (1995) Cellulose hydrolyses by bacteria and fungi. Adv Microb Physiol 37: 1-81

Trevors JT & Van Elsas JD (1997) Microbial interactions in soil. In: Van Elsas JD, Trevors JT, Wellington JMH (eds) Modern soil microbiology.Marcel Dekker, Inc. New York, pp 215-243

Vassilev N, Vassileva M, Azcon R, Medina A (2001) Application of free and Ca-alginate-entrapped *Glomus deserticola* and *Yarrowia lipolytica* in a soil–plant system. J Biotechnol 91: 237–242

Vishniac HS (1995) Simulated *in situ* competitive ability and survival of a representative soil yeasts, *Cryptococcus albidus*. Micro Ecol 30: 309 – 320

Vital MJS, Abranches J, Norton A, Mendonca-Hagler LC (2002) Mycocinogenic yeasts isolated from Amazon soils of the Maracá Ecological Station, Roraima-Brazil. Braz J Microbiol

Wang Z, Crawford DL, Magnuson TS, Bleakley BH, Hertel G (1990) Effects of bacterial lignin peroxidase on organic carbon mineralization in soil, using recombinant *Streptomyces* strains. Can J Microbiol 37: 287-294

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, Van der Putten WH, Wall DH (2004) Ecological linkages between aboveground biota. Science 304: 1629-1633

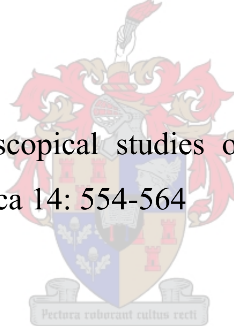
Williams ST (1985) Oligotrophy in soil: fact or fiction. In: Fletcher M & Floodgate GD (eds) Bacteria in their natural environments, London Academic Press, pp 81 - 110

Wynn-Williams DD (1982) Simulation of seasonal changes in microbial activity of maritime Antarctic peat. Soil Biol Biochem 14:1-12

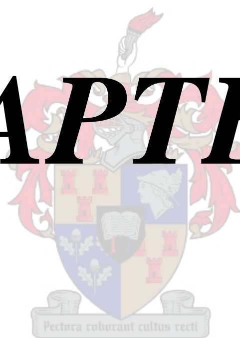
Yamanaka S, Kanbe S, Fudo R (1993) Lysis of basidiomycetous yeast, Rhodotorula glutinis caused by myxobacteria. J Gen Appl Microbiol 39: 419-427

Yeates GW (1971) Feeding types and feeding groups in plant and soil nematodes. Pedobiologia 11: 173-170

Yuen PH (1968) Electron microscopical studies on the anterior end of Panagrellus silusiae (Rhabditidae). Nematologica 14: 554-564



# *CHAPTER 2*



*A Method for the Selective Enumeration of Fermentative Yeasts In Soil*

## 2.1 Introduction

The presence and distribution of fermentative yeasts have mostly been studied in relation to breweries, vineyards and winemaking (Kurtzman & Fell 1998; Jolly et al. 2003). During these studies a variety of selective and enumeration techniques were used to determine fermentative yeasts in different habitats. Some workers used enrichment methods to determine the presence of yeasts, while others employed selective or differential agar media to enumerate yeasts in habitats associated with oenology. However, typical fermentative yeasts have also been found in habitats not associated with winemaking (Do Carmo-Sousa 1969). For example, it was found that representatives of the genus *Saccharomyces* occur in soil. Recently, an enrichment procedure was used to obtain *Saccharomyces cerevisiae* from soil associated with oak trees (Sniegowski et. al. 2002).

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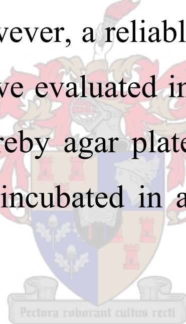
The isolation from and enumeration of yeasts in soil is particularly challenging, because of potential contamination by a wide spectrum of filamentous fungal species in soil samples. To counter this contamination, enumeration plates may be prepared using highly selective media containing a cocktail of selected nutrients and antimicrobial agents (Cornelissen et al. 2003). However, some yeasts, e.g. *S. cerevisiae,* require a medium composition that also supports growth of a diversity of fungi. This complicates the selective enumeration of the yeast under investigation, especially in complex habitats such as soil.

Although the fermentative ability of yeasts has been used in enrichment cultures to isolate these fungi (Beech & Davenport 1969), a characteristic feature not often used to isolate this yeast from environmental settings, is the ability to grow in an atmosphere containing low oxygen levels (Beech & Davenport 1969; De Jong & Put 1980). Using

plates prepared from complex media supplemented with ergosterol and Tween 80 and incubated in anaerobic jars, it was found that *S. cerevisiae* may be selectively enumerated among contaminating yeasts associated with the brewing industry (Longley et al. 1978). However, to our knowledge, complex agar media incubated in anaerobic jars has not been used to enumerate viable fermentative yeasts in soil.

Since it is known that *S. cerevisiae* may exert a positive effect on legume nodulation, on arbuscular mycorrhizal development, and as a result of ethanol production may even enhance the pathogenicity of *Acinetobacter s*pecies towards soil borne nematodes (see: Chapter 1, Yeasts interactions in soil), the occurrence of viable fermentative soil yeasts should be evaluated as a indicator of soil health. In addition, an ever increasing need exits to monitor genetically modified organisms (GMO's) including genetically modified strains of *S. cerevisiae*, in the environment (see Chapter 1, Genetically modified organisms in the environment). However, a reliable method to enumerate these yeasts in soil does not exist. Consequently, we evaluated in this study an enumeration procedure for fermentative yeasts in soil whereby agar plates, prepared with a complex medium containing antibacterial agents, are incubated in anaerobic jars before the colonies are enumerated.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## 2.2 Materials and Methods

### 2.2.1 Yeasts strains

*Saccharomyces cerevisiae* Y294, a laboratory strain; *S. cerevisiae* abo 5, a commercial brewing yeast; *S. cerevisiae* VIN13, a commercial wine yeasts strain (Anchor Yeast, South Africa); as well as *S. cerevisiae* S92 and a genetically modified strain originating from this wild-type (ML01), were used in the experimentation. *S.cerevisiae* ML01 is capable of malolactic fermentation, whereby malic acid is converted to lactic acid and $CO_2$ (Redzepovic et al. 2002). The latter two strains were obtained from Lesaffre International, France. The strains were maintained at 4°C on agar slopes prepared with yeast malt extract (YM) agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and

1.6% agar), in the Culture Collection at the Department of Microbiology of Stellenbosch University, South Africa. Every three weeks the yeast cultures were subcultured on YM agar, allowed to grow for three days at 25°C and then stored aerobically at 4°C.

### 2.2.2 Survival of yeasts incubated in anaerobic jars

The viability of the yeasts selected for soil inoculation under anaerobic conditions was tested in the following manner. For each strain, a culture in stationary growth phase was obtained by cultivating the yeast at 25°C for 48 h in 250 ml conical flasks, each containing 30 ml YM broth. The cultures were subsequently harvested using centrifugation (4000 ×g; 15 min) and the resulting pellet originating from each culture was washed and re-suspended in sterile physiological salt solution (PSS). The concentration of yeasts in this suspension was enumerated using a haemacytometer.

A dilution series in sterile water was prepared from each yeast suspension and spread plates were made using YM agar supplemented with 0.2g/l chloramphenicol and 0.5g/l streptomycin. The inoculated plates were incubated at 25°C for 96 h in an anaerobic atmosphere. For this purpose, anaerobic jars (Oxoid, Hants in UK) were used in combination with gas generating kits (Anaerobic System BR0038B, Oxoid) as instructed by the manufacturers. A control in which spread plates were incubated in a normal aerobic atmosphere was included for each yeast strain. All experiments were conducted in triplicate.

### 2.2.3 Enumeration of yeast strains in soil

Soil was collected from a sampling site in the Western Cape, South Africa. The soil represents a typical soil and is a Clovelly form with an orthic A-horizon, a yellow-brown apedal B-horizon and a sapprolite C-horizon. After the organic matter at the surface was removed, the top 30 cm of soil was collected. Approximately 100 kg soil was allowed to dry for 2 weeks at 30 °C, after which it was sieved (pore size 2 mm) and the whole batch thoroughly mixed to ensure homogeneous sub sampling. The sieved soil had a sandy

loam character with a pH of 5.02. Approximately 2 kg of the soil was sterilized using gamma- irradiation (25kGy min for 15 hours) and used to prepare a series of soil microcosms.

Each yeast strain was subsequently inoculated and enumerated in the soil in the following manner. A culture in stationary growth phase was obtained by cultivating the yeast at 25°C for 48 h in 250 ml conical flasks, each containing 30 ml YM broth. The cultures were subsequently harvested using centrifugation (4000 ×g; 15 min), the resulting pellet originating from each culture was washed and re-suspended in sterile physiological salt solution (PSS). The concentration of yeasts in this suspension was enumerated using a haemacytometer and appropriate dilutions were prepared containing predetermined yeast concentrations that were used as inocula for soil microcosms. The soil microcosms were prepared from the soil sample by inoculating 10 g sub-samples contained in 50 ml glass jars, each with $7.5 \times 10^5$ stationary-phase cells of a different yeast strain. Distilled water was added to each microcosm to result in a soil moisture content of *ca.* 30% (v/w); thereafter the microcosms were incubated at 25 °C for 30 min before soil dilution plates were prepared. The same YM agar supplemented with chloramphenicol and streptomycin as described above was used to prepare the plates. The inoculated plates were incubated at 25°C for 96 h in the same anaerobic jars as described above, before yeasts colonies (0.5 – 2 mm in diameter) were enumerated using a colony counter. Uninoculated microcosms were included as controls in the experimentation and all experiments were conducted in triplicate. The experiments were subsequently repeated using microcosms each inoculated with $1.5 \times 10^6$ or $3.5 \times 10^5$ stationary-phase cells of each of the yeast strains.

**2.2.4 Enumeration of fermenting wild yeasts in soil samples**

Soil microcosms, each consisting of 10 g soil, were prepared in a similar manner as described above, but without the yeast inoculum. The microcosms were subsequently incubated at 25 °C for 7 days. Dilution plates, prepared from YM agar supplemented with antibacterial agents and incubated in anaerobic jars as described above, were then

used to enumerate the yeasts in each microcosm. All colonies were purified by successive streaking out and incubation on YM agar supplemented with 0.2g/l chloramphenicol and 0.5g/l streptomycin at 25 °C.

The fermentative ability of each isolate was determined according to the method described by Van der Walt and Yarrow (1984) using test tubes containing inverted Durham tubes suspended in a carbohydrate containing complex medium. This medium consisted of 2% (w/v) glucose, 0.3% (w/v) malt extract, 0.5% (w/v) bacteriological peptone and 0.3% (w/v) yeast extract. After inoculation, the tubes were incubated at 25 °C for 7 days, during which period the Durham tubes were monitored for gas formation.

**2.3 Results and Discussion**

**2.3.1 Survival of yeasts incubated in anaerobic jars**

The results obtained for each yeast cell suspension in PSS, of the plate counts conducted after incubation under anaerobic and aerobic conditions, as well the total counts determined using a haemacytometer, are depicted in Figure 1 (p 49). Interestingly, in all cases the total counts obtained for each of the yeast suspensions (*ca.* log 4.9 yeasts/ml) were higher than the viable plate counts. This could be explained by the fact that the yeasts were in stationary phase and that it is known that some microbial cells in this growth phase may start to lyse as a result of nutrient limitation and other growth inhibiting factors (Fiechter et al. 1987). When plate counts, determined after incubation in anaerobic jars, were compared to viable yeast counts of which the plates were incubated under aerobic conditions, lower counts were obtained under anaerobic conditions for the laboratory strain *S. cerevisiae* Y294. However, no significant difference between aerobic and anaerobic counts was observed for the genetically modified strain (ML01), or for the industrial strains tested (Figure 1, p 49). Incubation under anaerobic conditions therefore had no significant effect on the results of plate counts conducted on these industrial yeast strains. It was therefore decided to evaluate

this technique using plates incubated in anaerobic jars to selectively enumerate each of these strains in soil.

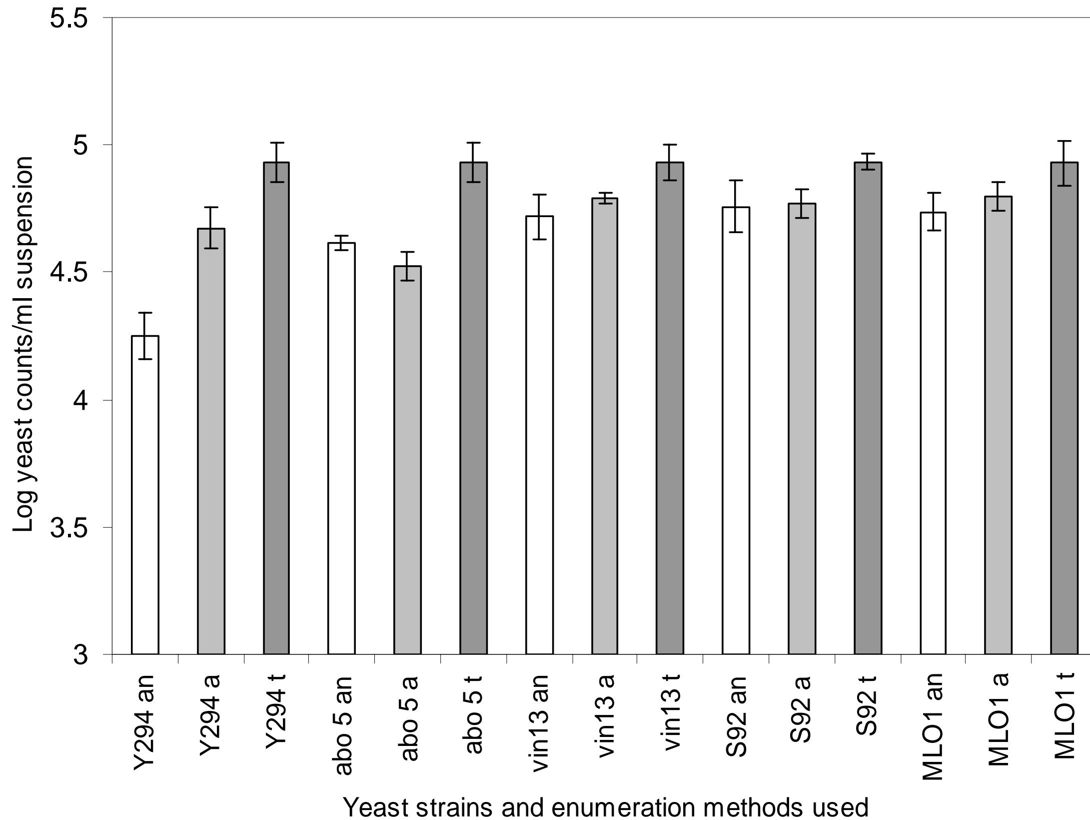


**Figure 1.** Using different enumeration methods counts were obtained for a suspension in sterile water for each of five *Saccharomyces cerevisiae* strains (i.e. Y294, abo 5, VIN13, S92 and MLO1). For each strain, plate counts were conducted after incubation under anaerobic (epithet, an) and aerobic (epithet, a) conditions. In addition, total counts (epithet, t) were made using a haemacytometer. Bars represent standard deviation for triplicate counts.

### 2.3.2 Enumeration of yeasts in soil

To compare the recovery and counting of yeasts mixed in soil with the enumeration of yeasts in a suspension of sterile water, soil microcosms containing *ca.* log 4.9 yeasts/g soil were prepared for each yeast strain. The counts subsequently obtained, using the anaerobic jars as incubation chambers, were compared with the results obtained when

similar numbers of yeasts were suspended in sterile water (Figure 2, p 51). It was concluded that the soil had no inhibitory effect on the numbers obtained for the yeast strains, since no significant difference was observed between the counts obtained for each strain regardless of whether it was suspended in sterile water or mixed into soil. No filamentous fungal colonies or bacterial colonies were observed that could have interfered with the enumeration of yeast colonies. Antibiotics were used to prevent growth of bacterial colonies. Furthermore, colony morphology was used to distinguish between yeast colonies and any bacterial colonies that may grow on the plates.

To verify our technique, anaerobic plate counts were conducted on another two series of soil microcosms, the first inoculated with ca. log 5.2 yeasts/g soil (Figure 3, p 52) and the second inoculated with ca. log 4.6 yeasts/g soil (Figure 4, p 53). In all cases, lower counts were observed for the viable yeasts recovered from the soil than for the total numbers of yeasts inoculated into the soil. Since soil had no detrimental effect on the enumeration process (Figure 2, p 51) and anaerobic incubation of plates only impacted negatively on enumeration of the laboratory strain *S. cerevisiae* Y294 (Figure 1, p 49), the lower counts of viable industrial yeasts (compared to total yeast counts) obtained in these latter two series of soil microcosms, may again be ascribed to the fraction of non-viable cells in yeast populations in stationary phase.

Pectora roborant cultus recti

### 2.3.3 Enumeration of fermenting wild yeasts in soil samples

When dilution plates, prepared from YM agar supplemented with antibacterial agents and incubated in anaerobic jars, were used to enumerate fermenting wild yeasts in one-week-old soil microcosms, 100% of the yeast colonies on the plates were able to ferment carbohydrates in a complex liquid medium. The enumeration method may therefore also be used to isolated and selectively enumerate fermentative yeasts in soil. Furthermore, no bacterial colonies or filamentous fungal colonies were observed, demonstrating the selective nature of the isolation procedure.

## 2.4 Conclusions

The results indicate that the enumeration method, utilizing plate counts in combination with anaerobic jars, is a reliable method to enumerate fermentative yeasts, such as industrial strains of *S. cerevisiae*, in soil. Consequently, this method was used in the following study to monitor growth of industrial strains of *S. cerevisiae* in soil under different environmental conditions.

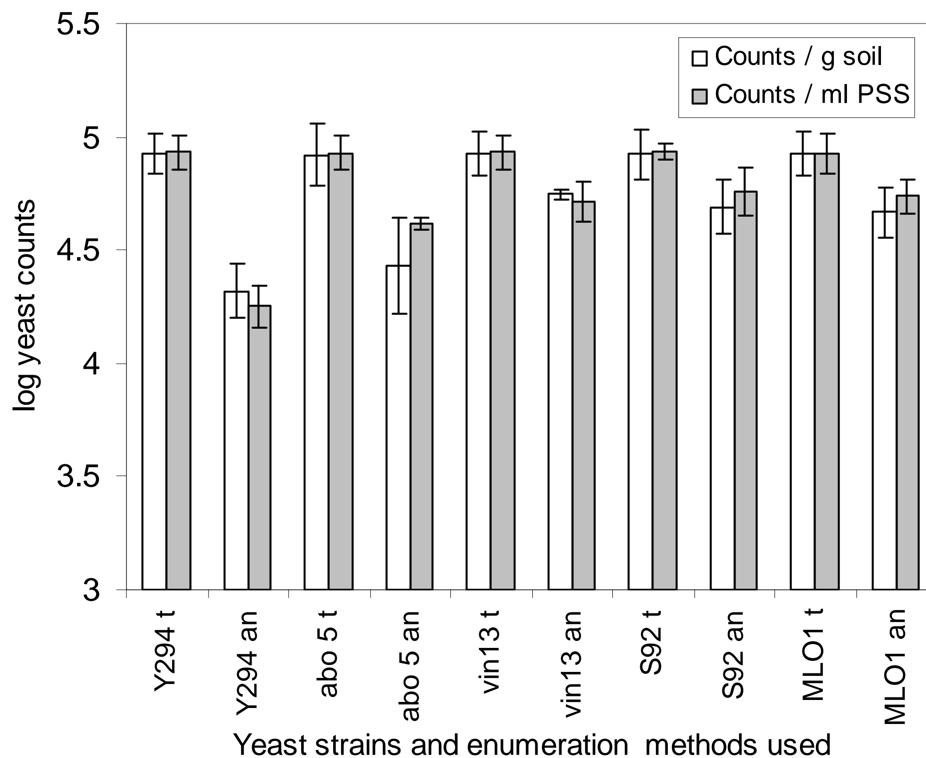


**Figure 2.** Viable yeast numbers for the five *Saccharomyces cerevisiae* strains (i.e. Y294, abo 5, VIN13, S92 and MLO1) in soil and in sterile water, obtained after incubation of dilution plates in anaerobic jars (epithet, an), as well as the total concentrations of the five strains (epithet, t) in the different soil microcosms and the suspensions in sterile water. Bars represent standard deviation for triplicate counts.

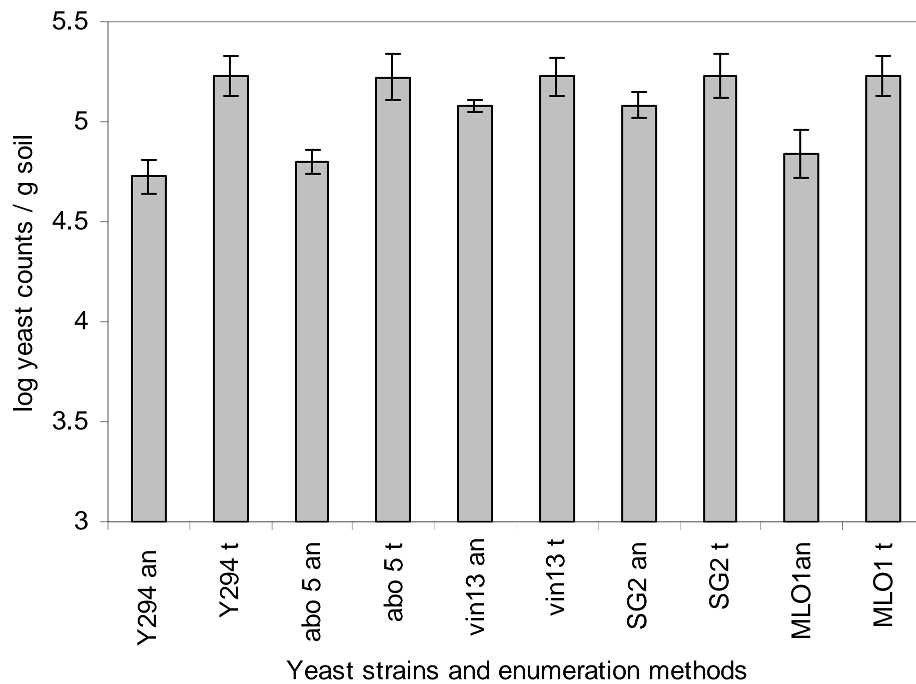


**Figure 3.** Viable yeast numbers for the five *Saccharomyces cerevisiae* strains (i.e. Y294, abo 5, VIN13, S92 and MLO1) in soil microcosms, obtained after incubation of dilution plates in anaerobic jars (epithet, an). The total concentration of yeasts in each microcosm was ca. $1.5 \times 10^5$ / g soil and is indicated for each microcosm (epithet, t). Bars represent standard deviation for triplicate counts.

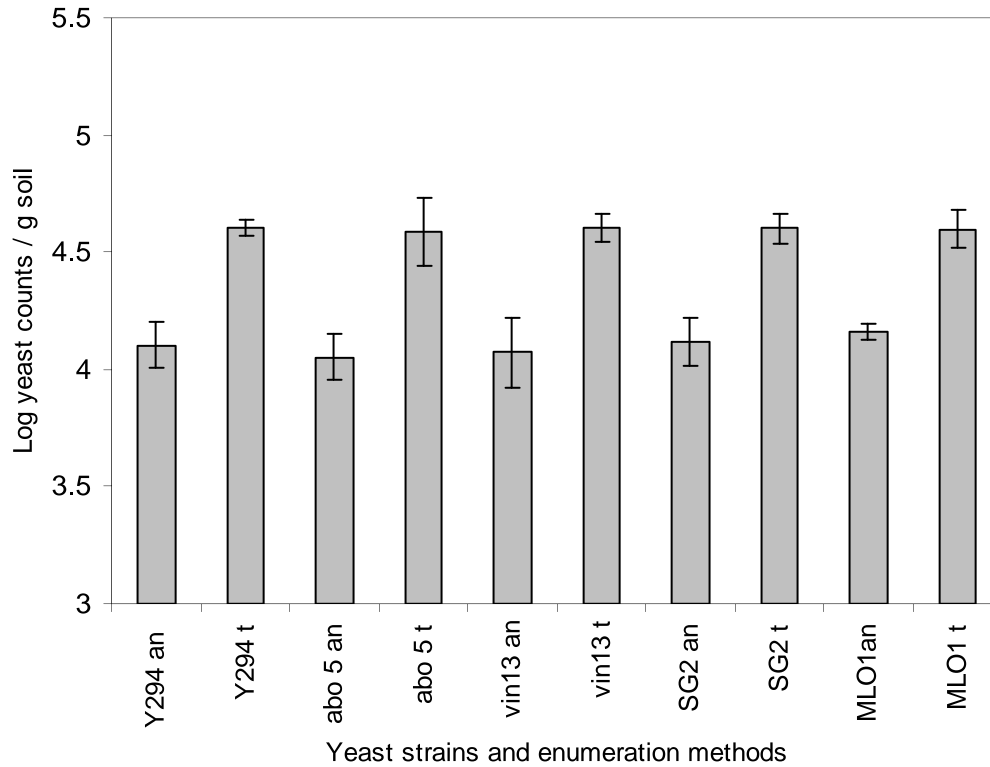


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**Figure 4.** Viable yeast numbers for the five *Saccharomyces cerevisiae* strains (i.e. Y294, abo 5, VIN13, S92 and MLO1) in soil microcosms, obtained after incubation of dilution plates in anaerobic jars (epithet, an). The total concentration of yeasts in each microcosm was ca. $3.5 \times 10^4$ / g soil and is indicated for each microcosm (epithet, t). Bars represent standard deviation for triplicate counts.

**2.5 References**


Beech FW & Davenport RR (1969) The isolation of non-pathogenic yeasts. In: Shapton DA, Gould GW (eds) Isolation methods for microbiologists, Academic Press, New York, pp. 71-88

Cornelissen S, Botha A, Conradie WJ, Wolfaardt GM (2003) Shifts in community composition provide a mechanism for maintenance of activity of soil yeasts in the presence of elevated copper levels. Can J Microbiol 49: 425 – 432

De Jong J & Put HMC (1980) Enumeration of yeasts in the presence of moulds. In: Skinner FA, Passmore SM, Davenport RR (eds) Biology and activities of yeasts. Academic Press, London, pp 289- 292

Do Carmo-Sousa L (1969) Yeasts Distribution of yeasts in nature. In: Rose AH & Harrison JS (eds) The yeasts, biology of yeasts, 1st edn, vol 1. Academic Press, London, pp 79 – 105

Fiechter A, Kappeli O, Meussdoerffer F (1987) Batch and continuous culture. In: Rose AH & Harrison JS (eds) The yeasts vol. 2, Yeasts and the environment. Academic Press, London, pp 99 – 129

Jolly NP, Augustyn OPH, Pretorius IS (2003) The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. S Afr J Enol Vitic 24: 35- 42

Kurtzman CP & Fell JW (1998) The Yeasts a taxonomic study, 4th edn. Elsevier, Amsterdam

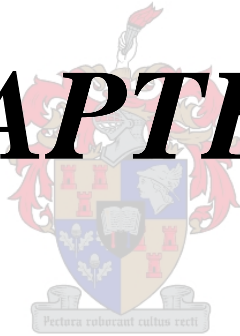
Longley RP, Dennis RR, Heyer MS, Wren JJ (1978) Selective *Saccharomyces* media containing ergosterol and Tween 80. J Inst Brew 84: 341 – 345

Redzepovic S, Orlic S, Majdak A, Kozina B, Volschenk H, Viljoen-Bloom M (2002) Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation.

Sniegowski PD, Dombrowski PG, Fingerman E (2002) *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. FEMS Yeast Research 1 299-306

Van der Walt JP & Yarrow D (1984) Methods for the isolation, maintenance, classification and identification of yeasts. In: Kreger-van Rij NJW (ed) The yeasts, a taxonomic study. Elsevier Science Publishers B.V., Amsterdam, pp 45 – 104

# *CHAPTER 3*



# *Growth of* Saccharomyces cerevisiae *in Soil under Different Environmental Conditions*

## 3.1 Introduction

It is generally accepted that the fermentative yeast *Saccharomyces cerevisiae* usually occurs in man-made environments such as wineries and fermentation plants (Vaughan-Martini & Martini 1995). In addition, it has been recovered from vineyards and vineyard soil, although it always occurred in relatively low numbers in the latter habitats. Interestingly, *Saccharomyces* strains were isolated in the 1950's from soil associated with oak and pine trees (Do Carmo-Sousa 1969). More recently, it was demonstrated that *S. cerevisiae* and *Saccharomyces paradoxus* can be isolated from oak-associated soils (Sniegowski et al. 2002). These findings are therefore in contrast to the view of *S. cerevisiae* being a fermentative yeast associated with habitats rich in carbohydrates, since soil is generally viewed as an oligotrophic environment (Williams 1985). Other yeasts that are known to have a metabolism adapted for growth in such habitats, are usually perceived as autochthononous soil yeasts; e.g. members of the genera *Cryptococcus* and *Rhodotorula* (Phaff & Starmer 1987; Spencer & Spencer 1997; Kimura et al. 1998; Joubert et al. 2003).

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Despite *S. cerevisiae* not generally being perceived as an autochthonous soil yeast, *in vitro* studies indeed revealed that this yeast does interact with a range of different organisms originating from soil. It was shown that the nematode *Panagrellus redivivus* is able to feed on *S. cerevisiae* (Hechler 1970) and ultimately that *P. redivivus* could be cultured on a monoxenic *S. cerevisiae* culture (Ricci et al. 2004). Interestingly, it was found that ethanol production by *S. cerevisiae* enhanced the growth of *Acinetobacter*, a bacterium which may be found in the same soil habitat as the yeast (Smith et al. 2004). The ethanol led to increased pathogenicity of the bacterium towards the predacious nematode *Caenorhabditis elegans*. Through this interaction, *S. cerevisiae* indirectly reduces the number of its nematode predators. Yeasts were also found to act as prey of

other soil organisms. A number of bacteria were isolated from Brazilian soil that was found to agglutinate with *S. cerevisiae* and cause lysis of the yeast cells (Goto-Yamamoto et al. 1993). The yeast *S. cerevisiae* has also been found to be preyed upon by other yeasts (Kreger-van Rij & Veenhuis 1973; Lachance & Pang 1997). *Arthroascus javanensis* and *Saccharomycopsis fibuligera*, both previously isolated from soil (Kurtzmann & Fell 2000), were found to be predacious to *S. cerevisiae*. During this process, the predatory yeasts produce outgrowths which penetrate the prey in search of nutrients (Kreger-van Rij & Veenhuis 1973; Lachance & Pang 1997).

From the above it is apparent that *S. cerevisiae* does occur in soil and evidence exists that it is able to interact with a wide range of soil organisms. Yet, very little is known of how growth and survival of this yeast compares with that of so-called autochthonous soil yeasts such as members of the genus *Cryptococcus*. Survival of *S. cerevisiae* in environmental settings has previously been studied in relation to the survival of genetically modified strains of this yeast, for example the fate of microbial species of potential use in genetic engineering was studied in model ecosystems such as sewage, lake water and soil (Liang et al. 1982). However, the survival of *S. cerevisiae* was only monitored in sewage and waste water and no insight was gained regarding the fate of this organism in soil. In a later study, the fate of a genetically engineered and a wild-type strain of *S. cerevisiae* was studied under simulated environmental conditions (Fujimura et al. 1994). Strains were tested in a natural soil/water suspension, soil/medium suspension and in waste water, but growth and survival of *S. cerevisiae* in soil *per se* were not studied.

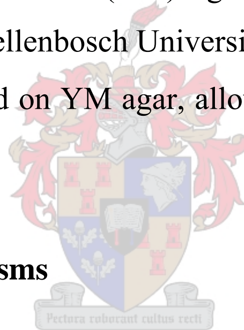
We were interested in the growth and survival of *S. cerevisiae* in soil under different environmental conditions. Consequently, we decided to monitor the fate of *S. cerevisiae* in soil microcosms differing in moisture content, nutrient composition and the presence of biota. For this purpose, two strains of *S. cerevisiae* were studied, a wild type and a genetically engineered strain of which the metabolism has been changed to be capable of malolactic fermentation. For comparison, two yeasts known to occur in soil (Kurtzman &

Fell 2000), i.e. *Cryptococcus laurentii* and *Cryptococcus podzolicus* were included in the experimentation.

## 3.2 Materials and methods

### 3.2.1 Yeast strains used

*Saccharomyces cerevisiae* S92 and a genetically modified strain originating from this wild-type (ML01) were used in the study. These two strains were obtained from Lesaffre International, France. *Cryptococcus laurentii* 1a, *Cryptococcus laurentii* 1f, *Cryptococcus podzolicus* 5a and *Cryptococcus podzolicus* 3f were kindly donated by Mr O.H.J. Rhode who isolated these strains from virgin soil. The strains were maintained at 4°C, on agar slopes prepared with yeast malt extract (YM) agar, in the Culture Collection at the Department of Microbiology of Stellenbosch University, South Africa. Every three weeks the yeast cultures were subcultured on YM agar, allowed to grow for three days at 25°C and then stored at 4°C.




### 3.2.2 Preparation of soil microcosms

Soil was collected from a sampling site in the Western Cape, South Africa. The soil represents a typical soil and is a Clovelly form with an orthic A-horizon, a yellow-brown apedal B-horizon and a sapprolite C-horizon (Fry 1987; Soil Classification Working Group 1991). After the organic matter was removed from the surface, the top 30 cm of soil was collected. Approximately 100 kg soil was allowed to dry for 2 weeks at 30 °C, after which it was sieved (pore size 2 mm) and the whole batch thoroughly mixed using a concrete mixer to ensure homogeneous sub sampling. The sieved soil, of which the physical and chemical properties are listed in Table 1 (p 59), had a sandy loam character and a pH of *ca*. 5.0. Approximately 2 kg of the soil was sterilized using gamma-irradiation (25kGy min for 15 hours) and used to prepare some of the series of soil microcosms.

**Table 1.** Characteristics of the soil used.

| | Characteristics of soil | |
|---|---|---|
| | **Unsupplemented** | **Supplemented with nutrients** |
| [a]**Classification** | Sandy | Sandy |
| [b]Stone (%) | 7.00 | 7.00 |
| **Chemical Characteristics** | | |
| [c]Organic Carbon (%) | 1.67 | 1.66 |
| [d]Total Nitrogen (%) | 0.11 | 0.096 |
| [e]Phosphorous (ppm) | 6.00 | 35 |
| [f]Copper (ppm) | 1.52 | 1.18 |
| [g]Zinc (ppm) | 2.60 | 2.9 |
| [h]Manganese (ppm) | 45.6 | 33.2 |
| [i]Boron (ppm) | 0.24 | 0.64 |
| [k]**Exchangeable cations** | | |
| Calcium (cmol $kg^{-1}$) | 2.51 | 2.94 |
| Potassium (cmol $kg^{-1}$) | 0.37 | 0.33 |
| Sodium (cmol $kg^{-1}$) | 0.08 | 0.06 |
| Magnesium (cmol $kg^{-1}$) | 1.23 | 1.07 |
| [l]pH (KCl). | 4.8 | 5.1 |

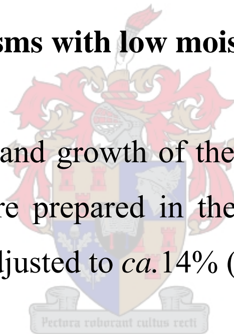
[a] Classification according to Soil Classification Working Group (1991)
[b] Determined by using the hydrometer method (Van der Watt 1966).
[c] Determined by using the Walkey-Black method (Nelson & Sommers 1982).
[d] Determined by through digestion in a LECO FP-528 nitrogen analyser.
[e]Determined in a Bray-2 extract (Thomas & Peaslee 1973).
[f-h] Determined in a di-ammonium EDTA extract (Beyers & Coetzer 1971).
[i] Determined in a hot water extract according to the methods of the Fertilizer Society of South Africa (1974).
[k] Determined in a 1M ammonium acetate extract (Doll & Lucas 1973).
[l] Determined according to the method of McClean (1982).

Yeast inocula for the soil microcosms were prepared in the following manner: Yeast cultures in stationary growth phase were obtained by cultivating each yeast strain at 25°C for 48 h in 250 ml conical flasks, each containing 30 ml yeast malt (YM) extract (Wickerham

1951). The cultures were subsequently harvested using centrifugation (4000 ×g; 15 min) and the resulting pellet originating from each culture was washed and re-suspended in physiological salt solution (PSS). The concentration of yeasts in this suspension was enumerated using a haemacytometer and an appropriate dilution was prepared containing a predetermined yeast concentration that was used as inocula for the soil microcosms. Soil microcosms were prepared from the soil sample by inoculating 10 g sub-samples contained in 50 ml glass jars, each with $1.5 \times 10^6$ stationary-phase cells of a yeast strain. Distilled water was added to each microcosm resulting in a soil moisture content of *ca.* 30% (v/w); thereafter the microcosms were incubated at 25 °C in damp chambers, which consisted of a Petri dish containing a moistened paper towel that was enclosed in a 2 l plastic bag. While being incubated in the damp chambers the metabolic activity and growth of the yeasts in the soil microcosms were monitored.

### 3.2.3 Preparation of soil microcosms with low moisture content

To monitor the metabolic activity and growth of the yeasts in soil with a relatively low moisture content, microcosms were prepared in the same manner as described above, except that the soil moisture was adjusted to *ca.*14% (v/w).



### 3.2.4 Preparation of microcosms from soil amended with nutrients

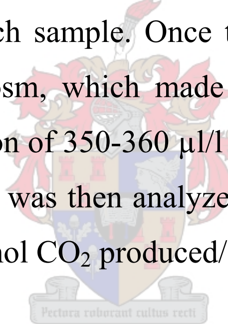
Soil microcosms were prepared to monitor the metabolic activity and growth of the yeasts in soil amended with nutrients used for sustaining growth of agricultural crops. Consequently, the following nutrients were added to the sieved soil (per kg); 0.25g super phosphate; 1.25g calcite agricultural lime and 0.25g limestone ammonium nitrate. The chemical composition of the amended soil is presented in Table 1 (p 59). The soil was subsequently sterilized as described above using gamma-irradiation and microcosms with a soil moisture content of *ca.* 30% (v/w) were prepared in the same manner as described above.

### 3.2.5 Preparation of microcosms from non-sterile soil

Soil microcosms were prepared to monitor growth of the yeast strains in soil in the presence of natural soil biota. For this purpose, microcosms containing a moisture content of *ca.* 30% (v/w), were prepared as described above, except that the sieved soil was not sterilized.

### 3.2.6 Monitoring yeast metabolic activity

The metabolic activity of the yeasts inoculated into sterile soil was periodically measured with: Carbon dioxide emissions using a LI 6262 infrared $CO_2$ analyzer (LI-COR Inc., Nebraska). The experimental setup consisted of a controlled temperature water bath, variable air pump and a flow meter (Smith 2003). During analysis, a stopper was placed on each microcosm and fresh air was pumped through the sample for 3 minutes in order to flush $CO_2$ present from the headspace of each sample. Once the sample had been flushed, a second stopper was fitted to the microcosm, which made it possible to monitor the $CO_2$ being produced. Once a $CO_2$ concentration of 350-360 µl/l was reached, readings were taken every 2s over a period of 100s. The data was then analyzed using Statistica 6.1 analyses software (Statsoft, Inc.) and converted to µmol $CO_2$ produced/g/h.




### 3.2.7 Monitoring growth of yeast inoculated into sterile soil

To periodically enumerate the yeasts in the microcosms, soil dilution plates using YM-agar were prepared for each sampling interval. A spatula was used to sample one gram of soil from each microcosm. The *Saccharomyces* strains were enumerated using YM-agar consisting of 1% (w/v) glucose, 0.3% (w/v) malt extract, 0.5% (w/v) bacteriological peptone, 0.3% (w/v) yeast extract, 0.2g/l chloramphenicol and 0.5g/l streptomycin. When the medium was used to enumerate cryptococci, the latter component was replaced with 0.01g/l cycloheximide. The inoculated plates were incubated for four days at 25°C before yeast colonies, ranging one millimeter and more in diameter, were counted. All experiments were conducted in triplicate.

### 3.2.8 Monitoring yeast growth inoculated into non-sterile soil

To selectively enumerate *Saccharomyces* strains in the soil microcosms the same isolation medium was used as described above, however, the plates were incubated at 25°C in anaerobic chambers as described in Chapter 2. Antibiotics in the isolation medium and anaerobic incubation ensured selective isolation of *Saccharomyces* strains (Chapter 2). The cryptococci were enumerated using the same selective conditions as described above. Cycloheximide and antibiotics in the isolation medium ensured the isolation of cryptococci. All experiments were conducted in triplicate.

### 3.2.9 Impact of soil cryptococci on growth of *Saccharomyces*

This aspect of the study was performed in gamma-irradiated soil. As described above, each microcosm contained 10g soil and had a moisture content of ca. 30% (v/w), but was inoculated with two yeast strains. Consequently, each microcosm received $7.5 \times 10^5$ stationary-phase cells of each yeast strain. Co-inoculations of the yeasts were made in the following combinations: *Cryptococcus laurenti* 1f and *Saccharomyces cerevisiae* ML01; *Cryptococcus laurenti* 1f and *Saccharomyces cerevisiae* S92; *Cryptococcus podzolicus* strain 3f and *Saccharomyces cerevisiae* ML01; *Cryptococcus podzolicus* strain 3f and *Saccharomyces cerevisiae* S92.

The microcosms were incubated at 25 °C and the numbers of each of the strains in each microcosm were selectively determined at different time intervals using soil dilution plates. The plates were incubated for four days before the colonies were counted. The compositions of the two isolation media used to enumerate the respective yeast from the co-cultures, were the same as described above, where the medium selective for the cryptococci contained cycloheximide instead of streptomycin. Preliminary studies showed that 0.01g/l cycloheximide was sufficient to inhibit growth of *Saccharomyces*. Enumeration of *Saccharomyces* was performed after incubation of the soil dilution plates in anaerobic jars, as described in Chapter 2.

### 3.2.10 Impact of soil protista on growth of Saccharomyces

The impact of protista on the numbers of *Saccharomyces* in soil was studied as follow:

*3.2.10.1 Preparation of sterile soil extract*

One hundred grams of soil was mixed in 900ml of distilled water and allowed to settle for 48 hours. The supernatant was filtered (Whatman No.1) and the filtrate was autoclaved for 20 min at 121°C.

*3.2.10.2 Isolation of protista*

Ten grams of soil was mixed in 90ml of distilled water and allowed to settle for 48-96 hours. One milliliter of the supernatant was extracted from approximately three millimeters above the soil sediment and transferred to a 250ml conical flask. Twenty milliliters of sterile soil extract was then added to the conical flask, along with one milliliter of a suspension of *S. cerevisiae*, cultivated in YM-broth. The conical flask was incubated at 25°C and the suspension was daily monitored microscopically for the occurrence of a bloom in the numbers of ciliates (*Tetrahymena* sp.). This protistan culture served as inoculum for microcosms.

*3.2.10.3 Preparation of soil microcosms containing protista*

Soil microcosms were prepared to monitor the numbers of the *Saccharomyces* strains in soil in the presence of protista. For this purpose the soil microcosms, each inoculated with $1.5 \times 10^6$ stationary-phase yeast cells containing a moisture content of *ca.* 30% (v/w), were prepared from sterile soil as described above. In addition, each microcosm received 400µl of the protistan inoculum. The numbers of *Saccharomyces* in these microcosms were then monitored, using soil dilution plates incubated in anaerobic jars, as described in Chapter two.

### 3.2.11 Analyses of data

A series of statistical analyses were conducted to determine the effect of different environmental factors on the metabolism, growth and survival of the yeasts in soil. Experimental data was analyzed using ANOVA as indicated below.

*3.2.11.1 Effect of soil moisture content and nutrients*

To determine the effect of soil moisture content and nutrient composition on growth of the different yeasts, viable yeast counts periodically obtained for the different series of soil microcosms, each prepared from sterile soil but differing in moisture content and/or nutrient composition, was analyzed using ANOVA. Statistica 6.1 software (Statsoft, Inc.) was used to perform a three-way cross classification variance analyses on the data. The three main components compared were yeast strains (*S. cerevisiae* S92; *S. cerevisiae* ML01; *C. laurentii* 1a; *C. laurentii* 1f; *C. podzolicus* 5a; *C. podzolicus* 3f), soil composition [moisture content *ca.* 30% (v/w), no additional nutrients; moisture content *ca.* 15% (v/w), no additional nutrients; moisture content *ca.* 30% (v/w), with additional nutrients] and days of observation (day 1; day 7; day 14; day 28; day 48). All two-way and three-way interactions were studied and found to vary significantly. Bonferroni multiple comparisons of these interactions were studied along with least-square estimates of these interactions. Subsequently, the main effect least square (LS) means plots were interpreted.

To determine the impact of soil differing in moisture content and nutrient composition on the metabolic activity of the different yeast strains, the data on $CO_2$ emissions periodically obtained for the different series of soil microcosms, was also analyzed using ANOVA. Statistica 6.1 software (Statsoft, Inc.) was again used to perform a three-way cross classification variance analyses on the data. The three main components compared were yeast strains, soil composition and days of observation (day 1; day 7; day 14; day 28). As was done during analyses of the viable yeast counts mentioned above, two-way

and three-way interactions were studied and were found to vary significantly. Also, Bonferroni multiple comparisons of these interactions were studied along with least-square estimates of these interactions and the main effect least square (LS) means plots were interpreted.

*3.2.11.2 Effect of soil biota*

ANOVA was applied to determine the effect of soil biota on the test yeast strains. For this purpose, viable yeast counts were periodically determined in different soil microcosms, each prepared from non-sterile soil with a moisture content of ca. 30% (w/v). Statistica 6.1 software (Statsoft, Inc.) was used to perform a two-way cross classification variance analyses on the data. The two main components compared were yeast strains (*S. cerevisiae* S92; *S. cerevisiae* ML01; *C. laurentii* 1a; *C. laurentii* 1f; *C. podzolicus* 5a; *C. podzolicus* 3f) and days of observation (day 1; day 7; day 14; day 28; day 48). Bonferroni multiple comparisons of the interactions were studied along with least-square estimates of these interactions. Bootstrap methods were applied to analyze the interaction plots of the LS means when there were indications of non-normality in the residuals. These plots are better for interpretation in non-normal residual cases.

*3.2.11.3 Impact of soil cryptococci on growth of Saccharomyces*

The effect of cryptococci on the growth of *S. cerevisiae* (and *visa versa*) in microcosms prepared from sterile soil with a moisture content of *ca.* 30% (v/w) and inoculated with ca. $7.5 \times 10^5$ cells of each yeast, was determined as follows: The viable counts obtained for a particular yeast in the presence of another were doubled to compare the data with the viable counts obtained for the particular yeast on its own in the absence of another, but under identical conditions (see above: Preparation of soil microcosms). The latter was included as controls in an ANOVA. Statistica 6.1 software (Statsoft, Inc.) was used to perform a two-way cross classification variance analyses on the data. The two main components compared for each of the yeast strains (*S. cerevisiae* S92; *S. cerevisiae* ML01; *C. laurentii* 1f; *C. podzolicus* 3f) were treatment with viable yeasts (*S. cerevisiae*

S92; *S. cerevisiae* ML01; *C. laurentii* 1f; *C. podzolicus* 3f) and days of observation (day 1; day 7; day 14; day 28). Bonferroni multiple comparisons of the interactions were also studied along with least-square estimates of these interactions. The main effect least square (LS) means plots were subsequently interpreted.

*3.2.11.4 Effect of different environmental conditions on growth of Saccharomyces*

To determine the effect of different environmental conditions on growth and survival of *Saccharomyces,* the data on viable yeast counts periodically obtained for the different series of soil microcosms, prepared from sterile soil, but differing in moisture content and/or nutrient composition, prepared from non-sterile soil, and prepared from sterile soil but inoculated with protista, was combined and analyzed using ANOVA. Statistica 6.1 software (Statsoft, Inc.) was used to perform a three-way cross classification variance analyses on the data. The three main components compared were yeast strains (*S. cerevisiae* S92; *S. cerevisiae* ML01), soil composition [moisture content *ca.* 30% (v/w), no additional nutrients, prepared from sterile soil; moisture content *ca.* 15% (v/w), no additional nutrients, prepared from sterile soil; moisture content *ca.* 30% (v/w), with additional nutrients, prepared from sterile soil; moisture content *ca.* 30% (v/w), no additional nutrients, prepared from non-sterile soil; moisture content *ca.* 30% (v/w), no additional nutrients, prepared from sterile soil but inoculated with protista] and days of observation (day 1; day 7; day 14; day 28; day 48). Two-way interactions were also studied, as well as Bonferroni multiple comparisons of these interactions along with least-square estimates of the interactions. Subsequently, the main effect least square (LS) means plots were interpreted.

## 3.3 Results and Discussion

### 3.3.1 Effect of soil moisture content and nutrients

The ability of the yeasts to grow and survive in soil with a moisture content of *ca.* 30% (v/w) with no additional nutrients, is depicted Figure 1a (p 67). Generally, all the yeasts

were able to survive in the soil over a period of 48 days. Despite the microcosms being inoculated with the same number of cells, resulting in an initial concentration of *ca.* log 5.2 yeasts/g soil, the cryptococci reached greater numbers than the *Saccharomyces* strains. This may be ascribed to the superior ability of the cryptococci to utilize available soil nutrients, since it is known that when a range of carbon sources are tested for assimilation by the two groups of yeasts, these *Cryptococcus* species are able to utilize notably more carbon compounds than *S. cerevisiae* (Kurtzman & Fell 2000). With the exception of day 48, no significant difference was observed between the numbers of the wild (S92) and genetically modified (ML01) *Saccharomyces* strains. The observed increase in the numbers of *S. cerevisiae* S92, compared to *S. cerevisiae* ML01, may be ascribed to

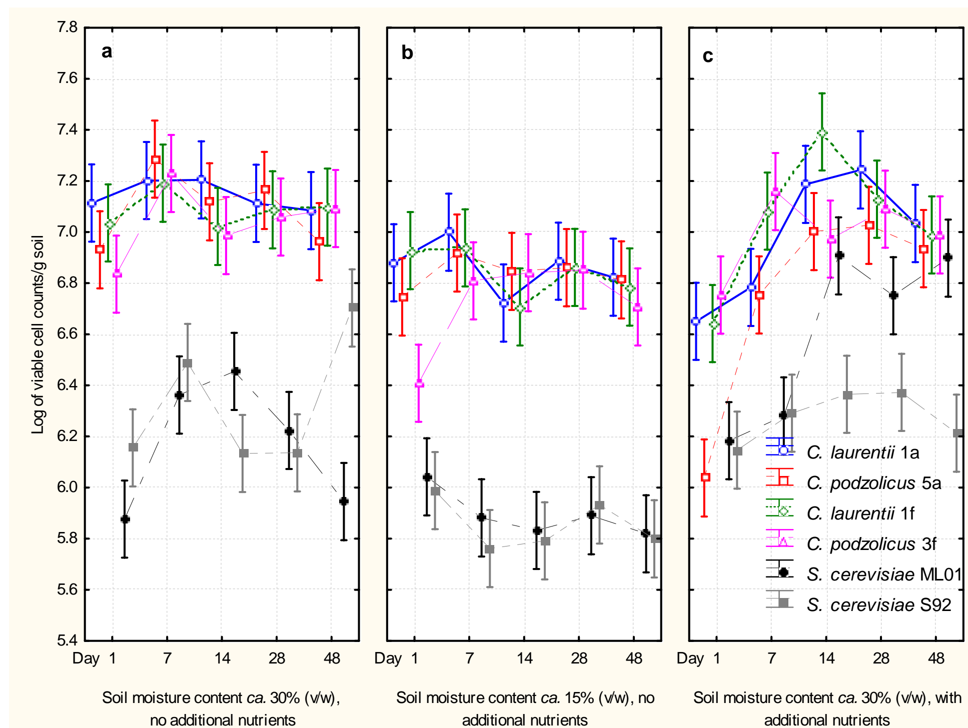



**Figure 1.** Viable counts of the different yeast strains periodically determined for soil microcosms prepared from soil differing in moisture content and nutrient composition (see Table 1 p59). Each value represents the mean of three repetitions; vertical bars denote 0.95 confidence intervals. **In all cases an inoculum of log 5.2 yeasts/g soil was used. The values obtained for day one, therefore represent growth over the initial 24 hours of incubation.**

differences in yeast metabolism that resulted in different interactions with the physico-chemical environment and should be investigated further. Despite increased yeast numbers beyond the first day of incubation, the rate of $CO_2$ emission from the different microcosms declined during this period (Figure 2a, p 69), indicating that the metabolic rate of all the yeasts started to decline after the first 24 hours of growth and this decline was maintained throughout the 28 day monitoring period. Adjustment of metabolic activity according to changes in soil conditions most probably contributed to the survival of the yeasts in this environment. Interestingly, the $CO_2$ emission from the microcosms inoculated with *S. cerevisiae* did not reach the same levels as that of the microcosms inoculated with the cryptococci during the initial period of rapid growth between days 1 and 7. This, as in the case of the superior numbers obtained by the cryptococci, may indicate that *S. cerevisiae* was not able to utilize the nutrient resources in the soil to the same extent as the cryptococci.

As was found for the soil microcosms with a moisture content of *ca.* 30% (v/w), all the yeast strains were able to grow and survive in soil with moisture content of *ca.* 15% (v/w) (Figure 1b, p 67). However, lower numbers were generally observed in this soil with the lower moisture content. This is not surprising, since it is known that decreased availability of water may inhibit growth of soil yeasts (Vishniac 1995). The fact that higher numbers were also recorded in this case for the cryptococci than for *S. cerevisiae,* is expected, as semi-arid soils, low in nutrients and moisture, were mostly populated by cryptococci and related basidiomycetous yeasts (Spencer & Spencer 1997). The two *S. cerevisiae* strains showed nearly identical responses to low moisture conditions (Figure 1b, p 67) and never reached numbers similar to that of the *Cryptococcus* strains. However, the $CO_2$ emission curves from soil microcosms inoculated with the different yeasts all declined similarly towards the end of the monitoring period at day 28 (Figure 2b, p 69). Interestingly, despite lower yeast numbers (Figure 1, p 67), all soil microcosms with the lower moisture content showed higher $CO_2$ emission rates at day 28 (Figure 2b, p 69), compared to the soil microcosms with a higher moisture content (Figure 2a, p 69).

This may indicate increased metabolic rates and enhanced energy expenditure to maintain the

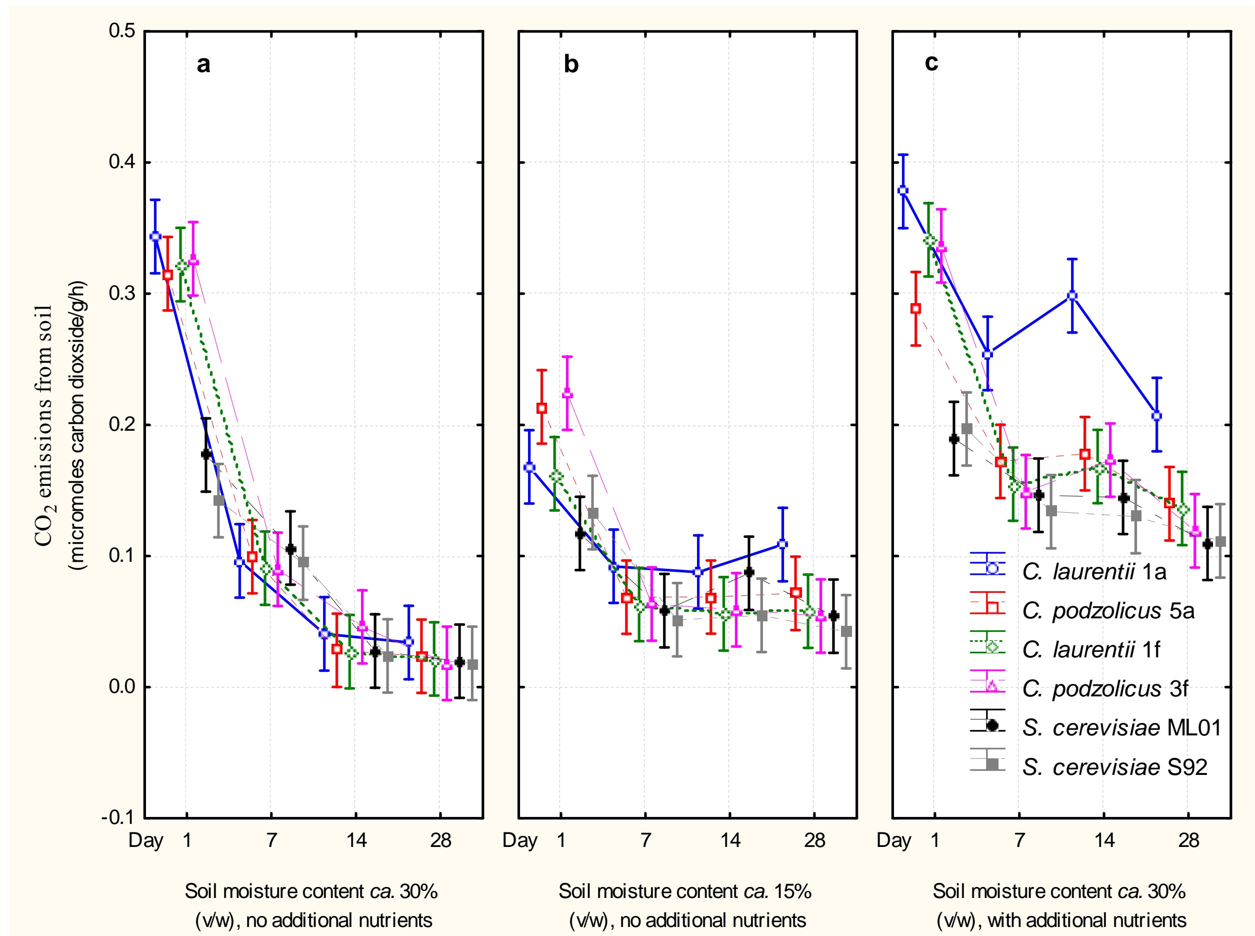
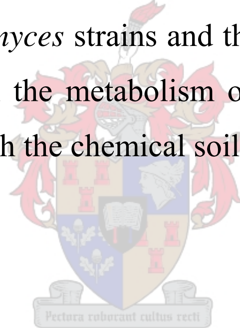



**Figure 2.** Carbon dioxide emissions periodically determined for soil microcosms, prepared from soil differing in moisture content and nutrient composition (see Table 1 p 59), and inoculated with different yeast strains. Each value represents the mean of three repetitions; vertical bars denote 0.95 confidence intervals.

yeast populations in soil with a lower water activity. The addition of nutrients, used for sustaining growth of agricultural crops, resulted in increased calcium, boron and phosphorous levels, as well as an increase in pH (see Table 1 p 59). Despite this increase in nutrient levels, the yeast population sizes generally took longer (14 days) to reach a maximum (Figure 1c, p 67), compared to the 7 days it took in the absence of additional

nutrients, but in soil with the same moisture content (Figure 1a, p 67). Also, beyond day one the $CO_2$ emission rates of the soil microcosms that received additional nutrients (Figure 2c, p 69) tended to be higher than the $CO_2$ emission rates of microcosms without additional nutrients (Figure 2a, p 69). It seems that a higher metabolic rate and energy expenditure was needed for the cryptococci to reach similar numbers after a longer period of time, than in the absence of these additional nutrients.

This apparent anomaly may be the result of the increased soil pH impacting negatively on nutrient availability; for example it is known that bioavailability of a number of divalent cations may decrease as a result of elevated soil pH (Agbenin & Olojo 2004). In contrast to the cryptococcal growth, growth of the *Saccharomyces* strains however, especially growth of *S. cerevisiae* ML01, was enhanced by the addition of nutrients, particularly beyond day 14 (Figure 1c, 67). The difference in growth between the two *Saccharomyces* strains and between the *Saccharomyces* strains and the cryptococci in these microcosms, may be ascribed to differences in the metabolism of these yeasts which also result in differences in their interactions with the chemical soil environment.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**3.3.2 Effect of soil biota**

In contrast to what was observed in the absence of other soil biota (Figure 1, p 67), the presence of natural soil organisms resulted in a decrease in viable numbers of all the test yeast strains towards the end of the incubation period (Figure 3, p 71). The decline may be ascribed to negative interactions with other soil biota, such as competition and predation (see Chapter 1).

**3.3.3 Impact of soil cryptococci on growth of *Saccharomyces***

To further investigate the negative interactions between *Saccharomyces* and soil organisms, the numbers of the two *Saccharomyces* strains were monitored in the presence of two cryptoccocci in different microcosms (Figures 4 and 5, pp 72, 73). The numbers of both *Saccharomyces* strains were significantly ($p \leq 0.05$) reduced, compared to the initial

concentrations of these strains, after 14 days of co-culturing with the cryptococci in the microcosms. This reduction may be the result of amensalism or competition (Botha 2005)

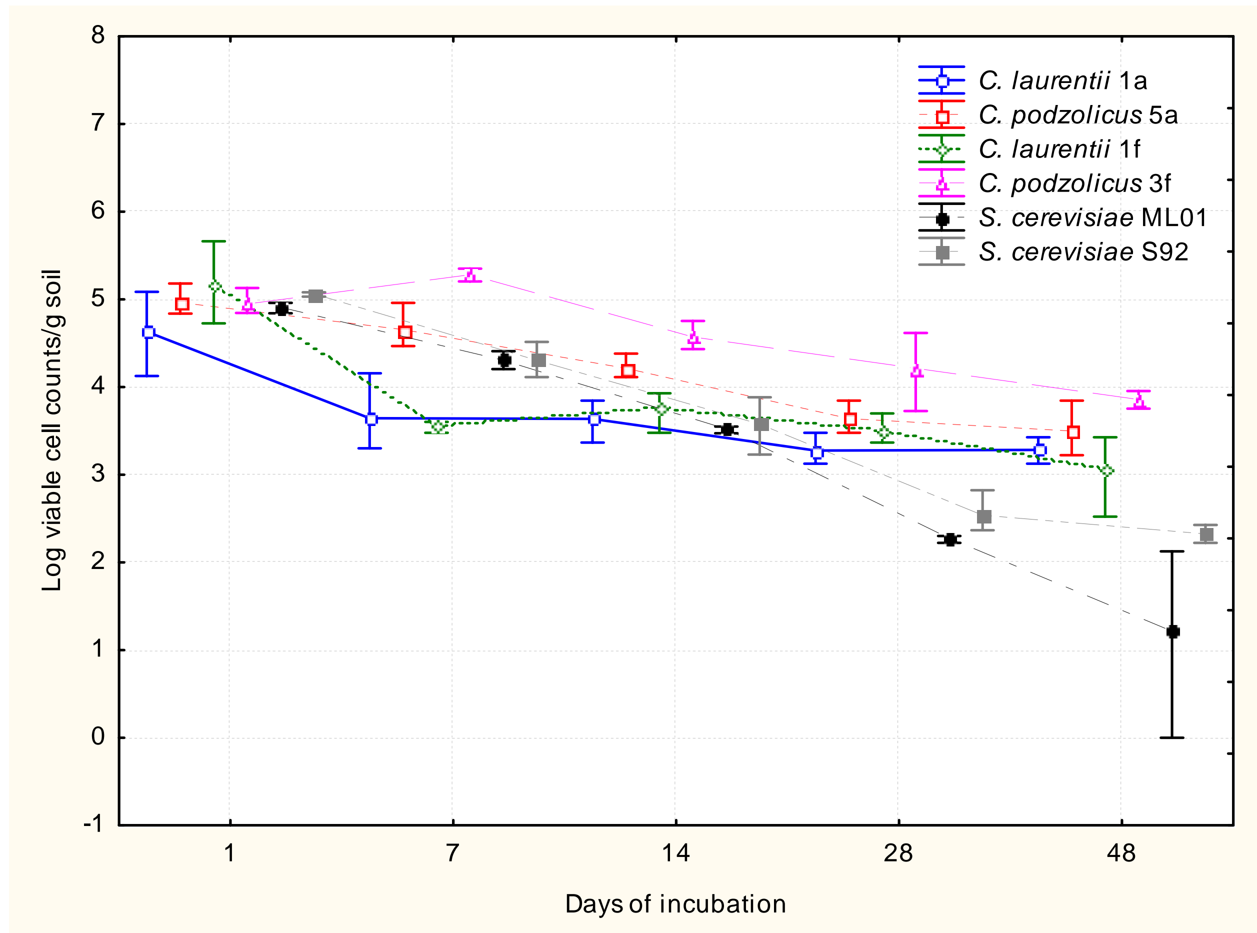



**Fig. 3** Viable counts of the different yeast strains periodically determined in soil microcosms, prepared from non-sterile soil with a moisture content of ca. 30 % (v/w). Each value represents the mean of three repetitions; vertical bars denote 0.95 bootstrap confidence intervals.

and there appears to be no significant difference between the *Saccharomyces* strains under these conditions. The genetically modified strain therefore seemed to have no competitive advantage over the wild-type under these conditions. Interestingly, the numbers of *C. laurentii* 1f took longer (28 days) before they were significantly ($p \leq 0.05$) reduced as a result of co-culturing with *Saccharomyces* strains in the microcosms (Figure 6, p 74). However, the numbers of *C. podzolicus* 3f co-cultured with the *Saccharomyces* strains, increased and remained constant, during the monitoring period (Figure 7, p 75).

Of the four yeast strains investigated, *C. podzolicus* 3f therefore seemed to the most competitive in co-cultures with the *Saccharomyces* strains. This *Cryptococcus* species occurs commonly in podzolic soils (Lachance & Starmer 1998) and in addition to its ability to utilize a wide diversity of carbon compounds (Fell & Statzell-Tallman 1998), it contains genes such the *CNLAC1* gene that encodes for laccase, which is known to exert a negative effect on other organisms (Petter et al. 2001).

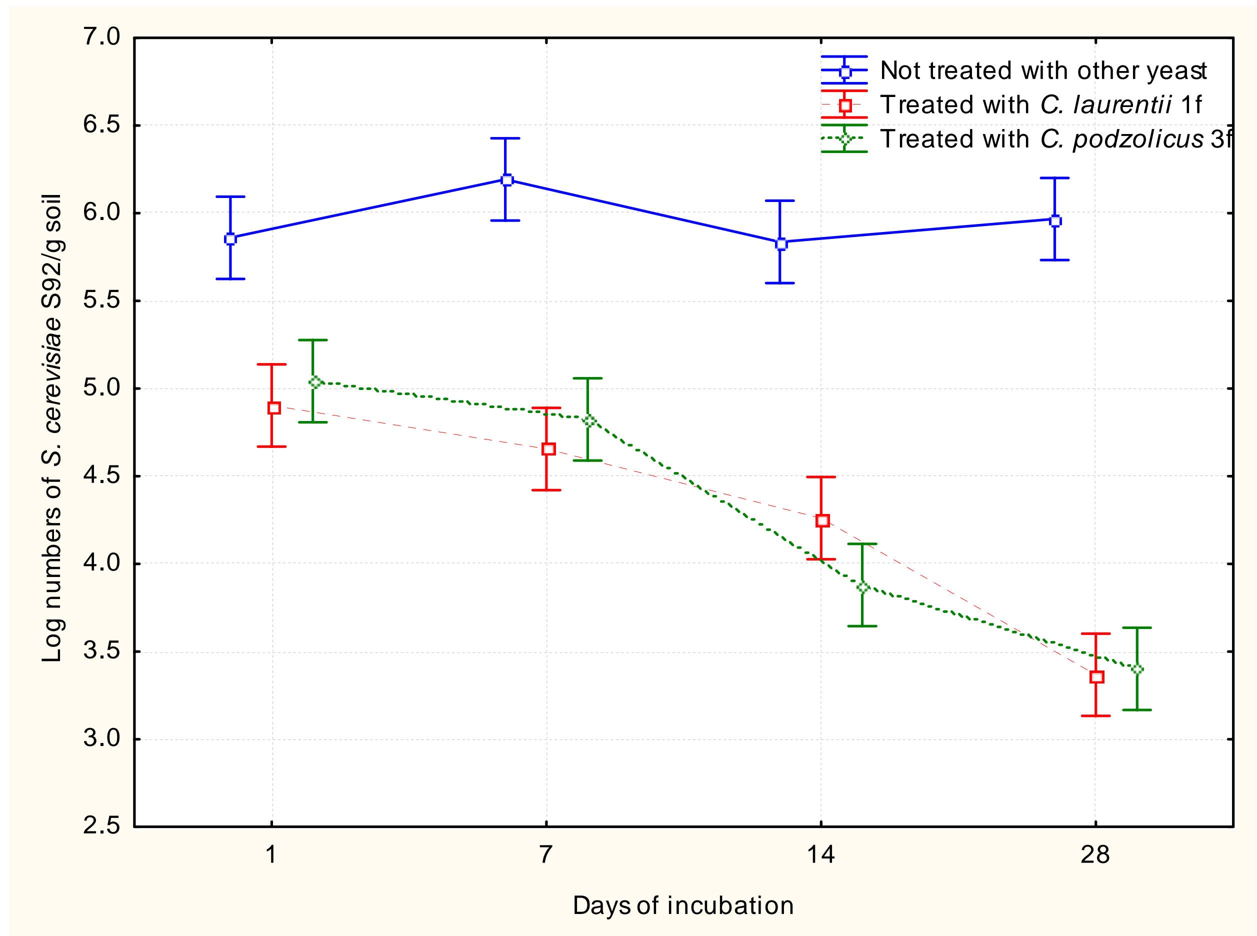



**Figure 4.** Viable numbers of *S. cerevisiae* S92 in microcosms prepared from sterile soil with a moisture content of *ca.* 30 (v/w) and treated with two different cryptococci. Each value represents the mean of three repetitions; vertical bars denote 0.95 confidence intervals. **In all cases an inoculum of log 5.2 yeasts/g soil was used. The values obtained for day one, therefore represent growth or the lack thereof over the initial 24 hours of incubation.**

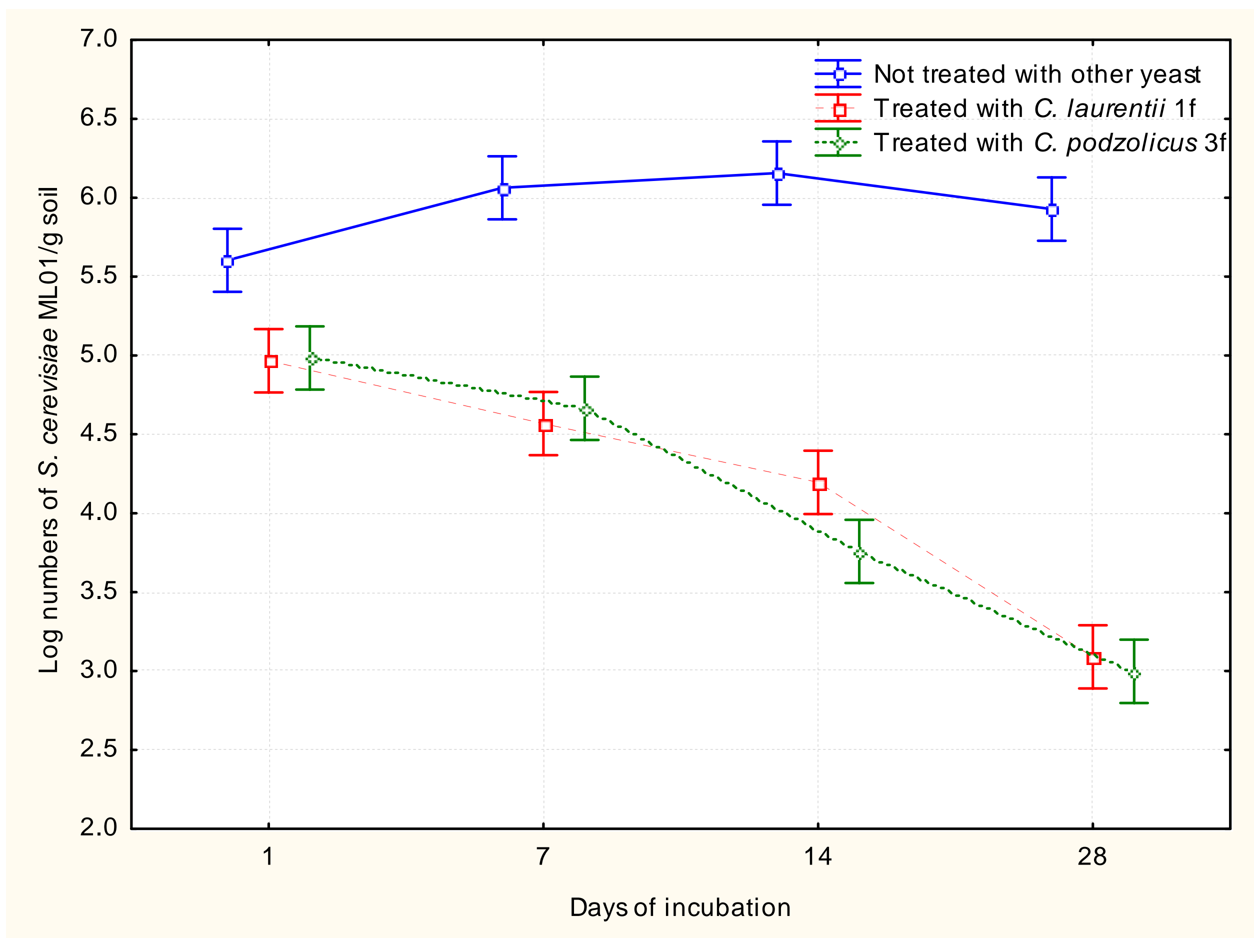



**Figure 5.** Viable numbers of *S. cerevisiae* ML01 in microcosms prepared from sterile soil with a moisture content of *ca.* 30 (v/w) and treated with two different cryptococci. Each value represents the mean of three repetitions; vertical bars denote 0.95 confidence intervals. **In all cases an inoculum of log 5.2 yeasts/g soil was used. The values obtained for day one, therefore represent growth or the lack thereof over the initial 24 hours of incubation.**

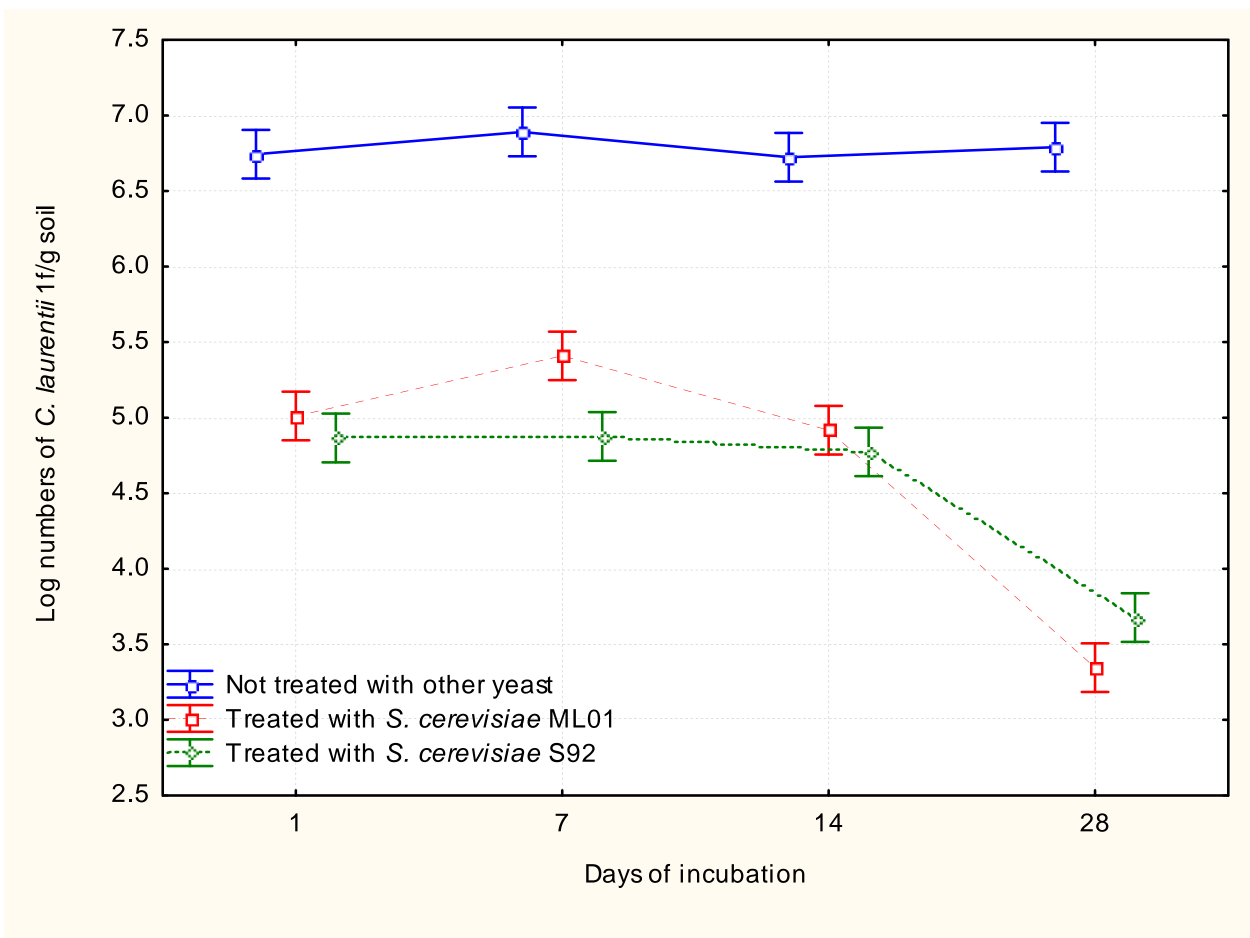



**Figure 6.** Viable numbers of *C. laurentii* 1f in microcosms prepared from sterile soil with a moisture content of *ca.* 30 (v/w) and treated with two *S. cerevisiae* strains. Each value represents the mean of three repetitions; vertical bars denote 0.95 confidence intervals. **In all cases an inoculum of log 5.2 yeasts/g soil was used. The values obtained for day one, therefore represent growth or the lack thereof over the initial 24 hours of incubation.**

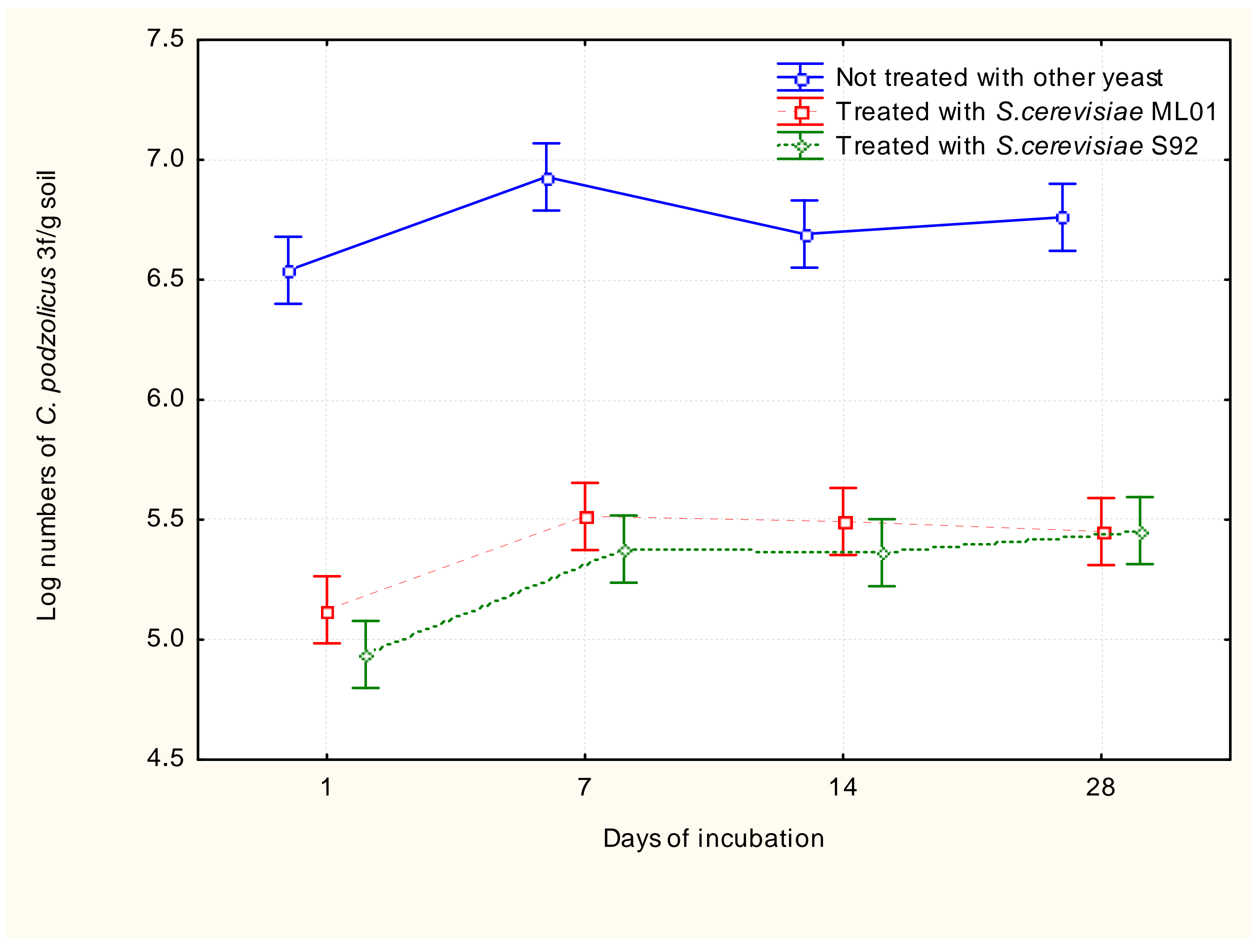



**Figure 7.** Viable numbers of *C. podzolicus* 3f in microcosms prepared from sterile soil with a moisture content of *ca.* 30 (v/w) and treated with two *S. cerevisiae* strains. Each value represents the mean of three repetitions; vertical bars denote 0.95 confidence intervals**. In all cases an inoculum of log 5.2 yeasts/g soil was used. The values obtained for day one, therefore represent growth or the lack thereof over the initial 24 hours of incubation.**

### 3.3.4 Effect of different environmental conditions on growth of *Saccharomyces*

The impact of different environmental conditions on growth and survival of *Saccharomyces* is illustrated in Figure 8 (p 78). As all the microcosms were initially inoculated with similar numbers of yeasts, resulting in an initial concentration of ca. log 5.2 yeasts /g soil, it is obvious that in all cases the yeast population grew within 1 day of incubation. The exception was when the yeasts were incubated in the presence of other soil biota (Figure 8d, p 78; see also Figure 3, p 71). As already mentioned, this decrease may be ascribed to competition and predation by the other soil organisms. However, when a known group of soil predators (i.e. protista, belonging to the genus *Tetrahymena*) were co-cultured with these yeasts, the populations of both yeast strains rapidly increased and remained at these relatively high levels until the end of the incubation period (Figure 8e, p 78). This was rather unexpected, as one would expect protistan predation to cause a decrease in yeast numbers, since protista are known to regulate the size of the microbial community (Bardgett & Griffiths 1997). However, protista are also known to accelerate the turnover of microbial biomass and soil organic matter, and excrete nutrients. Consequently, protista play a pivotal role in nutrient cycling within the soil environment (Griffiths 1994). The plots obtained for yeast numbers in the presence of protista that were for most part of the incubation period higher than any of the other plots (Figure 8 a,b, c and d; p 78), even in the presence of additional nutrients (Figure 8c, p 78), may therefore have been as a result of continual predation and liberation of nutrients, especially from senescent cells. The protista may therefore have been responsible for a continuous supply of nutrients to the yeast populations, thereby ensuring the yeast population sizes were maintained throughout the incubation period. Once again there was no significant difference between the *Saccharomyces* strains and the protistan predator did not exhibit a particular preference for either strain. Therefore, the nature of the genetic modification resulted in no competitive advantage of *S. cerevisiae* ML01 over the wild-type under these conditions. This is expected, since unlike laccase production by

cryptococcal strains (Steenbergen et al. 2001), the ability to degrade malic acid is not associated with a negative impact on protista. Since these yeasts were initially used as bait to isolate the protista, this interdependence of these two groups should be further investigated.

### 3.4 Conclusions

Both *S. cerevisiae* S92 and the genetically modified strain capable of malolactic fermentation, *S. cerevisiae* ML01, were able to grow and/or survive until the end of the incubation period in soil microcosms, differing in moisture content, nutrient composition and the presence of biota. In sterile soil, these yeasts reached smaller population sizes than common soil yeasts such as cryptococci, although the population sizes of both cryptococci and *Saccharomyces* were similarly reduced in the presence of other soil organisms. These results together with the evidence for the above-mentioned symbiosis between *S. cerevisiae* and a group of soil protista and the findings of Sniegowski et al. (2002) that *S. cerevisiae* may be isolated from soil, as well as the *in vitro* interactions of this yeast species with other soil organisms such as bacteria, nematodes and yeasts (Hechler, 1970; Goto-Yamamoto et al. 1993; Lachance and Pang, 1997; Smith et al. 2004), points to a natural niche for this species somewhere in the soil habitat. To reveal this niche is a challenge for future studies.

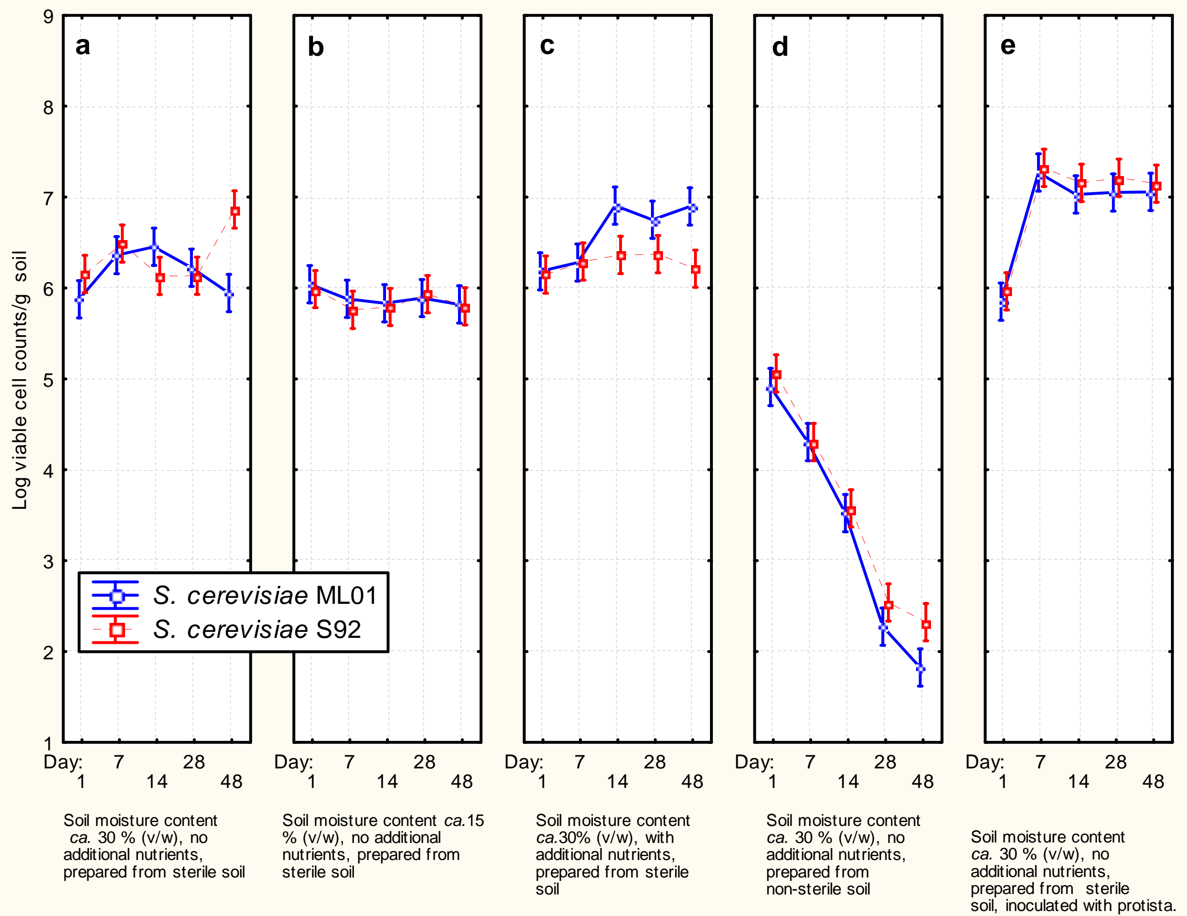



**Figure 8.** Viable counts of the *Saccharomyces* strains periodically determined for soil microcosms, prepared from soil differing in moisture content, nutrient composition, presence soil biota and presence of protista. Each value represents the mean of three repetitions; vertical bars denote 0.95 confidence intervals. **This figure represents the results obtained when the data for *Saccharomyces cerevisiae* was combined and analyzed using ANOVA. Statistica 6.1 was used to per form a three-way cross classification variance analyses on the data.**

### 3.5 References


Agbenin JO & Olojo LA (2004) Competitive adsorption of copper and zinc by a Bt horizon of a savanna Alfisol as affected by pH and selective removal of hydrous oxides and organic matter Geoderma 119: 85–95

Bardgett RD & Griffiths BS (1997) Ecology and Biology of Soil Protozoa, Nematodes and Microarthropods. In: Modern Soil Microbiology, eds. JD Van Elsas , JT Trevors, EMH Wellington, Marcell Dekker, Inc., New York, pp. 129-159

Beyers CP & Coetzer FJ (1971) Effect of concentration, pH and time on the properties of di-ammonium EDTA as a multiple soil extractant. Agrochemophysica 3, 49-54

Botha A (2005) Yeasts in soil. In: Ecophysiology of Yeasts, eds. C. A. Rosa & G. Péter. Springer-Verlag, Heidelberg (Accepted for publication)

Do Carmo-Sousa L (1969) Yeasts Distribution of yeasts in nature. In: The yeasts, biology of yeasts, 1st edn, vol 1, eds. Rose AH & Harrison JS. Academic Press, London, pp 79 – 105

Doll EC & Lucas RE (1973) Testing soils for potassium, calcium and magnesium. In Soil testing and plant analysis, eds. Walsh LM & Beatons JD pp. 133-152. Madison, Wisconsin: Soil Sci Soc Am

Fell JW & Statzell-Tallman A (1998) *Cryptococcus* Vuillemin. In: Kurtzman CP & Fell JW (eds) The yeasts, A taxonomic study, 4th ed. Elsevier, Amsterdam, pp 742 – 767

Fertilizer Society of South Africa (1974) Manual of soil analysis methods. FSSA Publication No 37

Fiechter A, Kappeli O, Meussdoerffer F (1987) Batch and continuous culture. In: Rose AH & Harrison JS (eds) The yeasts vol. 2, Yeasts and the environment. Academic Press, London, pp 99 – 129

Fry M St L (1987) A detailed characterization of soils under different fynbos-climate-geology combinations in the southwestern Cape. MSc Thesis. University of Stellenbosch, Stellenbosch, South Africa

Fujimura H, Sakuma Y, Amann E (1994) Survival of genetically-engineered and wild-type strains of the yeast *Saccharomyces cerevisiae* under simulated environmental conditions: a contribution on risk assessment. J Appl Microbiol 77: 689-693

Goto-Yamamoto N, Sato SI, Miki H, Park YK, Tadenuma M (1993) Taxonomic studies on yeast-lysing bacteria and a new species *Rarobacter Incanus*. J Gen Appl Microbiol 39: 261-272

Griffiths BS (1994) Soil nutrient flow. In Darbyshire JF (ed) Soil Protozoa. CAB International, Wallingford, pp. 65-91

Hechler HC (1970) Reproduction, chromosome number and postembryonic development of Panagrellus redivivus (Nematoda: Cephalobidae). J Nematol 2: 355-361

Joubert L-M, Botha A, Wolfaardt GM (2003) Feeding relationships in yeast-ciliate biofilms. In: McBain A, Allison D, Brading M, Rickard A, Verran J, Walker J (eds) BBC 6, Biofilm communities: Order from chaos? Bioline, Cardiff

Kimura Y, Nakano Y, Fujita K, Miyabe S, Imasaka S, Ishikawa Y, Sato M (1998) Isolation and characteristics of yeast able to grow at low concentrations of nutrients. Yeasts 14**:** 233 – 238

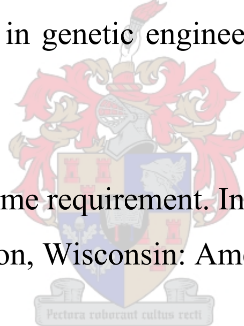
Kreger-van Rij NJW & Veenhuis M (1973) Electron microscopy of some special cell contacts in yeasts. J Bacteriol 113: 350-356

Kurtzman CP & Fell JW (2000) The yeasts: A taxonomic study, a 4th and enlarged edition. Elsevier, the Netherlands

Lachance MA & Pang WM (1997) Predacious yeasts. Yeast 13: 225-232

Lachance MA & Starmer WT (1998) Ecology of yeasts, In: Kurtzman CP, Fell JW, (eds) The Yeasts, A Taxonomic Study, 4th Ed., Amsterdam, Elsevier , pp. 21 – 30

Liang LN, Sinclair JL, Mallory LM, Alexander M (1982) Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl Env Mircobiol 44: 708-714



McClean EO (1982) Soil pH and lime requirement. In *Methods of soil analysis. Part 2.* ed. Page, A.L. pp. 570-571. Madison, Wisconsin: American Soc Agron

Nelson DW & Sommers LE (1982) Total carbon, organic carbon and organic matter. In *Methods of soil analysis. Part 2*. ed. Page, A.L. p.199. Madison, Wisconsin: American Soc of Agron

Petter R, Kanf BS, Boekhout T, Davis BJ, Kwon-Chung KJ (2001) A survey of heterobasidiomycetous yeasts for the genes homologous to virulence factors of *Filobasidiella neoformans*, *CNLAC1* and *CAP59*. Microbiol 147: 2029-2036

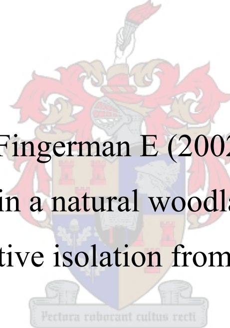
Phaff HJ & Starmer WT (1987) Yeasts associated with plants, insects and soil. In: Rose AH & Harrison JS (eds) The yeasts, biology of yeasts, 2nd edn, vol 1. Academic Press, London, pp 123 – 180

Pretorius IS (2003) The genetic analysis and tailoring of wine yeasts. In: De Winde JH (Ed) Functional genetics of wine yeasts. Springer Verlag, Heidelberg, Germany, pp 99-142

Ricci M, Fifi AP, Ragni A, Schlechtriem C, Focken U (2004) Development of a low-cost technology for mass production of the free-living nematode *Panagrellus redivivus* as an alternative live food for first feeding fish larvae. Appl Microbiol Biotech 60: 556-559

Smith MG, Des Etages SG, Snyder M (2004) Microbial synergy via an ethanol-triggered pathway. Mol Cell Biol 24: 3874-3884

Smith VR (2003) Soil respiration and its determinants on a sub-Antarctic island. Soil Biol Biochem 35: 77-91




Sniegowski PD, Dombrowski PG, Fingerman E (2002) *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. FEMS Yeast Res 1 299-306

Soil classification working group (1991) *Soil classification. A taxonomic system for South Africa.* Memoirs on the Agricultural Natural Resources of South Africa No 15. Pretoria, South Africa: Department of Agricultural Development

Spencer JFT & Spencer DM (1997) Ecology: Where yeasts live. In: Spencer JFT & Spencer DM (Eds) Yeasts in natural and artificial habitats. Springer, Berlin, pp 33 – 58

Steenbergen JN, Shuman HA, Casadevall A (2001) *Cryptococcus neoformans* interactions which suggest an explanation for its virulence and intracellular strategy in macrophages. Proc Natl Acad Sci 98: 15245-15250

Thomas GW & Peaslee DE (1973) Testing for soil phosphorus. In Soil testing and plant analysis. eds. Walsh, L.M. and Beatons, J.D. pp. 15-122. Madison, Wisconsin: Soil Sci Soc America

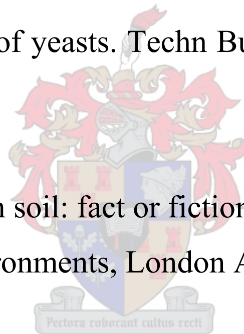
Van der Watt HVH (1966) Improved tables and a simplified procedure for soil particle size analysis by the hydrometer method. *S Afr J Agric Sci* 9**,** 911-916

Vaughan-Martini A & Martini A (1995) Facts, myths and legends on the prime industrial organism. J Ind Microbiol 14: 514 – 522

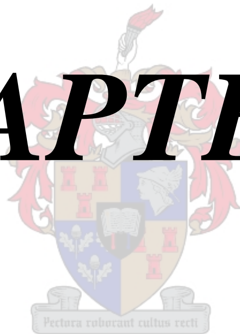
Vishniac HS (1995) Simulated *in situ* competitive ability and survival of a representative soil yeasts, *Cryptococcus albidus*. Microbial Ecol 30: 309 – 320

Wickerham LJ (1951) Taxonomy of yeasts. Techn Bull 1029, US Dept Agr,Washington, DC

Williams ST (1985) Oligotrophy in soil: fact or fiction. In: Fletcher M & Floodgate GD (eds) Bacteria in their natural environments, London Academic Press, pp 81 – 110



# *CHAPTER 4*



# *Biofilm Formation by* Saccharomyces cerevisiae *under Oligotrophic Conditions*

## 4.1 Introduction

Previously we found that *Saccharomyces cerevisiae* is able to survive and grow in soil under varying environmental conditions (Chapter 3). In order to obtain a better understanding about the mechanisms employed by *Saccharomyces* to adapt to the conditions in soil, which generally is viewed as an oligotrophic environment (Williams 1985), a variety of *in vitro* and *in situ* studies are needed.

It was found that a number of soil cryptococci are able to produce extracellular polymeric substances (EPS) and biofilms when cultivated in flowcells irrigated with oligotrophic growth media (Joubert et al. 2003). Biofilm formation is a known mechanism whereby microorganisms sequester and concentrate nutrients while growing under nutrient limited conditions (Decho 1990). Figure 1 (p 87) illustrates the ability of *Cryptococcus laurentii*, a known soil yeast, to form a biofilm on soil particles under oligotrophic conditions (Botha 2005). Therefore, we set out to determine whether *S. cerevisiae* is able to form biofilms when faced with oligotrophic conditions as is often found in soil. For this purpose we used epifluorescence microscopy to determine whether *S. cerevisisae,* similar to *Cryptococcus laurentii*, is also able to form biofilms in flow cells when challenged with an oligotrophic irrigation medium.

## 4.2 Materials and methods

### 4.2.1 Yeast strains used

*Saccharomyces cerevisiae* S92 and a genetically modified strain originating from this wild-type strain (ML01) were used in the experimentation. These two strains were obtained from Lesaffre International, France. *Cryptococcus laurentii* 1a, was kindly

donated by Mr. O.H.J. Rhode who isolated it from virgin soil. The strains were maintained at 4°C, on agar slopes prepared with yeast malt extract (YM) agar, in the Culture Collection at the Department of Microbiology of Stellenbosch University, South Africa. Every three weeks the yeast cultures were subcultured on YM agar, allowed to grow for three days at 25°C and then stored at 4°C.

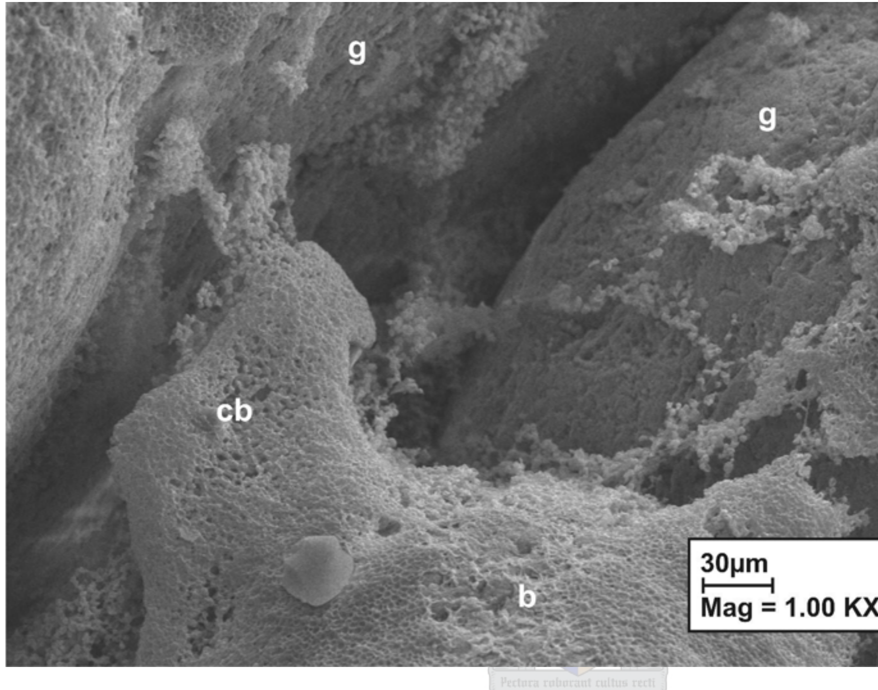



**Figure 1.** Scanning electron micrograph, taken from Botha (2005), illustrating biofilm formation (b) by a known soil yeast, *Cryptococcus laurentii,* under oligotrophic conditions on sand grains (g). Also visible is a connective bridge (cb) formed between the sand grains as a result of excessive EPS production by the yeast. Sand grains with attached yeast cells were mounted onto stubs, sputter-coated with gold and viewed unfixed and fully hydrated with a LEO 1430 VP Scanning Electron Microscope operated at 7 kV (Photo; L. Joubert, Department of Microbiology, University of Stellenbosch).

### 4.2.2 Cultivation of biofilm communities

Biofilm populations were prepared in flow cells (Wolfaardt et al. 1994) by inoculating a six channel flow cell with the two *Saccharomyces cerevisiae* strains. With the peristaltic pump (Watson Marlow 205S) turned off, three channels were inoculated with 200 µl of a 48 h old culture of each yeast strain, grown in yeast malt extract (YM) on a rotary shaker

at 25°C. Flow was resumed after 6 h and the yeast biofilms allowed to develop for an additional 72 h with an irrigation medium consisting of 2.5 mg/l sucrose and 6.7 mg/l Yeast Nitrogen Base (Difco) at a flow rate of 3 ml/h.

### 4.2.3 Epifluorescent microscopy

After three days, biofilms in individual flow cell channels were subjected to staining with the fluorescent yeast viability probe FUN-1™ from Molecular Probes. With the peristaltic pump turned off, the channels in the flow cell were incubated overnight with a single 200µl pulse of 40µM FUN-1™ according to the product information sheet. Flow was resumed for 1h to rinse the channels, whereafter live versus dead biofilm yeasts were visualized microscopically.

All microscopic observations and image acquisitions were performed with a Nikon Eclipse E400 epifluorescence microscope, equipped with a multipass filter set appropriate for viewing DAPI, as well as excitation/ barrier filter sets of 480/500nm and 490/635nm. Images were captured with a Nikon Coolpix 990 digital camera mounted on the same microscope. In the case of the yeast viability probe FUN-1™, metabolically active cells were marked with fluorescent red intravacuolar structures, while dead yeast cells exhibit yellow-green fluorescence.

## 4.3 Results and discussion

As is the case for *C. laurentii* 1a (Fig 2a, p 87), both *S. cerevisiae* S92 (Fig 2b, p 87) and ML01 (Fig 2c, p 87) were able to form viable (as indicated by the FUN-1™ probe) biofilms under oligotrophic conditions. No notable differences were observed between the two *Saccharomyces* strains, however, the *C. laurentii* strain proved to be metabolically more active than *Saccharomyces*. The latter was obvious from the red fluorescence indicating viable *Cryptococcus* cells.

This experiment demonstrated that, similar to the cryptococci, biofilm formation is one of the mechanisms that may be employed by *S. cerevisiae* to persist in oligotrophic environments such as soil. However, it was suggested that the survival and growth of yeast in soil do not to depend solely on the intrinsic abilities of the particular strain to maintain itself within the chemical environment (Botha 2005). Other factors than the ability to produce biofilms should also been studied, such as interactions with members of the soil microbial community. Therefore, future studies on the growth and survival of *S. cerevisiae* in flow cells under oligotrophic conditions should be conducted using mixed populations of soil organisms.

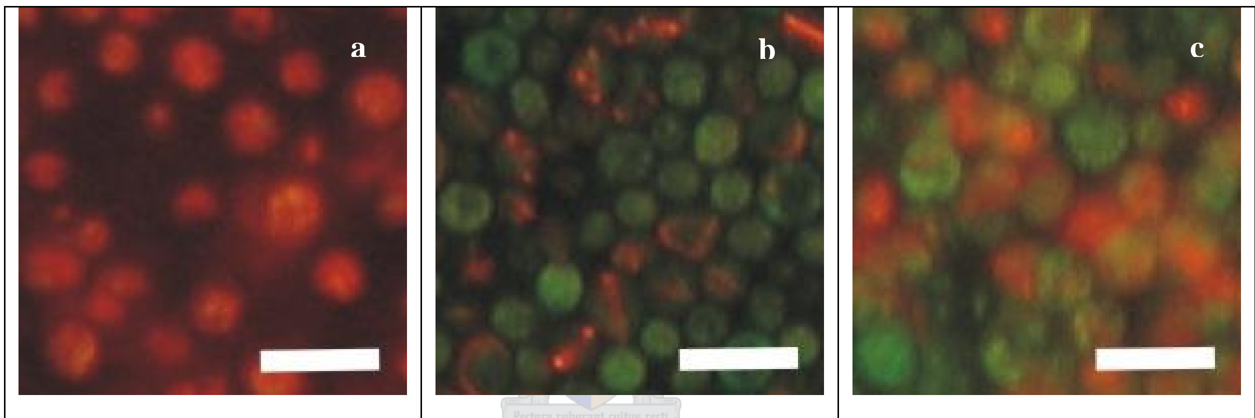



**Figure 2a, 2b and 2c.** Flow cell biofilm formation by *C. laurentii* 1a (a), *S. cerevisiae* S92 (b) and *S. cerevisiae* ML01 (c) under oligotrophic conditions. The white bar represents *ca.* 10µm. Metabolically active cells exhibit red intravacuolar structures as revealed by staining with FUN-1™ , while non-viable cells appear yellow-green.

## 4.4 References

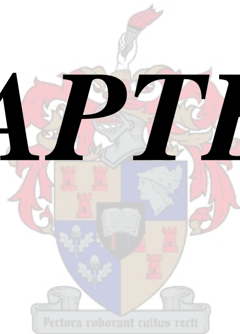

Botha A (2005) Yeasts in soil. In: Ecophysiology of Yeasts, eds. Rosa CA & Péter G Springer-Verlag, Heidelberg (Accepted for publication)

Decho AW (1990) Microbial exopolymer secretions in ocean environments: Their role(s) in food webs and marine processes. Oceanogr Mar Biol Annu Rev 28: 73 – 153

Joubert L-M, Botha A, Wolfaardt GM (2003) Feeding relationships in yeast-ciliate biofilms. In: Biofilm communities: Order from chaos? Eds. McBain A, Allison D, Brading M, Rickard A, Verran J, Walker J. BBC 6, Bioline, Cardiff

Williams ST (1985) Oligotrophy in soil: fact or fiction. In: Bacteria in their natural environments, eds. Fletcher M & Floodgate GD. London Academic Press, pp 81 – 110

# CHAPTER 5



# *General Conclusions and Hints on Future Research*

## 5.1 Conclusions and future research

*Saccharomyces cerevisiae* is commonly associated with the wine industry and aside from a few *in vitro* studies, the fate of *S. cerevisiae* in soil is largely unknown. This may be due to the lack of reliable methods to enumerate fermentative yeasts in soil. Thus, the purpose of this study was to develop a method for the isolation and enumeration of fermentative yeasts in soil and to determine the fate of a wild-type strain of *S. cerevisiae* (S92) and a genetically modified strain (ML01), capable of malolactic fermentation, in soil. Included in the experiments were two autochthonous soil yeasts, *Cryptococcus laurentii* and *Cryptococcus podzolicus*. The following observations were made:

1) Soil dilution plates, prepared from yeast malt extract (YM) agar, supplemented with 0.2g/l chloramphenicol and 0.5g/l streptomycin, incubated in anaerobic jars could be used to successfully enumerate and isolate fermentative yeasts, such as *S. cerevisiae,* from soil microcosms.

2) With the exception of growth in soil supplemented with nutrients, the genetically modified strain of *S. cerevisiae* (ML01) had no metabolic advantage over the wild-type strain (S92) in sterile soil. Furthermore, the cryptococci appeared to be better suited to this environment as they are known to occur in semi-arid soils (Spencer & Spencer 1997) and utilize notably more carbon compounds than *S. cerevisiae* (Kurtzman and Fell, 2000)

3) In the presence of the natural soil biota, the numbers of *Saccharomyces* in soil microcosms declined rapidly, probably as a result of competition and/or predation by the soil biota. No difference was observed between the two *Saccharomyces* strains regarding their performance under these conditions. Similarly, when incubated in co-culture with selected cryptococcal strains, the numbers of both

*Saccharomyces* strains were significantly reduced in soil microcosms, possible due to amensalism or competition (Botha 2005).

4) When the two *Saccharomyces* strains were cultured in soil microcosms inoculated with a protistan predator, populations of both strains increased and remained at high levels for the duration of the trial. These findings point to a possible symbiosis between *Saccharomyces* and the protista whereby the predators ensure continuous nutrient cycling (Griffiths 1994) within the soil microcosms. These findings are therefore indicative of a natural interdependency between these groups.

5) Epifluorescence microscopy revealed that, similar to known soil cryptococci, *Saccharomyces* is able to form biofilms. Both *Saccharomyces* strains were able to form biofilms and this may be a mechanism whereby *Saccharomyces* sequester and concentrate nutrients in oligotrophic environments, such as soil.

From the above results it can be concluded that *S. cerevisiae* S92 and the genetically modified strain capable of malolactic fermentation, *S. cerevisiae* ML01, were able to grow and/or survive until the end of the incubation period in soil microcosms, differing in moisture content, nutrient composition and the presence of biota. The genetically modified strain seemed to have no competitive advantage over the wild-type in the presence of the natural soil biota.

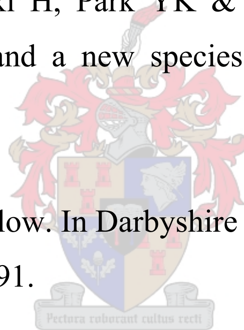
These observations together with indications of the above-mentioned symbiosis between *S. cerevisiae* and a group of soil protista, as well as evidence obtained from literature on the occurrence of *S. cerevisiae* in pristine soil (Sniegowski et al. 2002), and *in vitro* interactions of this yeast with other soil biota (Hechler, 1970; Goto-Yamamoto et al. 1993; Lachance and Pang, 1997; Smith et al. 2004), indicate that soil may act as habitat for this species. We now have the tools to determine the role of these fermentative yeasts in soil ecosystems by selectively monitoring their populations in natural settings, subjected to seasonal changes and perturbations by man. Also, viable populations of

genetically modified fermentative yeasts can selectively be monitored in natural settings. In addition, the interactions of fermentative soil yeast populations with the physicochemical environment can now be studied more effectively than in the past, where non-selective isolation media were the only viable option. Furthermore, correlations could be seeked between the number of naturally occurring fermentative yeasts and crop performance and/or soil fertility.

## 5.2 References

Botha A (2005) Yeasts in soil. In: Ecophysiology of Yeasts, eds. C A Rosa & G Péter. Springer-Verlag, Heidelberg (Accepted for publication).

Goto-Yamamoto N, Sato SI, Miki H, Park YK & Tadenuma M (1993) Taxonomic studies on yeast-lysing bacteria and a new species *Rarobacter Incanus*. J Gen Appl Microbiol 39: 261-272




Griffiths, BS (1994) Soil nutrient flow. In Darbyshire JF (ed) Soil Protozoa. CAB International, Wallingford, pp. 65-91.

Hechler HC (1970) Reproduction, chromosome number and postembryonic development of Panagrellus redivivus (Nematoda: Cephalobidae). J Nematol 2: 355-361

Kurtzman CP, Fell JW (2000) The yeasts: A taxonomic study, a $4^{th}$ and enlarged edition. Elsevier, the Netherlands.

Lachance MA & Pang WM (1997) Predacious yeasts. Yeast 13: 225-232

Smith MG, Des Etages SG, Snyder M (2004) Microbial synergy via an ethanol-triggered pathway. Mol Cell Biol 24: 3874-3884

Sniegowski PD, Dombrowski PG, Fingerman E (2002) *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. FEMS Yeast Research 1 299-306

Spencer JFT & Spencer DM (1997) Ecology: Where yeasts live. In: Spencer JFT & Spencer DM (Eds) Yeasts in natural and artificial habitats. Springer, Berlin, pp 33 – 58.

Wolfaardt GM, Lawrence GM, Robarts JR, Caldwell SJ, Caldwell DE (1994) Multicellular organization in a degradative biofilm community. Appl Environ Microbiol 60: 434-446