Yeast Biodiversity in Vineyards and Wineries and Its Importance to the South African Wine Industry. A Review

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The art of winemaking is as old as human civilization and the use of yeast in this complex ecological and biochemical process dates back to ancient times. Traditionally, yeasts associated with grape berries were simply allowed to ferment the sugars to ethanol, carbon dioxide and other minor, but important, metabolites. Spontaneous fermentations are still being used in *boutique* wineries that depend more on vintage variability. Various microbes found on the surface of grape skins and the indigenous microbiota associated with winery surfaces participate in these natural wine fermentations. Yeasts of the genera *Kloeckera, Hanseniaspora* and *Candida* predominate in the early stages, followed by several species of *Metschnikowia* and *Pichia* (including those species that were previously assigned to the genus *Hansenula*) in the middle stages when the ethanol rises to 3-4%. The latter stages of natural wine fermentations are invariably dominated by the alcohol-tolerant strains of *Saccharomyces cerevisiae*. However, other yeasts, such as species of *Brettanomyces, Kluyveromyces, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces* also may be present during the fermentation and can occur in the resultant wine. By contrast, the rule, rather than the exception, for modern wineries depending on reliable fermentation and the production of wines with predictable quality, is the use of specially selected starter cultures of *Saccharomyces*. However, the use of such cultures may not necessarily prevent the growth and metabolic activity of indigenous, winery associated strains of *S. cerevisiae* or other wild yeasts such as *Kloeckera apiculata, Hanseniaspora uvarum, Candida stellata* and *Torulaspora delbrueckii*. It is therefore clear that both spontaneous and inoculated wine fermentations are affected by the diversity of yeasts associated with the vineyard (natural habitat) and winery (man-made niche). In light of this, focused taxonomic surveys within an ecological framework are essential to preserve and exploit the hidden oenological potential of the untapped wealth of yeast biodiversity in our wine-producing regions. To achieve this, yeast taxonomists need to continue to isolate and characterize new yeast species and strains, while wine microbiologists develop improved identification techniques that differentiate more efficiently among individual strains. At the same time such biological surveys will complement strain development and the current international effort of molecular biologists to assign a biological function to the products of each of the 6000 genes identified by computer analysis of the nucleotide sequence of the 16 chromosomes of a laboratory strain of *S. cerevisiae*. Furthermore, only when we have a much better understanding of yeast biodeversity, biogeography, ecology and the interaction within yeast communities will we be able to optimally harness gene technology that will benefit both the wine producer and the consumer.

The art of winemaking is far older than recorded history and the development of fermentation technology underpinning this ancient process stretches over a period of nearly 7000 years. The fermentation of grape must and production of premium quality wines is a complex ecological and biochemical process involving the interaction of many microbial species, represented by fungi, yeasts, lactic acid bacteria and acetic acid bacteria, as well as the mycoviruses and bacteriophages affecting these grape-associated microorganisms (Fleet, 1993). Of all these different microbes and viruses, yeasts, being the primary catalysts of the bioconversion of grape juice into wine, represent the heart of the harmonious biochemical interaction with the musts derived from the various varieties of *Vitis* species which, in turn, are largely products of their respective genetic make-ups and the *terrior*.

This article, summarizing the most important aspects of yeast biodiversity and ecology, illustrates the importance of thorough biological surveys within the ecological framework of the wine-producing regions of South Africa.

Yeast taxonomy:

Yeast can be defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth predominantly results from budding or fission and which do not form their sexual states within or upon a fruiting body (Kurtzman & Fell, 1998b). The world's growing awareness of the importance of biodiversity re-

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focused the attention on new taxonomic surveys within an ecological framework (Lachance & Stramer, 1998).

The first level of yeast classification is based on aspects of yeasts' sexuality (Ascomycotina or Basidiomycotina) or the lack of a sexual phase in the life cycle (Deuteromycotina). The lower taxonomic subdivisions (*families, subfamilies, genera, species* and *strains*) are based on various morphological, physiological and genetic criteria, including sexual reproduction (Kurtzman & Fell, 1998a). Many of these criteria, as well as sexual compatibility studies used for the speciation of yeasts, are derived from a small portion of the genome, and are therefore often unreliable and time-consuming. Since several of the morphological and physiological characteristics can be reversed by a mutation in a single gene, these methods on their own are inadequate for routine yeast identification. Moreover, the biological concept, which delimits species on their ability to hybridize, is also problematic in yeast systematics - the lack of fertility does not preclude conspecificity, since only a few genes affect the ability to mate (De Barros Lopes *et al.*, 1999).

These phenotypic traits serve a purpose in yeast taxonomy, since not all of these characteristics are unstable and insignificant. However, they do not necessarily reflect genetic relatedness, since the same phenotype may be a result of convergent evolution. Conversely, the phylogenetic relationships should be reflected in similarities at the level of the deoxyribonucleic acid (DNA) sequence homology in different yeasts (Pretorius & Van der Westhuizen, 1991).

Limitations in using morphological and physiological criteria have led to the increasing use of nucleic acid methods in yeast taxonomy, including DNA base composition and reassociation studies and sequence analysis of the ribosomal ribonucleic acid (rRNA) genes (ribotyping). The recent emphasis on these DNA-based identification methods has helped yeast taxonomists to correlate taxonomy with phylogeny.

As an example the classification of ascomycetous yeasts and a list of the currently accepted yeast genera are given in Tables 1 and 2, respectively. Rules for taxonomy of the yeasts fall under the authority of the *International Code of Botanical Nomenclature* (Greuter *et al.*, 1994) and publication of a new species must include a description of essential characters as well as a diagnosis that distinguishes the taxon from a previously described species (Kurtzman & Fell, 1998b). Based on improved methods of yeast isolation, identification and classification, the number of yeast genera and species have more than doubled since the release of the second edition of the monographic series, *The Yeasts, A Taxonomic Study* (Lodder, 1970). The fourth and latest edition of the series describes 100 yeast genera representing over 700 species (Kurtzman & Fell, 1998a).

Yeast biodiversity and ecology:

Notwithstanding the spectacular growth in the number of described yeast species between 1970 and 1998, the wealth of yeast biodiversity is still largely untapped. This point is best illustrated by the fact that for ascomycetes in general, the numbers of undescribed genera and species have been calculated at 62 000 and 669 000, respectively (Hawksworth & Mouchassa, 1994). Therefore, in order to discover the hidden oenological potential with respect to the immense untapped yeast biodiversity, it is imperative to continue to develop ways of characterizing and preserving remaining species and strains. According to Lachance & Stramer (1998) these new taxonomic surveys, should, however, not be a meaningless catalogue or inventory of yeast names, devoid of real biological relevance, but rather a study of yeast species in the context of their environment. Several molecular biological taxonomic approaches are now assisting in the fundamental understanding of yeast communities in specific habitats and niches. This will undoubtedly provide improved means to preserve and exploit yeast biodiversity (Roberts & Wildman, 1995) and to track and monitor the spread of genetically modified yeasts when used for the production of fermented foods and beverages in future.

Yeast communities, habitats and niches: Yeasts occur widespread in nature although they are not as ubiquitous as bacteria (Phaff, Miller & Mrak, 1978). However, these chemo-organotrophic fungi, requiring fixed, organic forms of carbon for growth, do not occur randomly throughout the biosphere. They form communities of species and each community is defined by its *habitat*, the actual place where an assemblage of yeasts lives, and by the niches of its component species (for a recent review see Lachance & Stramer, 1998). The *niche* consists of the attritubtes that make a yeast capable of sharing a habitat with other *autochthonous* (essential components of the community) and *allochthonous* (components that are transient or present fortuitously) members of the community and is therefore the sum of all physical, chemical or biotic factors required for successful existence (Lachance & Stramer, 1998). For example, different yeasts are able to utilize different carbon sources and nutritional selectivity determines yeast species diversity in particular niches. Therefore, *generalist* yeasts are endowed with a broad niche and as a consequence occupy many habitats, whereas *specialist* yeasts exhibit great specialization for their habitat and thus occur in very unique habitats (Lachance & Stramer, 1998). The Atlas & Bartha (1993) classification of the various types of community interactions is outlined in Table 3.

Yeast flora of grapes: Being non-motile, yeasts rely on aerosols, animal vectors and human activity for their natural dispersal (Walker, 1998). They can be isolated from terrestrial, aquatic and aerial environments, but preferred habitats are plant tissues. The microflora of grapes are highly variable, with a predominance of the low alcohol-tolerant species of *Kloeckera* and its teleomorph *Hanseniaspora (e.g., K. apiculata* and *H. uvarum*) that account for about 50-75% of the total yeast population. Furthermore, significant by their presence, but at lesser numbers than the latter species, are species of *Candida* (especially *C. stellata* and *C. pulcherrima), Brettanomyces, Cryptococcus, Kluyveromyces, Pichia* (including those species that were previously assigned to the genus *Hansenula*) and *Rhodotorula* (for a review see Fleet & Heard, 1993). However, fermentative species of *Saccharomyces (e.g., S. cerevisiae)* occur at extremely low populations on sound, undamaged grapes and are rarely isolated from intact berries (Du Plessis, 1959; Peynaud & Domercq, 1959; Van Zyl & Du Plessis, 1961; Martini & Vaughan-Martini, 1990; Martini, 1993). In fact, Vaughan-Martini & Martini (1995) concluded that a natural origin for *S. cerevisiae* should be excluded. According to these authors *S. cerevisiae* strains present in spon-

TABLE 1

Classification of the ascomycetous yeasts (Kurtzman & Fell, 1998a).

Class Order Family Genus	 Family Genus
Phylum Ascomycota	Metschnikowiaceae
"Archiascomycetes"	*Clavispora*
Schizosaccharomycetales	***Metschnikowia****
Schizosaccharomycetaceae	Saccharomycetaceae
Schizosaccharomyces*	*Arxiozyma*
Taphrinales	*Citeromyces*
Taphrinaceae	*Cyniclomyces*
Taphrina	***Debaryomyces****
Lalaria (Anamorph of *Taphrina*)	***Dekkera****
Protomycetales	*Issatchenkia*
Protomycetaceae	***Kluyveromyces****
Protomyces	*Lodderomyces*
Saitoella (Anamorphic genus)	*Pachysolen*
Pneumocystidales	***Pichia****
Pneumocystidaceae	***Saccharomyces****
Pneumocystis	*Saturnispora*
Euascomycetes	***Torulaspora****
Endomyces (*E. scopularum*)	*Williopsis*
Oosporidium	***Zygosaccharomyces****
Hemiascomycetes	Saccharomycodaceae
Saccharomycetales (synonym Endomycetales)	***Hanseniaspora****
Ascoideaceae	*Nadsonia*
Ascoidea	***Saccharomycodes****
Cephaloascaceae	*Wickerhamia*
Cephaloascus	Saccharomycopsidaceae
Dipodascaceae	*Ambrosiozyma*
Dipodascus	*Saccharomycopsis*
Galactomyces	Candidaceae (Anamorphic)
Sporopachydermia	*Aciculoconidium*
Stephanoascus	*Arxula*
Wickerhamiella	*Blastobotrys*
Yarrowia	*Botryozyma*
Zygoascus	***Brettanomyces****
Endomycetaceae	***Candida****
Endomyces (*E. decipiens*)	*Geotrichum*
Helicogonium	***Kloeckera****
Myriogonium	*Myxozyma*
Phialoascus	*Schizoblastosporion*
Trichomonascus	*Sympodiomyces*
Eremotheciaceae	*Trigonopsis*
Eremothecium	
Coccidiascus	
Lipomycetaceae	
Babjevia	
Dipodascopsis	
Lipomyces	
Zygozyma	

*Genera that are frequently encountered in vineyards, wineries, grape must and/or wine are typed in bold.

TABLE 2

An overview of yeast genera according to Kurtzman and Fell (1998a).

Teleomorphic ascomycetous genera (Ascomycotina)	Anamorphic ascomycetous genera (Deuteromycotina)	Teleomorphic heterobasidiomycetous genera (Basidiomycotina)	Anamorphic heterobasidiomycetous genera (Basidiomycotina)
Ambrosiozyma	*Aciculoconidium*	*Agaricostilbum*	*Bensingtonia*
Arxiozyma	*Arxula*	*Bulleromyces*	*Bullera*
Ascoidea	*Blastobotrys*	*Chionosphaera*	***Cryptococcus****
Babjevia	*Botryozyma*	*Cystofilobasidium*	*Fellomyces*
Cephaloascus	***Brettanomyces****	*Erythrobasidium*	*Hyalodendron*
Citeromyces	***Candida****	*Fibulobasidium*	*Itersonilia*
Clavispora	*Geotrichum*	*Filobasidiella*	*Kockovaella*
Coccidiascus	***Kloeckera****	*Filobasidium*	*Kurtzmanomyces*
Cyniclomyces	*Lalaria*	*Holtermannia*	*Malassezia*
Debaryomyces*	*Myxozyma*	*Leucosporidium*	*Moniliella*
Dekkera*	*Oosporidium*	*Mrakia*	*Phaffia*
Dipodascopsis	*Saitoella*	*Rhodosporidium*	*Pseudozyma*
Dipodascus	*Schizoblastosporion*	*Sirobasidium*	*Reniforma*
Endomyces	*Sympodiomyces*	*Sporidiobolus*	***Rhodotorula****
Eremothecium	*Trigonopsis*	*Sterigmatosporidium*	*Sporobolomyces*
Galactomyces		*Tilletiaria*	*Sterigmatomyces*
Hanseniaspora*		*Tremella*	*Sympodiomycopsis*
Issatchenkia		*Trimorphomyces*	*Tilletiopsis*
Kluyveromyces*		*Xanthophyllomyces*	*Trichosporon*
Lipomyces			*Trichosporonoides*
Lodderomyces			*Tsuchiyaea*
Metschnikowia*			
Nadsonia			
Pachysolen			
Pichia*			
Protomyces			
Saccharomyces*			
Saccharomycodes*			
Saccharomycopsis			
Saturnispora			
Schizosaccharomyces*			
Sporopachydermia			
Stephanoascus			
Torulaspora			
Wickerhamia			
Wickerhamiella			
Williopsis			
Yarrowia			
Zygoascus			
Zygosaccharomyces*			
Zygozyma			

*Genera that are frequently encountered in vineyards, wineries, grape must and/or wine are typed in bold.

TABLE 3

Classification of the various types of community interactions, the interaction of yeasts and other microbes in specific habitats (Atlas & Bartha, 1993).

Type of interaction	Definition
Microbe-microbe interaction	
Neutralism	Sparse, independent populations sharing the same habitat
Commensalism	A population benefits from the activity of another, *synergism* (cooperative modification of resources leading to close spatial relationships)
Mutualism/symbiosis	Very strong synergistic association
Competition	Utilization of limited common resources
Amensalism	Chemical interference
Predation	Ingestion
Parasitism	Long term destructive contact
Animal-microbe interactions	
Grazing	
Food enrichment	
Epizoic relationships	
Plant-microbe interactions	
Rhizosphere	
Rhizoplane	
Mycorrhizae	Mutualistic interaction
Phyllosphere	
Epiphytes	
Pathogens	

taneously fermenting grape must originate from various surfaces in the winery. On the other hand, Török *et al.* (1996) supplied data indicating that the vineyard is in fact the primary source of this yeast. They also noted that each plant and grape cluster is different regarding the presence/absence of *S. cerevisiae*. To elude the problems associated with the recovery of such low numbers of yeast, the majority of surveys on the population kinetics and geographic distribution of natural-occurring yeast species invariably included an enrichment procedure after the isolation (Frezier & Dubourdieu, 1992; Fleet, 1993; Querol, Barrio & Ramon, 1994; Schütz & Gafner, 1994; Versavaud *et al.*, 1995; Constanti *et al.*, 1997). The combined effect of several factors affects the microflora of grapes (Table 4).

Winery yeast flora: In addition to natural habitats, some yeasts have found niches in man-made environments such as wine cellars. The surfaces of winery equipment that come into contact with grape juice and wine become locations for the development of a so-called *residential* or *winery yeast flora* (Peynaud & Domercq, 1959; Rosini, 1984). The extent of the development of a residential yeast flora (*e.g.*, species of *Saccharomyces, Candida* and *Brettanomyces*) will depend upon factors listed in Table 4. Unlike its low occurrence in natural habitats such as grapes, *S. cerevisiae* is prevalent on these surfaces (Rosini, 1984; Martini, Ciani & Scorzetti, 1996). In fact, *S. cerevisiae* is by far the most dominant yeast species colonizing surfaces in wineries demonstrating the selective effects of grape juice and wine as growth substrates (Martini, 1993).

Although the presence, and importance of winery yeasts have been known, or surmised, for many years (Peynaud & Domercq, 1959; Van Zyl & Du Plessis, 1961), their actual contribution to must fermentations has been all but ignored. Rosini (1984) supplied preliminary data indicating that these yeasts actually dominate spontaneous fermentations conducted in well-established wineries. Martini *et al.* (1996) indicated that fermentation of grape must aseptically prepared in the laboratory is often anomalous. They also speculated that resident winery yeasts will compete with pure *S. cerevisiae* cultures used to inoculate musts. This view is supported by data recently generated by Constanti *et al.* (1997). These authors found that a resident winery yeast completely dominated an inoculated fermentation in two-year old Spanish winery. Török *et al.* (1996), however, do not support the idea that yeasts resident on winery surfaces play an important, or even dominant, role during spontaneous fermentation of grape must. Clearly the origin (if present at all), composition and actual contribution to fermentation of resident winery yeasts need to be studied much more extensively.

TABLE 4

Factors influencing the microflora of vineyards, grapes, wineries and must.

Microflora of vineyards and grapes

Climatic influences
- temperature
- rainfall
- wind
- microclimate as affected by viticultural practices such as canopy management

Soil and viticultural practices
- soil type
- fertilization
- irrigation
- application of fungicides

Grape
- variety
- physical damage by mould, insect or bird attack

Microflora of surfaces of winery equipment

Nature of surfaces
- irregular, unpolished surfaces
- cracks and welds

Cleaning and sanitization

Microflora of grape must

Method of grape harvest
- handpicked or mechanical
- grape temperature

Transport from vineyard to wine cellar
- time
- initial grape temperature
- air temperature
- sulfite addition

Condition of grapes
- time
- temperature
- sulfite addition

Must treatment
- cellar hygiene
- aeration
- sulfite addition
- clarification method
- temperature
- inoculation with yeast starter cultures

Yeast flora in grape must: The microbiota of grape must are affected indirectly by all factors influencing the indigenous grape microflora and the winery flora as well as by aspects listed in Table 4.

Although grape must is considered to be relatively complete in nutrient content, it can only support the growth of a limited number of microbial species. According to Henschke (1997), the low pH and high sugar content of grape must exert strong selective pressure on the microorganisms, such that only several yeast and bacterial species can proliferate. Furthermore, the use of restrictive concentrations of sulfur dioxide as an anti-oxidant and anti-microbial preservative, imposes additional selection, particularly against undesirable oxidative microbes. The selectivity of fermenting must is further strengthened once anaerobic conditions are established, certain nutrients become depleted and the increasing levels of ethanol start to eliminate alcohol-sensitive microbial species (Henschke, 1997). Therefore, spontaneous fermentation of grape juice into wine can be regarded as a heterogeneous microbiological process involving the sequential development of various yeasts and other microbiological species, affected by the prevailing fermentation conditions in a particular vat or tank. The fermentation is usually started by yeasts of the genera

Kloeckera, Hanseniaspora and *Candida* and, to a lesser extent, *Metschnikowia* and *Pichia*. These yeasts predominate during the early and middle phases of fermentation until ethanol levels rise to around 3 to 4% (Fleet & Heard, 1993; Mortimer *et al.*, 1994). These ethanol-sensitive yeasts are then overtaken by the stronger fermenting and alcohol-tolerant species of *Saccharomyces* which carry out and complete fermentation, reaching ethanol levels in the range of 12 to 14%. It is also amply reported by numerous authors that other yeasts, such as species of *Brettanomyces, Kluyveromyces, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces* may also be present during the fermentation and can occur in the resultant wine, some of which are capable of adversely affecting the sensorial quality of the end product (Fleet & Heard, 1993; Henschke, 1997). From this it is clear that, when must is used as a culture medium, the abovementioned selective pressures always yield biased results in favour of the yeasts with the most efficient fermentative catabolism, particularly strains of *S. cerevisiae* (Martini, 1993) and perhaps strains of closely related species such as *S. bayanus* (Vaughan-Martini & Martini, 1998).

Wine yeast starter cultures:

Wine yeast strain variation: Over the years, strains of *S. cerevisiae* were isolated from vineyards and selected to be used as commercial starter cultures. The genetic variation normally present in all *Saccharomyces* populations also served as a gene pool for the breeding of improved starter strains. The natural genetic heterogeneity in wine yeast strains is mainly due to mitotic recombination and spontaneous mutation.

It is now believed that strains of *Saccharomyces* indigenous to vineyards and wineries, tend to be homozygous for most of the genes by a process known as *genome renewal* (Mortimer *et al.*, 1994). This phenomenon is based on the capability of homothallic haploid *Saccharomyces* cells to switch their mating-type from **a** to *x* and *vice versa* and to conjugate with cells of the same single-spore colony. According to Mortimer *et al.* (1994) continued propagation of yeast cells in their natural (*e.g.*, vineyards) or man-made (*e.g.*, wineries) habitats leads to a situation where strains of *Saccharomyces* accumulate heterozygous recessive mutations and the concomitant heterozygotes can change to completely homozygous diploids by sporulation and homothallic switching of individual haploid spores. This process would eliminate the recessive lethal or deleterious genes that adversely affect yeast fitness (*e.g.*, slower growth, lower fermentation rate, reduced spore viability, *etc.*). Furthermore, *genome renewal* could also be responsible for the replacement of the parental heterozygous strains by the new homozygous diploids bearing new recessive alleles that increase fitness. Perhaps this is the reason why most indigenous strains of *Saccharomyces* isolated from grapes, wineries and musts, are homothallic since homothallism, together with the capability to sporulate, would provide the yeast community with a mechanism by which cells carrying deleterious recessive mutations can be eliminated, thereby enabling them to efficiently adapt to changing environmental conditions.

The practical implications of *genome renewal* and yeast population dynamics in the vineyards and wineries, and even within yeast starter cultures, are far reaching, whether winemakers rely on spontaneous fermentation of grape juice or whether they inoculate grape must with selected or tailor-made wine yeast strains.

Spontaneous versus inoculated fermentations: Originally all wine was made by utilizing the natural microflora in spontaneous fermentations. The practice remained prevalent in *old world* wine-producing areas until the 1980s. Many *boutique* wineries, depending on vintage variability, still utilize this process today. However, the emergence of large-scale wine production where rapid, reliable, trouble free fermentations are essential for consistent wine flavour and predictable quality, necessitated the use of selected pure yeast inocula of known ability (Henschke, 1997). It is these large wineries that will be the main beneficiaries of programmes aimed at yeast strain development. These programmes will continually produce new yeast strains that have even more reliable performance so reducing processing inputs, and consequently facilitate the production of affordable high-quality wines (Henschke, 1997).

Besides the primary role of wine yeast to catalyze the rapid, complete and efficient conversion of grape sugars to alcohol without the development of off-flavours, yeast starter cultures should also possess properties such as high tolerance to sulfite, osmotic stress, ethanol and copper; genetic stability; production of glycerol and ß-glucosidase; minimal lag phase on rehydration; complete fermentation of sugar at low temperatures; and limited production of foam, sulfur dioxide, hydrogen sulfide, volatile acidity, acetaldehyde, pyruvate, ethyl carbamate precursors and polyphenol oxidase (for reviews see Pretorius & Van der Westhuizen, 1991; Degré, 1993; Henschke, 1997; Pretorius, 1999, 2000). The importance of these additional yeast characteristics largely depends on the type and style of wine to be made and the technical requirements of the winery.

Industrial-taxonomic relationship for wine yeast strains: Saccharomyces strains with specific characteristics are preferred when making different types of wine, such as dry white, dry red, sparkling, sweet and fortified wine, and flor sherry (Henschke, 1997). This led to a situation where these wine yeast strains of *Saccharomyces* were classified into several different species or varieties, including *S. bayanus, S. beticus, S. capensis, S. chevaleri, S. ellipsoideus, S. fermentati, S. oviformis, S. rosei* and *S. vini* (Lodder & Kreger-van Rij, 1952; Lodder, 1970). In fact, the characteristics of some of the yeasts used for the production of specific wine types were so marked that a strong taxonomic linkage was believed to exist (Henschke, 1997). For example, while *S. ellipsoideus* was widely used for the production of dry wine, ethanol-tolerant and flocculent strains with autolytic properties (*e.g., S. bayanus* and *S. oviformis*) were prefered for the production of bottle-fermented sparkling wine, film-forming strains with strong oxidative capabilities (*e.g., S. beticus* and *S. capensis*) for the production of flor sherry and osmotolerant strains forming little or no volatile acids (*e.g., S. rosei*) for sweet wines (Henschke, 1997). Despite this strong industrial-taxonomic relationship that has developed over a number of decades, and the considerable phenotypic differences among these yeasts, most of them are now, based on results obtained using sophisticated genetic taxonomic techniques, considered to be physiological strains of *S. cerevisiae* (Kreger-van Rij, 1984; Barnett, 1992; Vaughan-Martini & Martini, 1995; Kurtzman & Fell, 1998a). Of all these so-called *wine yeasts*, only *S. fermentati* and *S. rosei* were not re-

classified as *S. cerevisiae* but rather as *Torulaspora delbrueckii*. The current description of the genus *Saccharomyces* together with the list of the currently accepted species and strians of *S. cerevisiae* and *S. bayanus* are given in Tables 5, 6, 7 and 8, respectively. The morphology and growth patterns of *Saccharomyces* are depicted in Fig. 1.

The assignment of most traditional wine yeast strains (except for *S. fermentati* and *S. rosei*) to a single species does, however, not imply that all strains of *S. cerevisiae* are equally suitable for the various wine fermentations. These physiological strains of *S. cerevisiae* differ significantly in their fermentation performance and their ability to contribute to the final bouquet and quality of the various types of wine and distillates. Therefore, to ensure strain authenticity, security and proper strain management, it is of cardinal importance to have reliable taxonomic techniques available to identify and characterize individual strains of commercial cultures, and to continue to characterize and select new isolates and to genetically improve these wine yeast strains for the production of premium quality wines (Pretorius, 1999, 2000).

Strain identification methods: Contrary to yeast taxonomists whose aims are to classify yeasts to species level, identification of individual strains is more the focus of wine microbiologists. The combined use of two or more molecular techniques can usually discriminate among wine yeast strains with similar physiological properties (Querol *et al.*, 1992b; Cavalieri *et al.*, 1998; Van der Westhuizen, Augustyn & Pretorius, 1999). The most frequently used molecular methods include:

- pyrolysis-gas chromatography or gas-liquid chromatography of long-chain fatty acids resulting in fingerprinting peaks on chromatograms (Tredoux *et al.*, 1987; Augustyn, 1989; Augustyn & Kock, 1989; Rozes *et al.*, 1992);
- *p*oly*a*crylamide *g*el *e*lectrohoresis (PAGE) of total soluble proteins generating computer-analysable banding patterns/protein profiles (Van Vuuren & Van der Meer, 1987; Degré *et al.*, 1989; Van der Westhuizen & Pretorius, 1992);
- restriction enzyme analysis of total, ribosomal (rDNA) or mitochondrial DNA (mtDNA) for the detection of *r*estriction *f*ragment *l*ength *p*olymorphisms (RFLPs) (Querol *et al.*, 1992a; Van der Westhuizen & Pretorius, 1992; Vézinhet *et al.*, 1992; Versavaud *et al.*, 1995);
- pulse-field electrophoretic karyotype (CHEF-DNA) analysis for the detection of *c*hromosome *l*ength *p*olymorphisms (CLPs; Fig. 2) (Johnston & Mortimer, 1986; Degré *et al.*, 1989; Yamamoto *et al.*, 1991; Bidenne *et al.*, 1992; Van der Westhuizen & Pretorius, 1992; Naumov, Naumova & Gaillardin, 1993; Grando & Calato, 1994; Kishimoto, Soma & Goto, 1994; Lavallée *et al.*, 1994; Khan *et al.*, 2000; Van der Weshuizen *et al.*, 1999, 2000a, b);
- *r*andom *a*mplified *p*olymorphic DNA (RAPD) generated by the *p*olymerase *c*hain *r*eaction (PCR) technique (Huffman, Molina & Jong, 1992; Ness *et al.*, 1993; Lalvallée *et al.*, 1994; De Barros Lopes *et al.*, 1996; Quesada & Cenis, 1995);
- *a*mplified *f*ragment *l*ength *p*olymorphisms (AFLP) generated by selective PCR amplification of restriction fragments from a total digest of genomic DNA (De Barros Lopes *et al.*, 1999).

The DNA fingerprinting techniques (RFLP, CLP, RAPD and AFLP) have been used with great success in biogeographical surveys of yeast strains isolated from grapes and musts, as well as in wine yeast breeding programmes (Vézinhet, Blondin & Hallet, 1990; Yamamoto *et al.*, 1991; Frezier & Dubourdieu, 1992; Van der Westhuizen & Pretorius, 1992; Vézinhet *et al.*, 1992; Naumov *et al.*, 1993; Schütz & Gafner, 1993, 1994; Querol *et al.*, 1994; Versavaud *et al.*, 1995; De Barros Lopes *et al.*, 1996; Querol & Ramón, 1996; Constanti *et al.*, 1997; Cavalieri *et al.*, 1998; De Barros Lopes *et al.*, 1999; Van der Westhuizen *et al.*, 1999, 2000a, b; Khan *et al.*, 2000). Although such molecular identification approaches for wine yeast strains are considered to be more objective, sensitive and reproducible than traditional morphological and biochemical tests, they are still not rapid enough to satisfy the needs of modern wine production operations (Walker, 1998). Therefore, to ensure strain authenticity, security and proper strain management, it is of cardinal importance to continue to develop reliable and more rapid fingerprinting techniques.

Importance of yeast biodiversity to the wine industry:

Until quite recently, winemakers in *old world* wine-producing countries relied on spontaneous fermentations because of the long-held belief that superior yeast strains associated with specific vineyards, gave a distinctive quality to wine (Martini, 1993). More recent work has indicated that the contribution of resident winery yeasts to wine aroma is far superior to that of the indigenous microflora present on the grapes (Rosini, 1984; Constanti *et al.*, 1997). This view is not shared by all. Whereas Török *et al.* (1996) dismiss the importance of resident winery yeasts, Heard (1999) notes that it is now recognized that yeasts from both the vineyard and winery environment are important in the fermentation of grape must. On the other hand, a better understanding of wine aroma composition has led to the notion that wine flavour is more closely linked to the accumulation of secondary metabolites by the grape (Abbott, Williams & Coombe, 1993 as quoted by Henschke, 1997). While the adage *"the best wines are made in the vineyard"* is undoubtedly true, it is also true that different yeasts contribute differently to the aroma and quality of the final product. It is therefore not surprizing that there is an ever-increasing quest for new and improved wine yeast strains (for reviews see Pretorius & Van der Westhuizen, 1991; Barre *et al.*, 1993; Henschke, 1997; Pretorius, 1999, 2000).

Against this background, a comprehensive, long-term research programme has been launched by several microbiologists from the Wine and Fermentation Technology Division at the ARC-Fruit, Vine and Wine Research Institute, Nietvoorbij Centre for Vine and Wine, and the Institute for Wine Biotechnology at the University of Stellenbosch. The objectives of this research programme include the following:

(i) the systematic cataloging (isolation and characterization) of yeasts occurring in the wine-producing regions of the Western Cape of South Africa and the preservation of the natural yeast biodiversity;

(ii) a survey of the geographic distribution of the various yeast species and strains associated with the Cape's vineyards falling into different climatic zones;

TABLE 5

Diagnosis of the genus *Saccharomyces* Meyen ex Reess (adapted from Vaughan-Martini & Martini, 1998).

Vegetative reproduction is by multilateral budding.
Cells are globose, ellipsoidal or cylindroidal.
Pseudohyphae may be formed but not septate hyphae.
The vegetative phase is predominantly diploid (or of higher ploidy), conjugation occurs on or soon after germination of the ascospores; diploid ascospores may be formed.
Ascospores are globose to short ellipsoidal, with a smooth wall and usually one to four per ascus. Asci are persistent.
Vigorous fermentation of sugars.
Starch-like compounds are not produced.
Absence of growth with nitrate as a sole source of nitrogen.
Diazonium blue B reaction is negative.

TABLE 6

Key characteristics of species of the genus *Saccharomyces* (adapted from Vaughan & Martini, 1998).

Species	Fermentation			Assimilation									Growth				Fructose transport system
				Carbon source						Nitrogen source							
	Sucrose	Raffinose	Trehalose	Sucrose	Maltose	Raffinose	D-Ribose	Ethanol	D-Mannitol	Cadaverine 2HCl	Ethylamine HCl	L-Lysine	Cycloheximide 1000[a]	30°C	37°C	Vitamin-free medium	
S. barnettii	+	+	s	+	-	+	-	-	-	-	-	-	-	-	-	-	n
S. bayanus	+	+	-	+	+	+	-	+	v	-	-	-	-	+	-	+	+
S. castellii	-	-	-	-	-	-	+/v	-	-	-	-	-	-	+	v	-	n
S. cerevisiae	+	+	-	+	+	+	-	+	-	-	-	-	-	+	v	-	-
S. dairenensis	-	-	-	-	-	-	v	v	-	-	-	-	-	+	v	-	n
S. exiguus	+	s	+	+	-	+	-	s	-	-	-	-	v	+	-	-	n
S. kluyveri	+	+	-	+	+	+	-	+	+	+	+	+	-	+	+	-	n
S. paradoxus	+	+	-	+	+	+	-	+	+	-	-	-	-	+	+	-	-
S. pastorianus	v	+	-	+	+	+	-	+	-	-	-	-	-	+	-	-	+
S. rosinii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	n
S. servazzii	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	n
S. spencerorum	+	-	+	+	-	-	-	-	-	+	+	+	-	+	+	-	n
S. transvaalensis	-	-	-	-	-	-	-	v	-	v	-	v	-	+	+	-	n
S. unisporus	-	-	-	-	-	-	-	+	-	+	+	+	+	+	v	-	n

[a] Indicates resistance to 1000 ppm cycloheximide in the medium.

n, not determined / no data; +, positive; -, negative; v, variable; s, positive but slow.

TABLE 7

Classification and reclassification of *Saccharomyces* species* between 1952 and 1998 as depicted in major taxonomic reference works during this period.

1952 classification (Lodder & Kreger-van Rij, 1952)	1970 classification (Lodder, 1970)	1984 classification (Kreger-van Rij, 1984)	1998 classification (Kurtzman & Fell, 1998a)
S. bayanus	***S. bayanus*** (syn. ***S. beticus***, *S. cheriensis* ***S. oviformis***, *S. pastorianus*)	***S. cerevisiae***	***S. bayanus***
S. oviformis			*S. pastorianus*
S. pastorianus			***S. cerevisiae***
			S. paradoxus
S. uvarum	*S. uvarum*		
S. carlsbergensis			
S. logos			
S. cerevisiae (syn. ***S. vini***)	***S. cerevisiae***		
S.c. var. ***ellipsoideus***			
S. willianus			
S. chevalieri	***S. chevalieri***		
S. fructuum			
S. italicus	*S. italicus*		
S. steineri			
S. heterogenicus	*S. heterogenicus*		
	S. aceti		
	S. capensis		
	S. coreanus		
	S. diastaticus		
	S. globosus		
	S. hienipiensis		
	S. inusitatus		
	S. norbensis		
	S. oleaceus		
	S. oleaginosus		
	S. prostoserdovii		
S. exiguus	*S. exiguus*	*S. exiguus*	*S. barnettii*
			S. exiguus
			S. spencerorum
S. bailii	*S. bailii*	*Zygosaccharomyces bailii*	*Z. bailii*
S. acidifaciens			
S. elegans			
S. bisporus	*S. bisporus* var. *bisporus*	*Zygosaccharomyces bisporus*	*Z. bisporus*
S. mellis	*S. bisporus* var. *mellis*	*Zygosaccharomyces rouxii*	*Z. rouxii*
S. rouxii	*S. rouxii*		
S. rouxii var. *polymorphus*	*S. bailii* var. *osmophilus*		
	S. inconspicuus		
S. delbrueckii	*S. delbrueckii*		
S. fermentati (syn. ***S. beticus***)	***S. fermentati***		
S. rosei	***S. rosei***	***Torulaspora delbrueckii***	***T. delbrueckii***
	S. saitoanus		
	S. vafer		
	S. microellipsodes var. *osmophilus*		
S. marxianus	*Kluyveromyces marxianus*	*K. marxianus*	*K. marxianus*
S. fragilis	*Kluyveromyces fragilis*		*K. lactis*
S. lactis	*Kluyveromyces lactis*		
S. veronae	*Kluyveromyces veronae*	*K. thermotolerans*	*K. thermotolerans*
	S. amurcae	*Zygosaccharomyces cidri*	*Z. cidri*
	S. cidri		
S. microellipsodes	*S. microellipsodes*	*Zygosaccharomyces microellipsoides*	*Z. microellipsoides*
		S. servazzii	*S. servazzii*
S. pastori	*Pichia pastoris*	*Pichia pastoris*	*Pichia pastoris*
	S. dairenensis	*S. dairenensis*	*S. castellii*
			S. dairenensis
			S. rosinii
S. florentinus	*S. florentinus*	*Zygosaccharomyces florentinus*	*Z. florentinus*
	S. eupagycus		
	S. unisporus	*S. unisporus*	*S. unisporis*
	S. kluyveri	*S. kluyveri*	*S. kluyveri*
	S. telluris	*S. telluris*	*Arxiozyma telluris*
	S. kloeckerianus	*Torulaspora globosa*	*T. globosa*
	S. montanus	*Zygosaccharomyces fermentati*	*Z. fermentati*
	S. mrakii	*Zygosaccharomyces mrakii*	*Z. mrakii*
	S. transvaalensis	*Pachytichospora transvaalensis*	*S. transvaalensis*
	S. pretoriensis	*Torulaspora pretoriensis*	*T. pretoriensis*

*Author citations to the species are to be found in Lodder & Kreger-van Rij (1952), Lodder (1970), Kreger-van Rij (1984) and Kurtzman & Fell (1998a). (Meaning of abbreviations: syn. = synonym; var. = variety).

**Saccharomyces sensu stricto* have been separated into four species: *S. bayanus, S. cerevisiae, S. paradoxus* and *S. pastorianus.*