The Role and Use of Non-*Saccharomyces* Yeasts in Wine Production

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The contribution by the numerous grape-must-associated non-*Saccharomyces* yeasts to wine fermentation has been debated extensively. These yeasts, naturally present in all wine fermentations, are metabolically active and their metabolites can impact on wine quality. Although often seen as a source of microbial spoilage, there is substantial contrary evidence pointing to a positive contribution by these yeasts. The role of non-*Saccharomyces* yeasts in wine fermentation is therefore receiving increasing attention by wine microbiologists in Old and New World wine producing countries. Species that have been investigated for wine production thus far include those from the *Candida, Kloeckera, Hanseniaspora, Zygosaccharomyces, Schizosaccharomyces, Torulaspora, Brettanomyces, Saccharomycodes, Pichia* and *Williopsis* genera. In this review the use and role of non-*Saccharomyces* yeast in wine production is presented and research trends are discussed.

INTRODUCTION

Wine is the product of a complex biological and biochemical interaction between grapes (grape juice) and different microorganisms (fungi, yeasts, lactic acid bacteria and acetic acid bacteria) and the mycoviruses and bacteriophages affecting them (Fleet, 2003). The process starts in the vineyard, continues through fermentation and maturation, and concludes at packaging. It is affected by the various viticultural and oenological practices available to the grape-grower and winemaker, respectively (Regueiro *et al*., 1993). Of the microorganisms involved, it is the yeasts that play the most important role; they conduct the alcoholic fermentation (conversion of grape sugar to ethanol and CO_2). Furthermore, although wine flavour is directly determined by grape variety, yeasts also affect wine flavour and quality by the production and excretion of metabolites during growth and through autolysis (Fleet, 1993, 2003; Lambrechts & Pretorius, 2000; Swiegers & Pretorius, 2005; Swiegers *et al*., 2005). In some instances, yeasts can also act as spoilage organisms during wine production (including maturation) and after packaging (Loureiro & Malfeito-Ferreira, 2003). Yeasts present during fermentation are derived from grapes and the vineyard, the equipment used in the cellar, cellar surfaces and external sources such as selected cultures that are added to facilitate the fermentation process.

Since 1866, when Louis Pasteur first elucidated the bio-conversion of grape juice into wine, this complex process and the role of the yeast therein has been studied extensively. Yet, more than 130 years later, there are many areas that are still not well understood (Pretorius, 2000). This is especially the case for the roles of the numerous non-*Saccharomyces* yeasts normally associated with grape must and wine. These yeasts, naturally present in all wine fermentations to a greater or lesser extent, are metabolically active and their metabolites can impact on wine quality. While they were originally seen as a source of microbial related problems in wine production, winemakers, especially in Old World countries, saw indigenous yeasts as integral to the authenticity of their wines as these yeasts impart distinct regional and other desirable characteristics (Amerine *et al*., 1972; Jackson, 1994). Evidence supporting this view has been published (Fleet, 1990; Heard, 1999) and the role of the non-*Saccharomyces* yeasts in wine fermentation is receiving increasingly more attention by wine microbiologists in both Old and New World wine-producing countries.

YEAST CLASSIFICATION

Yeasts can be defined as unicellular fungi, either ascomycetous or basidiomycetous, that have vegetative states which predominantly reproduce by budding or fission and which do not form their sexual states within or on a fruiting body (Kurtzman & Fell, 1998a).

Current taxonomies recognise 100 genera comprising more than 700 species (Kurtzman & Fell, 1998b), of which approximately 20 are relevant to winemaking (Fleet, 1993). Yeast genera, with those non-*Saccharomyces* yeasts relevant to winemaking indicated in bold type, are listed in Table 1.

Rules for taxonomy of yeasts fall under the authority of the International Code of Botanical Nomenclature (Greuter *et al*., 1994). Publication of new species must include a description of essential characteristics, as well as a diagnosis that distinguishes

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the taxon from previously described species. Names of taxa must be given in Latin or modified in such a way that they follow the rules of Latin derivation, including appropriate designations.

The first level of yeast classification is based on the lack of a sexual phase during the life cycle (Deuteromycotina) or aspects of the sexual phase (Ascomycotina and Basidiomycotina). Further taxonomic subdivisions (orders, families, genera and species) are based on morphological, physiological, biochemical and genetic properties (Kreger-van Rij, 1984; Kurtzman & Fell, 1998b) that are elucidated by conducting 55 to 70 tests. Many of these tests can be used individually to characterise a selection of yeasts.

Some yeasts are found as a sexual (teleomorphic) type and produce ascospores. A similar form of the same yeast is the asexual (anamorphic) type that does not form ascospores. To complicate matters, the ability to form ascospores can be lost during long-term storage (Yarrow, 1998; M. Th. Smith, personal communication, 2000). Sporulation is also difficult to induce for some yeasts. Whether a newly isolated yeast is subsequently identified as teleomorphic or anamorphic can therefore largely depend on the time lapsed between isolation and identification as well as adherence to methodology. This can lead to confusion when authors report on isolates as essentially the same yeast species, but refer to them by different names. If any uncertainty exists in determining sporulation it is therefore preferable to use the anamorphic name where applicable. Some of the more commonly encountered anamorphic yeasts and their teleomorphic counterparts in must and wine are given in Table 2.

TABLE 1

A list of yeast genera[1] according to Kurtzman & Fell (1998b).

Teleomorphic ascomycetous genera (Ascomycotina)	**Anamorphic ascomycetous genera (Deuteromycotina)**	**Teleomorphic heterobasidio-mycetous genera (Basidiomycotina)**	**Anamorphic heterobasidio-mycetous 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			

[1] Non-Saccharomyces genera that can be encountered in vineyards, on winery surfaces, in grape musts and/or in wine are indicated in bold type.

TABLE 2

Anamorphs, teleomorphs and synonyms of some of the non-*Saccharomyces* yeasts in the Ascomycetous genera encountered in wine fermentations (Kurtzman & Fell, 1998b).

Anamorphic form	**Teleomorphic form**	**Synonyms[1]**
Brettanomyces bruxellensis	*Dekkera bruxellensis*	
Candida colliculosa	*Torulaspora delbrueckii*	*Saccharomyces rosei*
Candida famata	*Debaryomyces hansenii*	
Candida globosa	*Citeromyces matritensis*	
Candida guilliermondii	*Pichia guilliermondii*	
Candida hellenica	*Zygoascus hellenicus*	
Candida lambica	*Pichia fermentans*	
Candida pelliculosa	*Pichia anomala*	*Hansenula anomala*
Candida pulcherrima	*Metschnikowia pulcherrima*	*Torulopsis pulcherrima*
Candida reukaufii	*Metschnikowia reukaufii*	
Candida sorbosa	*Issatchenkia occidentalis*	
Candida stellata	-[2]	*Torulopsis stellata*
Candida valida	*Pichia membranifaciens*	
Kloeckera africana	*Hanseniaspora vineae*	
Kloeckera apiculata	*Hanseniaspora uvarum*	
Kloeckera apis	*Hanseniaspora guilliermondii*	
Kloeckera corticis	*Hanseniaspora osmophila*	
Kloeckera javanica	*Hanseniaspora occidentalis*	
-[3]	*Issatchenkia terricola*	*Pichia terricola*
-[3]	*Kluyveromyces thermotolerans*	
-[3]	*Saccharomyces kluyveri*	
-[3]	*Saccharomycodes ludwigii*	
-[3]	*Zygosaccharomyces bailii*	*Saccharomyces bailii*
-[3]	*Pichia farinosa*	

[1] Names sometimes found in older literature.
[2] No teleomorphic form.
[3] No anamorphic form.

ECOLOGY OF YEASTS

Yeasts are found throughout nature. However, they do not occur randomly, but are found in specific habitats where different species form communities (Lachance & Starmer, 1998). The different species found in a habitat can either be autochothonous (those that are essential components of the community) or allochothonous (those that are transient, or there by chance). The component species within yeast communities are further defined by niches, i.e. the physical, chemical and biotic attributes required by the yeast to survive and grow (Lachance & Starmer, 1998).

Yeasts found in many different habitats are considered generalists (broad niche), while those found in unique habitats are considered specialists (narrow niche) (Lachance & Starmer, 1998). Within the winemaking environment (habitat), the vineyard (grape surfaces) and cellar (equipment surfaces and must) can be considered specialised niches where the wine related yeasts can form communities (Polsinelli *et al*., 1996). These niches differ broadly. The surface of the grape berry before ripeness presents limitations regarding nutrients. These are alleviated as berries ripen and/or are damaged. External factors such as fungicides and pesticides used in the vineyard will have a negative impact on populations. However, it has been reported that some pesticides can stimulate certain yeasts, e.g. *K. apiculata*, when tested in laboratory fermentations (Cabras *et al.*, 1999).

Grape must is a rich nutritive environment, but low pH and high osmotic pressure of the must and the use of SO_2 detracts from this otherwise ideal yeast niche. Surfaces of cellar equipment can also harbour numerous microorganisms due to constant contact with grape must. Cellar hygiene consequently plays a big role in this niche.

NON-*SACCHAROMYCES* YEASTS ASSOCIATED WITH GRAPES, FERMENTING MUST AND WINE

The yeast species found in different niches associated with grape growth (vineyards) and wine production (wineries, grape must, fermentation and wine) can be arbitrarily divided into two groups, i.e. the *Saccharomyces* group and the non-*Saccharomyces* group.

The *Saccharomyces* group with its primary representative, *Saccharomyces cerevisiae*, is present on grape skins in low numbers (Van Zyl & Du Plessis, 1961; Rankine, 1972; Török *et al*., 1996), and on winery equipment and in fermenting must in greater numbers (Peynaud & Domercq, 1959; Vaughan-Martini & Martini, 1995). *S. cerevisiae* is the most important yeast for wine production and is responsible for the metabolism of grape sugar to alcohol and CO_2 (Reed & Peppler, 1973; Fleet, 1993; Pretorius *et al*., 1999; Pretorius, 2003; Swiegers & Pretorius, 2005; Swiegers *et al*., 2005). It has an equally important role to play in the formation of secondary metabolites of importance to wine (Fleet, 1993; Pretorius, 2003), as well as in the conversion of grape aroma precursors to varietal aromas in wine (Darriet *et al*., 1995; Dubourdieu, 1996; Ribéreau-Gayon *et al*., 2000; Howell *et al*., 2004). For these reasons *S. cerevisiae* is often simply referred to as "the wine yeast". The knowledge pertaining to *S. cerevisiae* during wine fermentation can often be applied to non-*Saccharomyces* yeasts under the same conditions and in the same environment. In addition, as most fields of research are focussed primarily on *S. cerevisiae*, non-*Saccharomyces* research can be-

nefit from the techniques developed by the *S. cerevisiae* researchers.

The non-*Saccharomyces* yeasts contain numerous species, dominated numerically by the apiculate yeasts, e.g. *Kloeckera* spp. and *Candida* spp. that are found predominantly on grapes and in freshly processed must. Lesser numbers are found on winery equipment.

The microflora of grapes is affected by a number of factors. These include vineyard altitude and aspect, climatic conditions (temperature, rainfall, humidity, maritime influences), grape variety (cultivar, thickness of grape skin), viticultural practices (fertilisation, irrigation, canopy management, use of fungicides, use of elemental sulphur), developmental stage of grapes, health of grapes (physical damage to berries, insect pests) and winery waste-disposal practices (Bisson & Kunkee, 1991; Regueiro *et al*., 1993; Boulton *et al*., 1996; Epifanio *et al*., 1999; Pretorius, 2000). The manner in which grapes are sampled (e.g. the berries or bunches) and processed (washing vs. crushing) can also determine what yeasts are isolated (Martini *et al*., 1980; 1996), as the number of yeast cells is greater close to the peduncle than it is at the centre and lower part of the bunch (Rosini *et al*., 1982).

At harvest, grape temperature, method of harvest (manual vs. mechanical), method of transport to the cellar (picking crates/baskets, tipsters), time of transport to the cellar, time lapse before crushing, and sulphite and enzyme addition can all affect yeast populations (Pretorius *et al*., 1999; Pretorius, 2000). Yeasts found on the surface of grapes are introduced into the must at crushing (Bisson & Kunkee, 1991; Lonvaud-Funel, 1996). Other strains found on the surface of cellar equipment can also be transferred to the must (Boulton *et al*., 1996). Populations are further affected by the method of crushing, i.e. pressing whole bunches vs. berries, sulphite addition, enzyme addition, cellar hygiene, type of equipment used, clarification method and temperature control (Regueiro *et al*., 1993; Epifanio *et al*., 1999; Pretorius *et al*., 1999; Pretorius, 2000).

The specific environmental conditions in the must, i.e. high osmotic pressure, presence of SO_2, and temperature, all play a role in determining what species can survive and grow (Bisson & Kunkee, 1991; Longo *et al*., 1991). The fermentation rapidly becomes anaerobic and the alcohol levels increase, which further affects yeast populations.

Despite all the variables in grape harvest and wine production, the yeast species generally found on grapes and in wines are similar throughout the world (Amerine *et al*., 1967). However, the proportion (or population profile) of yeasts in the different regions shows distinct differences (Amerine *et al*., 1967; Longo *et al*., 1991).

In areas with high rainfall during harvest the numbers of non-*Saccharomyces* yeasts increase (Querol *et al*., 1990). Pesticides and other chemical sprays used in the vineyard can also affect yeast populations (Monteil *et al*., 1987; Cabras *et al*., 1999; Guerra *et al*., 1999).

The range of non-*Saccharomyces* species isolated often depends on the place from which, and the stage in the winemaking process at which, the samples are taken (see Tables 3 & 4). The methods of isolation and enumeration can also impact on the type of yeasts that are isolated, as evident from Table 3. Such methods include shaking grape berries in a broth or crushing whole berries and plating on nutrient agar media. The technique of crushing berries before plating is closer to practical winemaking protocols than is shaking in a broth, but the objective of the investigation will determine which method is chosen.

The type of growth medium used can also play an important role by limiting the growth of specific yeasts. The use of Lysine Medium, for example, does not allow the growth of *S. cerevisiae* due to the inability of this yeast to utilise lysine as the sole carbon source (Fowell, 1965; Heard & Fleet, 1986). However, some non-*Saccharomyces* yeasts might also not be able to utilise lysine and, therefore, will not be detected. On a general nutrient agar medium, fast-growing yeasts can also overgrow slow growers. For yeasts with similar growth rates, only yeasts present in the same numerical order will be detected, and more specific techniques and media are needed to isolate slower growing yeasts or yeasts found in low numbers.

The aforementioned limitations can be overcome to a degree by using culture-independent techniques. These include the use of epi-fluorescence microscopy (Du Toit *et al*., 2005), PCR (polymerase chain reaction) based DGGE (denaturing gradient gel electrophoreses) (Cocolin *et al*., 2000; Prakitchaiwattana *et al*., 2004) and FT-IR (Fourier-transform infrared) spectroscopy (Wenning *et al*., 2002).

Non-Saccharomyces *yeasts in vineyards and on grapes*

Low numbers of yeasts (10-10^3 cfu/g) are found on unripe grapes, but as the grapes ripen the numbers increase to 10^4-10^6 cfu/g (Fleet, 2003). This is due to sugars that leach or diffuse out from inner tissue to the grape skin surfaces, providing nutrition for the yeasts. Damaged berries increase the leaching effect. Therefore, the maturity of the grapes and/or the degree of damage to grape berries will largely determine the population numbers.

Generally, between nine and 15 culturable yeast species are found on grapes (Du Plessis, 1959; Van Zyl & Du Plessis, 1961; Parish & Caroll, 1985; Yanagida *et al*., 1992; Regueiro *et al*., 1993; Zahavi *et al*., 2002; Jolly *et al*., 2003a; Rementeria *et al*., 2003). These include *Hanseniaspora/Kloeckera* spp., *Metschnikowia/Candida* spp., *Rhodotorula* spp. and *Cryptococcus* spp. Unfortunately, comparisons between different studies are difficult, as different approaches have been used for grape sampling and yeast isolation (see Table 3). In addition, the state of ripeness and berry damage is never given. Further factors that can influence a yeast population include specific meso- and microclimates in vineyards. Notwithstanding, there is general agreement that the most frequently occurring species in vineyards are usually the apiculate yeasts, *K. apiculata/Hanseniaspora uvarum* (50-75% of isolates) (Van Zyl & Du Plessis, 1961; Yanagida *et al*., 1992; and a recent review by Pretorius, 2000). It has been reported that in warm to hot regions the teleomorphic form (*H. uvarum*) tends to replace the anamorphic form (*K. apiculata*), while the anamorphic form is present in greater numbers in cooler regions (Bisson & Kunkee, 1991; Jackson, 1994; Boulton *et al*., 1996). In moderate climates both types occur in equal numbers. However, this distribution between the teleomorphic and anamorphic forms might be region dependent. Altitude also appears to play a role as it has been reported that *Kloeckera* spp. are found more frequently at high altitudes and *Hansenia-*

spora spp. more frequently at low altitudes. This might be linked to temperature. Identification of *Kloeckera* vs. *Hanseniaspora* yeasts also depends on how long the yeasts have been conserved before identification (Yarrow, 1998; M. Th. Smith, personal communication, 2000).

According to Van Zyl & Du Plessis (1961), the following yeasts occurred in highest frequency in South African vineyards: *K. apiculata, Rhodotorula glutinis, Candida krusei, Candida pulcherrima, Candida laurentii, Cryptococcus albidus*, and *Candida stellata* (*T. bacillaris*). In a more recent, but limited, investigation (Jolly *et al*., 2003a), *K. apiculata, C. pulcherrima* and a *Rhodotorula* sp. were still found in dominant numbers but *Kluyveromyces thermotolerans* and *Zygosaccharomyces bailii* were also isolated. Studies by Le Roux *et al*. (1973) showed that *Botrytis cinerea* infection of grapes influenced the non-*Saccharomyces* populations – *C. krusei* and *K. apiculata* increased and *R. glutinis* decreased. Other non-*Saccharomyces* yeasts found on grapes and in vineyards are shown in Table 3.

Non-Saccharomyces *yeasts associated with fermenting must*

During crushing, the non-*Saccharomyces* yeasts on the grapes, on cellar equipment and in the cellar environment (air- and insect-borne) are carried to the must (Peynaud & Domercq, 1959; Bisson & Kunkee, 1991; Boulton *et al*., 1996; Lonvaud-Funel, 1996; Török *et al*., 1996; Constantí *et al*., 1997; Mortimer & Polsinelli, 1999; Fleet, 2003). Non-*Saccharomyces* species that have been isolated from cellar surfaces include *Pichia anomala, Pichia membranifaciens, Candida* spp., *Cryptococcus* spp. and, more rarely, *Rhodotorula* spp., *Debaryomyces hansenii, K. apiculata* and *Metschnikowia pulcherrima* (Loureiro & Malfeito-Ferreira, 2003). However, cellar surfaces play a smaller role than grapes as a source of non-*Saccharomyces* yeasts, as *S. cerevisiae* is the predominant yeast inhabiting such surfaces (Peynaud & Domercq, 1959; Rosini, 1984; Lonvaud-Funel, 1996; Pretorius, 2000). Furthermore, hygienic procedures used in most modern cellars should minimise contamination of must by resident cellar flora (Jackson, 1994; Pretorius, 2000). It might therefore be expected that the dominant yeasts in must after crushing will be the same as are found on grapes (Rementeria *et al*., 2003).

The specific environmental conditions in grape must are limiting and hostile to yeasts due to low pH, high sugar (high osmotic pressure), an equimolar mixture of glucose and fructose, presence of SO_2 and a non-optimal growth temperature during cold settling (Bisson & Kunkee, 1991; Longo *et al*., 1991; Pretorius, 2000). Furthermore, the environment rapidly becomes anaerobic, with increasing levels of ethanol that is toxic to yeasts. Nitrogen levels are usually sufficient at the start of fermentation (Bisson & Kunkee, 1991), but can be limiting towards the end of fermentation unless supplemented. The clarification of white must (centrifugation, enzyme treatments, cold settling) can also reduce the initial population of yeasts (Fleet, 1990; Lonvaud-Funel, 1996; Pretorius, 2000).

Non-*Saccharomyces* yeasts found in grape must and during fermentation (see Table 4) can be divided into three groups:

(i) yeasts that are largely aerobic, e.g. *Pichia* spp., *Debaryomyces* sp., *Rhodotorula* spp., *Candida* spp. (e.g. *C. pulcherrima and C. stellata*), and *Cryptococcus albidus*;

(ii) apiculate yeasts with low fermentative activity, e.g. *K. apiculata* (*H. uvarum), Kloeckera apis, Kloeckera javanica;* and

(iii) yeasts with fermentative metabolism, e.g. *Kluyveromyces marxianus, Torulaspora* spp. (e.g. T. *globosa* and *T. delbrueckii*) and *Zygosaccharomyces* spp. (Fleet *et al*., 1984; Querol *et al*., 1990; Bisson & Kunkee, 1991; Longo *et al*., 1991; Lonvaud-Funel, 1996; Lorenzini, 1999; Torija *et al*., 2001; Combina *et al*., 2005).

During fermentation, and especially in spontaneous fermentations, there is a sequential succession of yeasts. Initially, species of *Kloeckera* (*Hanseniaspora*), *Rhodotorula, Pichia, Candida* (*C. stellata, C. pulcherrima* [*M. pulcherrima*], *Candida sake*) and *Cryptococcus* are found at low levels in the fresh must (Parish & Caroll, 1985; Bisson & Kunkee, 1991; Frezier & Dubourdieu, 1992; Jackson, 1994; Granchi *et al*., 1998; Fleet, 2003; Combina *et al*., 2005). Of these, *K. apiculata* is usually present in the highest numbers, followed by various *Candida* spp.

In a study of South African musts, however, very few *K. apiculata* yeasts were found (Van Zyl & Du Plessis, 1961). This was attributed to the addition of large quantities of SO_2 to the must to aid settling. In another study on muscadine (*Vitis rotundifolia*) grapes from North Carolina *H. uvarum* (*K. apiculata*) was absent, but *Hanseniaspora osmophilia* and *P. membranifaciens* predominated during the initial stages of the fermentation (Parish & Caroll, 1985). This might be an association for that particular geographic area or grape type. However, viticultural practices could have affected the normal non-*Saccharomyces* population. At the start of fermentation an initial proliferation of apiculate yeasts (*Kloeckera* and *Hanseniaspora*) normally occurs. This is usually more apparent in red must than in white, possibly due to the higher pH of the former.

In the past it was generally believed that all non-*Saccharomyces* yeasts died soon after the commencement of an alcoholic fermentation due to the increasing ethanol concentration and added SO_2. However, more recent work has shown that some non-*Saccharomyces* yeasts can survive to a later stage of fermentation (up to 12 days) than initially believed (Fleet *et al*., 1984; Heard & Fleet, 1985; Fleet, 1990; Longo *et al*., 1991; Todd, 1995; Gafner *et al*., 1996; Granchi *et al*., 1998; Zohre & Erten, 2002; Fleet, 2003; Combina *et al*., 2005). Other non-*Saccharomyces* yeasts might be present throughout the fermentation, reaching cell densities of 10^6 to 10^8 cells/mL (Fleet *et al*., 1984; Combina *et al*., 2005). This sustained growth of non-*Saccharomyces* spp. is more evident in spontaneous fermentations, which lack the initial high density inoculum of *S. cerevisiae*. Abnormal vintages due, for example, to excessive rainfall during grape ripening, also contribute to greater numbers of non-*Saccharomyces* yeasts in the initial stages and later in the fermentation (Querol *et al*., 1990). Non-*Saccharomyces* yeasts have also been observed to grow to levels of *ca*. 10^4 cells/mL in red wines during malo-lactic fermentations (Fleet *et al*., 1984).

Despite the sustained presence of certain non-*Saccharomyces* yeasts, the majority disappear during the early stages of a vigorous fermentation (Fleet *et al*., 1984; Jackson, 1994; Henick-Kling *et al*., 1998). This might be due to their slow growth and inhibition by the combined effects of SO_2, low pH, high ethanol content and oxygen deficiency (Jackson, 1994; Combina *et al*., 2005).

TABLE 3

Non-*Saccharomyces* yeasts isolated from grapes.

Yeast species Anamorph / Teleomorph [Synonym][1]	Form isolated A/T/S[2]	Region or country	Isolation material	Brief description of method of isolation	Reference
Candida albicans		North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
Candida edax / Stephanoascus smithiae	A	North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
Candida famata / Debaryomyces hansenii	A	Israel	Muscat d' Alexandrie	Twenty to fifty berries shaken in sterile distilled water and plated onto basal yeast agar	Zahavi *et al*., 2002
	A	Israel	Cabernet Sauvignon	Twenty to fifty berries shaken in sterile distilled water and plated onto basal yeast agar	Zahavi *et al*., 2002
Candida glabrata [Torulopsis glabrata]	S	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Candida globosa / Citeromyces matritensis	T	Israel	Cabernet Sauvignon, Colombard	Twenty to fifty berries shaken in sterile distilled water and plated onto basal yeast agar	Zahavi *et al*., 2002
Candida guilliermondii / Pichia guilliermondii or Pichia ohmeri	A	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
	A	Israel	Cabernet Sauvignon	Twenty to fifty berries shaken in sterile distilled water and plated onto basal yeast agar	Zahavi *et al*., 2002
Candida inconspicua [Torulopsis inconspicua]	S	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Candida krusei / Issatchenkia orientalis	A	Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium	Van Zyl & Du Plessis, 1961
Candida lambica / Pichia fermentans	T	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Candida melinii / Pichia canadensis	A	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Candida pulcherrima / Metschnikowia pulcherrima [Torulopsis pulcherrima]	A	Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium	Van Zyl & Du Plessis, 1961
	A	Israel	Cabernet Sauvignon	Twenty to fifty berries shaken in sterile distilled water and plated onto basal yeast agar	Zahavi *et al*., 2002
Candida reukaufi / Metschnikowia reukaufi	T	Israel	Muscat d' Alexandrie	Twenty to fifty berries shaken in sterile distilled water and plated onto basal yeast agar	Zahavi *et al*., 2002
Candida sake		North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
Candida stellata [Torulopsis bacillaris]	S	Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium	Van Zyl & Du Plessis, 1961
	S	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Candida valida / Pichia membranifaciens	T	North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
Candida vini [Candida mycoderma]	S	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Candida zeylanoides		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Van Zyl & Du Plessis, 1961
Cryptococcus albidus [Cryptococcus diffluens]		Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium	Van Zyl & Du Plessis, 1961
		Japan	Zenkoji and Koshu	Berries crushed; dilutions plated	Yanagida *et al*., 1992
		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
	S	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973

TABLE 3 (continued)

Non-*Saccharomyces* yeasts isolated from grapes.

Yeast species Anamorph / Teleomorph [Synonym][1]	Form isolated A/T/S[2]	Region or country	Isolation material	Brief description of method of isolation	Reference
Cryptococcus humicolus *[Candida humicola]*		Spain	Traditional grape varieties	Ten berries washed in saline and plated on Sabouraud dextrose agar	Rementeria *et al*., 2003
	S	North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
Cryptococcus laurentii		Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium	Van Zyl & Du Plessis, 1961
		Japan	Chardonnay, Zenkoji and Koshu	Berries crushed; dilutions plated	Yanagida *et al*., 1992
		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Cryptococcus neoformans / *Filobasidiella neoformans*	A	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Kloeckera apiculata / *Hanseniaspora uvarum*	A	Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium	Van Zyl & Du Plessis, 1961
	A	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
	A	Japan	Niagra, Zenkoji and Koshu	Berries crushed; dilutions plated	Yanagida *et al*., 1992
	T	California	Vineyard	Fermentations at 13°C and 18°C; dilutions plated on WL medium	Pallmann *et al*., 2001
Kloeckera corticis / *Hanseniaspora osmophila* *[K. magna]*	T	North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
Kloeckera javanica / *Hanseniaspora occidentalis*	T	Japan	Niagra	Berries crushed; dilutions plated	Yanagida *et al*., 1992
Lodderomyces elongisporus		North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
Rhodotorula aurantiaca		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Rhodotorula glutinus		North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
		Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium	Van Zyl & Du Plessis, 1961
		Japan	Chardonnay and Zenkoji	Berries crushed; dilutions plated	Yanagida *et al*., 1992
		Spain	Traditional grape varieties	Ten berries washed in saline and plated on Sabouraud dextrose agar	Rementeria *et al*., 2003
		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973
Rhodotorula minuta		North Carolina	Muscadine *(Vitis rotundifolia)*	Ten berries shaken in peptone buffer and plated on potato dextrose agar	Parish & Carroll, 1985
		Japan	Zenkoji	Berries crushed; dilutions plated	Yanagida *et al*., 1992
Rhodotorula mucilaginosa		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar	Le Roux *et al*., 1973

[1] Where applicable, the anamorph and teleomorph designations are given, and also the synonym or alternate name used in older literature. Yeast nomenclature according to Kurtzman & Fell (1998).

[2] A = anamorph; T = teleomorph; S = synonym.

This is consistent with their oxidative or weak fermentative metabolism. Nutrient limitation and size of *S. cerevisiae* inoculum would also have a suppressive effect. Granchi *et al*. (1998) reported that numbers of *K. apiculata* declined once *S. cerevisiae* became dominant rather than when the fermentation temperature and ethanol concentration reached values known to inhibit apiculate yeast growth. It has also been reported that *T. delbrueckii* and *K. thermotolerans* are less tolerant to low oxygen levels and it is this, rather than ethanol toxicity, that affects their growth and leads to their death during fermentation (Hansen *et al*., 2001). It was also shown that a cell-cell contact mechanism in the presence of high concentrations of viable *S. cerevisiae* yeasts played a role in the inhibition of these two non-*Saccharomyces* species (Nissen *et al*., 2003).

The non-*Saccharomyces* spp. that survive and are present until the end of fermentation may also have a higher tolerance to ethanol. It has been documented that *C. stellata* (*Torulopsis stellata*) can tolerate up to 12% ethanol (Combina *et al*., 2005), which would account for its sustained presence during fermentation. Other species reported throughout fermentation are *Z. bailii* (*Saccharomyces acidifaciens*) (Peynaud & Domercq, 1959) and *Pichia* sp. (Bisson & Kunkee, 1991).

The extent to which different factors affect the non-*Saccharomyces* yeasts are dependent on the characteristics of the individual species. Growth parameters for one species will not necessarily be the same for others. Variations can also occur for strains within a species.

Non-Saccharomyces *yeasts associated with wine*

Non-*Saccharomyces* yeasts in wine are usually associated with wines in barrels and post-fermentation spoilage (Van der Walt & Van Kerken, 1958; Amerine & Cruess, 1960; Van Zyl, 1962; Heresztyn, 1986; Grbin, 1999; and a recent review by Loureiro & Malfeito-Ferreira, 2003). However, only a small number are able to tolerate the adverse conditions in wine, and multiply (Van Kerken, 1963). These include *Brettanomyces* spp. (*Dekkera* spp.), *Z. bailii*, *P. membranifaciens*, *C. krusei* and *C. valida* (Van Kerken, 1963; Fleet *et al*., 1984; Parish & Caroll, 1985; Bisson & Kunkee, 1991; Grbin, 1999). Some of these species, e.g. *Brettanomyces* spp. and *Zygosaccharomyces* spp. are as ethanol tolerant as *S. cerevisiae* and may be found in bottled wine. Their presence is influenced by the degree of filtration that precedes bottling and cellar hygiene during bottling.

THE ROLE OF NON-*SACCHAROMYCES* YEASTS IN WINE PRODUCTION

The role of non-*Saccharomyces* yeasts in wine production has been debated extensively (Castor, 1954; Van Zyl *et al*., 1963; Fleet *et al*., 1984; Heard & Fleet, 1985; Fleet, 1990; Herraiz *et al*., 1990; Longo *et al*., 1991; Romano *et al*., 1992; Todd, 1995; Gafner *et al*., 1996; Gil *et al*., 1996; Lema *et al*., 1996; Granchi *et al*., 1998; Henick-Kling *et al*., 1998; Lambrechts & Pretorius, 2000; Fleet, 2003; Rementeria *et al*., 2003; Combina *et al*., 2005). As already discussed, grape musts contain a mixture of yeast species. Wine fermentation is therefore not a single-species fermentation (Fleet, 1990), although the dominance of *S. cerevisiae* (inoculated or indigenous) in the fermentation is expected and desired. However, the indigenous non-*Saccharomyces* yeasts, already present in the must, and often in greater numbers than *S. cerevisiae*, are adapted to the specific environment and are in an active growth state, giving them a competitive edge.

Despite a long-held belief among winemakers in Old World wine regions that spontaneous fermentations (comprising mixed cultures of non-*Saccharomyces* and *Saccharomyces* yeasts) produce superior wines compared with pure culture fermentations, earlier authors usually refer to the non-*Saccharomyces* yeasts as spoilage organisms or 'wild yeasts' (Amerine & Cruess, 1960; Van Zyl & Du Plessis, 1961; Van Kerken, 1963; Rankine, 1972; Le Roux *et al*., 1973). This was substantiated by their frequent isolation from stuck fermentations and from spoiled bottles of wine.

Furthermore, although it was known that some non-*Saccharomyces* yeasts could form metabolites, e.g. esters, leading to aromas not always detrimental to wine quality (Castor, 1954; Amerine & Cruess, 1960; Van Zyl *et al*., 1963), this was outweighed by the high levels of volatile acids and other undesirable compounds produced (Castor, 1954; Amerine & Cruess, 1960; Van Zyl *et al*., 1963; Amerine *et al*., 1967; 1972). Some yeasts, e.g. *Candida*, *Pichia* and *Hansenula* spp. are capable of forming films on the surface of wine exposed to oxygen. Off-odours, including acetic acid, ethyl acetate and acetaldehyde are also associated with their growth (Grbin, 1999). *Brettanomyces* spp. (*Dekkera* spp.) can contribute to 'animal/farmyard/mousy' taints in wines (Parish & Caroll, 1985; Grbin, 1999, Grbin & Henschke, 2000; Arvik & Henick-Kling, 2002; Du Toit *et al*., 2005). It has also been reported that *Brettanomyces bruxellensis* can form biogenic amines (Caruso *et al*., 2002) that can lead to undesirable physiological effects in sensitive humans. Other non-*Saccharomyces* yeasts such as *Saccharomycodes ludwigii*, more commonly a contaminant of sulphated musts due to its high resistance to SO_2, produce large amounts of ethyl acetate and acetaldehyde that negatively affect wine aroma and quality (Ciani & Maccarelli, 1998).

Authors of earlier publications also considered non-*Saccharomyces* yeasts to be sensitive to SO_2 in must and added SO_2 primarily to control their growth and that of spoilage bacteria (Amerine & Cruess, 1960; Van Zyl & Du Plessis, 1961; Amerine *et al*., 1972). Non-*Saccharomyces* yeasts were also known to be poor fermenters of grape must and intolerant to ethanol (Castor, 1954), especially in the presence of SO_2 (Amerine *et al*., 1972). It was therefore accepted that those non-*Saccharomyces* yeasts not initially inhibited by the SO_2 died during fermentation due to the combined toxicity of the SO_2 and alcohol. Consequently, the non-*Saccharomyces* yeasts were seen to be of little significance in normal wine production and it was recommended that only proven strains of the wine yeast *S. cerevisiae* be used in commercial fermentations (Amerine & Cruess, 1960; Amerine *et al*., 1972).

As already mentioned, non-*Saccharomyces* yeasts can survive and reach high cell densities, similar to *S. cerevisiae* (10^6 to 10^8 cells/mL), during fermentation. More recently reported higher numbers of non-*Saccharomyces* yeasts might be the result of improved cellar technology and hygiene in modern cellars that has led to a reduction in SO_2 usage, presumably resulting in the survival of a greater number and diversity of non-*Saccharomyces* yeasts. Coupled to this is the use of modern laboratory techniques that makes the detection of non-*Saccharomyces* yeasts easier.

TABLE 4

Non-*Saccharomyces* yeasts isolated from grape must.

Yeast species Anamorph / Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material (must variety)**	**Brief description of method of isolation**	**Reference**
Brettanomyces bruxellensis / Dekkera bruxellensis [Brettanomyces vini]	T	Tenerife	White Listan	Random harvesting; further details not given	De Cos *et al*., 1999
	A	Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
	T	Spain	Ribeiro	Dilution and plating on YPD	Cansado *et al*., 1989
Candida sp.		Spain	Ribeiro	Dilution and plating on YPD	Cansado *et al*., 1989
Candida colliculosa / Torulaspora delbrueckii [Torulaspora rosei] [Saccharomyces fermentati]	A	Australia	Hermitage (red)	Spread inoculation on malt extract and Lysine agar	Heard & Fleet, 1985
	A	Catalonia	Macabeo (white) and Grenache (red)	Dilution and plating on YPD	Torija *et al*., 2001
	T	Bordeaux	White variety	Not given	Peynaud & Domercq, 1959
	T	Italy	Variety not given	Not given	Castelli, 1955
	T	Tenerife	White Listan	Random harvesting; further details not given	De Cos *et al*., 1999
	S	Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al*., 1984
	T	Bordeaux	White variety	Not given	Peynaud & Domercq, 1959
Candida famata / Debaryomyces hansenii	T	Tenerife	White Listan	Random harvesting; further details not given	De Cos *et al*., 1999
	A	Israel	Muscat d' Alexandrie	Twenty to fifty berries shaken in sterile distilled water and plated on basal yeast agar	Zahavi *et al*., 2002
	T	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
	T	Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
Candida glabrata		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Candida glucosophila		Spain	White and red (traditional varieties)	Samples plated on Sabouraud dextrose agar	Rementeria *et al*., 2003
Candida guilliermondii / Pichia guilliermondii or Pichia ohmeri	A	Spain	Abarino, Godello(white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Candida hellenica / Zygoascus hellenicus [Candida steatolytica]	S	Majorca	Prensal blanc (white)	Dilutions plated onto YM and lysine agar	Mora & Mulet, 1991
Candida kefyr / Kluyveromyces marxianus	T	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Candida krusei / Issatchenkia orientalis	A	Bordeaux	Semillon	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
[Saccharomyces krusei]	S	Bordeaux	Merlot	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
	T	Argentina	Malbec	Plated on malt extract agar	Combina *et al*., 2005
Candida lambica / Pichia fermentans	T	Bordeaux	Red and white varieties	Not given	Peynaud & Domercq, 1959
Candida lusitaneae / Clavispora lusitaneae	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Candida pelliculosa / Pichia anomala [Hansenula anomala]	S	Australia	Hermitage (red)	Spread inoculation on malt extract and lysine agar	Heard & Fleet, 1985
	S	Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
	S	Majorca	Chenin blanc	Dilutions plated on YM and lysine agar	Mora & Mulet, 1991

TABLE 4 (continued)

Non-*Saccharomyces* yeasts isolated from grape must.

Yeast species Anamorph / Teleomorph [Synonym][1]	Form isolated A/T/S[2]	Country or region	Isolation material (must variety)	Brief description of method of isolation	Reference
Candida pulcherrima / Metschnikowia pulcherrima [Torulopsis pulcherrima]	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
	T	Catalonia	Macabeo (white) and Grenache (red)	Dilution and plating on YPD	Torija *et al*., 2001
	A	Australia	Riesling (white) and Malbec (red)	Spread inoculation on malt extract and lysine agar	Heard & Fleet, 1985
	A	Bordeaux	Red grape variety	Not given	Peynaud & Domercq, 1959
	T	Bordeaux	Semillon	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
	A	Majorca	Prensal blanc (white)	Dilutions plated on YM and lysine agar	Mora & Mulet. 1991
	T	Italy	Nebbiola (red)	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	T	Germany	Pinot noir (red)	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	S	Switzerland	Pinot noir (red)	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	T	Italy	Variety not given	Details not given	Castelli, 1955
	A	Argentina	Malbec	Plated on malt extract agar	Combina *et al*., 2005
Candida rugosa		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Candida stellata [Torulopsis stellata; Torulopsis bacillaris]		Australia	Riesling, Semillon (white), Malbec and Hermitage (red)	Spread inoculation on malt extract and lysine agar	Heard & Fleet, 1985
		Catalonia	Garnatxa	Dilutions plated on malt extract agar	Constantí *et al*., 1997
		Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al*., 1990
	S	Bordeaux	Merlot	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
	S	Bordeaux	Semillon	Dilutions plated on malt extract agar	Fleet *et al*., 1984
		Catalonia	Macabeo (white) & Grenache (red)	Dilution and plating on YPD	Torija *et al*., 2001
	S	Bordeaux	Red & white variety	Not given	Peynaud & Domercq, 1959
		Majorca	Chenin blanc & Prensal blanc	Dilutions plated on YM and lysine agar	Mora & Mulet, 1991
		Argentina	Malbec	Plated on malt extract agar	Combina *et al*., 2005
Candida valida / Pichia membranifaciens	T	Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al*., 1999
	T	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
	T	North Carolina	Muscadine *(Vitis rotundifolia)*	Direct plating on potato dextrose agar	Parish & Carroll, 1985
	T	Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al*., 1990
	T	Majorca	Prensal blanc	Dilutions plated on YM and lysine agar	Mora & Mulet, 1991
	T	Bordeaux	Red and white varieties	Not given	Peynaud & Domercq, 1959
	T	Argentina	Malbec	Plated on malt extract agar	Combina *et al*., 2005
Candida vini		Argentina	Malbec	Plated on malt extract agar	Combina *et al*., 2005
Debaryomyces etchellsii [Pichia etchelsii]	S	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Issatchenkia terricola		Majorca	Chenin blanc	Dilutions plated on YM and lysine agar	Mora & Mulet, 1991
Kloeckera sp.		Spain	Ribeiro	Dilution and plating on YPD	Cansado *et al*., 1989

TABLE 4 (continued)

Non-*Saccharomyces* yeasts isolated from grape must.

Yeast species Anamorph / Teleomorph [Synonym][1]	Form isolated A/T/S[2]	Country or region	Isolation material (must variety)	Brief description of method of isolation	Reference
Kloeckera africana / Hanseniaspora vineae	A	Bordeaux	Red grape variety	Not given	Peynaud & Domercq, 1959
	A	Italy	Variety not given	Not given	Castelli, 1955
	T	Tenerife	White Listan	Random harvesting; further details not given	De Cos *et al*., 1999
Kloeckera apiculata / Hanseniaspora uvarum	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
	A	Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al*., 1990
	T	Catalonia	Macabeo (white) and Grenache (red)	Dilution and plating on YPD	Torija *et al*., 2001
	T	Catalonia	Garnatxa	Dilutions plated on malt extract agar	Torija *et al*., 2001
	A	Majorca	Chenin blanc & Prensal blanc	Dilutions plated onto YM and lysine agar	Mora & Mulet, 1991
	A	Australia	Riesling, Semillon (white), Malbec and Hermitage (red)	Spread inoculation on malt extract and lysine agar	Heard & Fleet, 1985
	T	Bordeaux	Semillon	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
	A	Bordeaux	Merlot	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
	A	Bordeaux	Red & white varieties	Not given	Peynaud & Domercq, 1959
	T	Italy	Nebbiola (red)	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	T	Germany	Pinot noir	Dilutions plated on yeast extract agar	Schütz & Gafner, 1993
		Bordeaux	Red varieties	Not given	Peynaud & Domercq, 1959
	T	Switzerland	Pinot noir	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	T	Switzerland	Pinot noir	Details not given	Lorenzini, 1999
	A	Italy	Different varieties	Details not given	Castelli, 1955
	A	Argentina	Malbec	Plated on malt extract agar	Combina *et al*., 2005
Kloeckera apis / Hanseniaspora guilliermondii	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Kloeckera corticis / Hanseniaspora osmophila [K. magna]	T	Italy	Variety not given	Details not given	Castelli, 1955
Kloeckera javanica / Hanseniaspora occidentalis [Kloeckera jensenii]	S	Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
	A	Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al*., 1990
	T	North Carolina	Muscadine *(Vitis rotundifolia)*	Direct plating on potato dextrose agar	Parish & Carroll, 1985
	T	Tenerife	White Listan	Random harvesting; further details not given	De Cos *et al*., 1999
Kluyveromyces thermotolerans		Catalonia	Macabeo (white) & Grenache (red)	Dilution and plating on YPD	Torija *et al*., 2001
		Tenerife	White Listan	Random harvesting; further details not given	De Cos *et al*., 1999
Pichia farinosa		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Pichia kluyveri		Bordeaux	Merlot	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
Pichia terricola / Issatchenkia terricola	A	Bordeaux	Merlot	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
Rhodotorula sp.		Bordeaux	Merlot	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984

TABLE 4 (continued)

Non-*Saccharomyces* yeasts isolated from grape must.

Yeast species Anamorph / Teleomorph [Synonym][1]	Form isolated A/T/S[2]	Country or region	Isolation material (must variety)	Brief description of method of isolation	Reference
Rhodotorula glutinis		Bordeaux	Merlot	Dilutions plated on malt extract agar and grape juice agar	Fleet *et al*., 1984
		Tenerife	White Listan	Random harvesting; further details not given	De Cos *et al*., 1999
		Spain	White and red (traditional varieties)	Samples plated on Sabouraud dextrose agar	Rementeria *et al*., 2003
Rhodotorula graminis		Bordeaux	Semillon	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al*., 1984
Rhodotorula minuta		Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al*., 1990
Rhodotorula mucilaginosa		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Saccharomycodes sp. *Saccharomycodes ludwigii*		Spain	Ribeiro	Dilution and plating on YPD	Cansado *et al*., 1989
[Saccharomyces ludwigii]	S	Tenerife	White Listan	Random harvesting; no further details.	De Cos *et al*., 1999
Schizosaccharomyces spp.		Catalonia	Macabeo (white) and Grenache (red)	Dilution and plating on YPD	Torija *et al*., 2001
Torulaspora sp.		Spain	Ribeiro	Dilution and plating on YPD	Cansado *et al*., 1989
		Bordeaux	Red & white varieties	Not given	Peynaud & Domercq, 1959
Torulaspora globosa		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Torulopsis famata		Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
Zygosaccharomyces bailii		Spain	White and red varieties	Samples of must diluted and plated on agar malt and Sabouraud plates	Regueiro *et al*., 1993
Zygosaccharomyces florentinus		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al*., 1991
Zygosaccharomyces rouxii		Argentina	Malbec	Plated on malt extract agar	Combina *et al*., 2005

[1] Where applicable, the anamorph and teleomorph designations are given, and also the synonym or alternate name used in older literature. Yeast nomenclature given according to Kurtzman & Fell (1998).
[2] A = anamorph; T = teleomorph; S = synonym.

The high numbers and sustained presence of non-*Saccharomyces* yeasts in modern wine fermentations have resulted in wine microbiologists revisiting the role of these yeasts in wine fermentation. Spontaneously fermented wines, although carrying a higher risk of spoilage, are generally regarded as having improved complexity, mouth-feel (texture) and integration of flavours relative to inoculated wines (Heard & Fleet, 1985; Bisson & Kunkee, 1991; Gil *et al*., 1996; Lema *et al*., 1996; Grbin, 1999; Soden *et al*., 2000). This is due to specific metabolic end products.

Documented cases include the study by Lema *et al*. (1996) on Albariño wine aroma components. These authors concluded that the predominance of inoculated *S. cerevisiae*, along with a notable growth rate of indigenous non-*Saccharomyces* yeasts during the first days of the wine fermentation, contributed significantly to the desirable aromatic properties of the wines. Herraiz *et al*. (1990) and Gil *et al*. (1996) also reported that wines produced by pure and mixed cultures of *S. cerevisiae* and apiculate yeasts (*K. apiculata* and *H. uvarum*) differ regarding their aromatic compounds. The low frequency of *Kloeckera* spp. during fermentation has also been suggested as a reason for the lack of aroma complexity of Folle blanche wines in the Basque region in Spain (Rementeria *et al*., 2003).

The range of flavour compounds produced by different yeasts is well documented (Castor, 1954; Suomalainen & Lehtonen, 1979; Soles *et al*., 1982; Nykänen, 1986; Rauhut, 1993; Romano & Suzzi, 1993a; Lambrechts & Pretorius, 2000; Rojas *et al*., 2003; Romano *et al*., 2003; Moreira *et al*., 2005; Swiegers & Pretorius, 2005; Swiegers *et al*., 2005). The metabolic products resulting from non-*Saccharomyces* growth include glycerol, acetaldehyde, acetic acid, succinic acid, higher alcohols and fatty acid esters (Fleet *et al*., 1984; Bisson & Kunkee, 1991; Jackson, 1994; Boulton *et al*., 1996; Lonvaud-Funel, 1996; Heard, 1999; Zohre & Erten, 2002; Clemente-Jimenez *et al*., 2004).

Glycerol, after ethanol, is the next major yeast metabolite produced during wine fermentation and is important in yeast metabolism for regulating the redox potential in the cell (Scanes *et al*., 1998; Prior *et al*., 2000). Glycerol contributes to smoothness (mouth-feel), sweetness and complexity in wines (Ciani & Maccarelli, 1998), but the grape variety and wine style will determine the extent to which glycerol impacts on these properties. It appears that the quality of Chardonnay, Sauvignon blanc and Chenin blanc is not improved by increased glycerol concentrations (Nieuwoudt *et al*., 2002). However, as all wine sensory profiles are unique, some wines might benefit from increased glycerol levels.

Spontaneously fermented wines have higher glycerol levels, indicating a possible contribution by non-*Saccharomyces* yeasts (Romano *et al*., 1997; Henick-Kling *et al*., 1998). It is known that *C. stellata* produces elevated glycerol concentrations of between 10 and 14 g/L (Ciani & Picciotti, 1995; Ciani & Ferraro, 1998), compared with 4 to 10.4 g/L by *S. cerevisiae* (Radler & Schütz, 1982; Ciani & Maccarelli, 1998; Prior *et al*., 2000). Unfortunately, increased glycerol production is usually linked to increased acetic acid production (Prior *et al*., 2000), which can be detrimental to wine quality. This makes the growth of *C. stellata* during fermentation problematic, unless a balance can be achieved between glycerol and acetic acid production, which will not detract from wine quality.

Apiculate yeasts (*K. apiculata* and *Hanseniaspora guilliermondii*) have also been implicated in glycerol production. These yeasts can generally be divided into two groups, i.e. high-glycerol (3 g/L), low ethanol (0.9% $^{v}/_{v}$) producers, and low- glycerol (1 g/L), high-ethanol (2.4% $^{v}/_{v}$) producers (Romano *et al*., 1997). Apiculate yeasts are also known as high producers of acetic acid, making them undesirable for wine production (Ciani & Picciotti, 1995). However, it has been reported that large strain variability exists and that not all strains of *Kloeckera* spp. form high levels of acetic acid (Romano *et al*., 1992). Some strains form less than 1 g/L and are comparable to *S. cerevisiae*. If these strains also form higher levels of glycerol, their use can benefit a wine for which higher glycerol levels are needed.

The primary flavour of wine is derived from the grapes. However, secondary flavours are derived from ester formation by yeasts during wine fermentation (Nykänen, 1986; Lambrechts & Pretorius, 2000). Over 160 esters have been distinguished in wine (Jackson, 2000). These esters can have a positive effect on wine quality, especially in wine from varieties with neutral flavours that are consumed shortly after production, e.g. some Chenin blanc wines (Lambrechts & Pretorius, 2000). Non-*Saccharomyces* yeasts isolated from South African musts during 1961 could be divided into two groups, viz. neutral yeasts (producing little or no flavour compounds) and flavour producing species (both desired and undesired) (Van Zyl *et al*., 1963). Flavour producing yeasts included *P. anomala* (*Hansenula anomala*) and *K. apiculata*. *C. pulcherrima* is also known to be a high producer of esters (Bisson & Kunkee, 1991; Clemente-Jimenez *et al*., 2004). However, *C. pulcherrima* has an antagonistic effect on several yeasts, including *S. cerevisiae* (Panon, 1997; Nguyen & Panon, 1998). When aerobic growth of *C. pulcherrima* cultures was followed by inoculation with *S. cerevisiae*, delays in fermentation occurred. This was due to a killer effect, but it was not the same as the classical *S. cerevisiae* killer phenomenon; it was linked to pulcherrimin pigment produced by *C. pulcherrima*. Contrary reports (Jolly *et al*., 2003b, 2003c) indicate that when using a *C. pulcherrima* isolate for wine production there was no effect on fermentation. This contradiction might be due to different distinct biotypes within the *C. pulcherrima* species (Pallmann *et al*., 2001), but this still needs to be investigated further.

Other yeasts that can play a role in wine production are those of the genus *Brettanomyces* (*Dekkera*). Different *Brettanomyces* strains can develop flavours ranging from pleasantly fruity and toffee, to volatile and unpleasant (Eschenbruch & Wong, 1993). The 'mousy' and 'medicinal-like' character in wine (known as "Brett"), is linked to the growth of *Brettanomyces* spp. and is due to the formation of tetrahydropyridines and 4-ethyl phenol, respectively (Grbin *et al*., 1995; Grbin & Henschke, 2000; Arvik & Henick-Kling, 2002). This 'Brett' character is considered to be an off-flavour. However, anecdotal evidence indicates that the 'Brett' character, at low levels in a red wine with an overall complex aroma, can be a positive addition.

Some non-*Saccharomyces* yeasts, e.g. *T. delbrueckii* and *C. stellata* are able to form succinic acid (Ciani & Maccarelli, 1998; Ferraro *et al*., 2000). This correlates with high ethanol production and ethanol tolerance. Succinic acid production could positively influence the analytical profile of wines by contributing to the total acidity in wines with insufficient acidity. However,

succinic acid has a salty/bitter-acid taste (Amerine *at al*., 1972) and excessive levels will negatively influence wine quality.

Different non-*Saccharomyces* yeasts produce different levels of higher alcohols (n-propanol, isobutanol, isoamyl alcohol, active amyl alcohol) (Romano *et al*., 1992; Lambrechts & Pretorius, 2000). This is important during wine production, as high concentrations of higher alcohols are generally not desired, but lower values can add to wine complexity (Romano & Suzzi, 1993b). Non-*Saccharomyces* yeasts often form lower levels of these alcohols than *S. cerevisiae,* but there is great strain variability (Romano *et al*., 1992, 1993; Zironi *et al*., 1993).

Other non-*Saccharomyces* metabolites can act as intermediates in aroma metabolic pathways. Romano *et al*. (1993) showed that *H. guilliermondii* and *K. apiculata* strains produced 50.3 to 258.1 mg/L and 55.8 to 187.4 mg/L acetoin, respectively, in grape must. Acetoin is considered a relatively odourless compound in wine, with a threshold value of approximately 150 mg/L (Romano & Suzzi, 1996). However, diacetyl and 2,3-butanediol (potentially off-flavours in wine) can be derived from acetoin by chemical oxidation and yeast-mediated reduction, respectively. This indicates that acetoin can play a role in off-flavour formation in wines. High concentrations of acetoin produced by non-*Saccharomyces* yeasts can also be utilised by *S. cerevisiae.* This was shown by Zironi *et al*. (1993) in their chemical analysis results of wines fermented from pure cultures, compared to mixed and sequential culture fermentations. They could not, however, confirm what metabolites were formed from acetoin by *S. cerevisiae*.

These, and other compounds not discussed in this article (e.g. volatile fatty acids, carbonyl and sulphur compounds), are known to play a role in the sensory quality of wine (Nykänen, 1986; Lambrechts & Pretorius, 2000; Moreira *et al*., 2005). However, as stated by Guth (1997), there are over 680 documented compounds in wine and a large number of these can, depending on their concentrations, contribute either positively or negatively to wine aroma and flavour. It is not known how these compounds relate to the metabolism of the different yeast species found in fermentation.

Certain flavour and aroma compounds are present in grapes as glycosidic precursors with no sensory properties (Todd, 1995; Pretorius, 2003). These compounds may be hydrolysed by the enzyme β-glucosidase to form free volatiles that can improve the flavour and aroma of wine, but this enzyme is not encoded by the *S. cerevisiae* genome (Ubeda-Iranzo *et al*., 1998; Van Rensburg *et al*., 2005). However, certain non-*Saccharomyces* yeasts belonging to the genera *Debaryomyces, Hansenula, Candida, Pichia* and *Kloeckera* possess various degrees of β-glucosidase activity (Rosi *et al*., 1994; Todd, 1995; Spagna *et al*., 2002; Fernández-Gonzáles *et al*., 2003; Rodriguez *et al*., 2004) and can play a role in releasing volatile compounds from non-volatile precursors. An intracellular β-glucosidase has also been isolated and purified from *Debaryomyces hansenii.* This enzyme, which is not inhibited by glucose and ethanol, was used during fermentation of Muscat grape juice, resulting in an increase in concentration of monoterpenols in the wine (Yanai & Sato, 1999).

As already mentioned, ester formation by yeast plays an important role in secondary flavours. However, the net accumulation of esters in wine is determined by the balance between the yeast's ester-synthesising enzymes and esterases (responsible for cleavage and, in some cases, formation of ester bonds) (Swiegers & Pretorius, 2005). Although extracellular esterases are known to occur in *S. cerevisiae* (Ubeda-Iranzo *et al*., 1998), the situation for non-*Saccharomyces* needs further investigation.

Other non-*Saccharomyces* extracellular enzymatic activity (e.g. proteolytic, polygalacturonase) might also be of use in winemaking. For example, proteolytic activity of some non-*Saccharomyces* yeasts could lead to a reduction in protein levels, with an accompanying increase in protein stability. However, Dizy & Bisson (2000) reported to the contrary, namely that increased yeast proteolytic activity did not lead to a reduction in haze formation. Some extracellular enzymes produced by non-*Saccharomyces* species are shown in Table 5. Species found to produce the greatest number of extracellular enzymes are *C. stellata, H. uvarum/K. apiculata* and *M. pulcherrima/C. pulcherrima*.

New research by Carrau *et al.* (2005) on the formation of aroma compounds by yeasts suggests that *S. cerevisiae* can synthesise monoterpenes (floral aroma in wine), compounds previously thought to be solely derived from grapes. They have also proposed a new metabolic pathway (MCC pathway). It is unknown whether any non-*Saccharomyces* yeasts have these capabilities. However, considering the large variety of non-*Saccharomyces* yeast species found in grape must, it is likely that there will be some.

FACTORS AFFECTING GROWTH OF NON-*SACCHAROMYCES* YEASTS DURING WINE FERMENTATION

The contribution by non-*Saccharomyces* yeasts to wine flavour will depend on the concentration of metabolites formed. Therefore, any factors affecting the growth rate of individual non-*Saccharomyces* species will determine the extent of their contribution to flavour development (Heard, 1999). These factors include intrinsic components of grape juice (pH, sugar concentration and clarity), processing methods (method of clarification, use of preservatives and/or SO_2), fermentation conditions (temperature, oxygen content/aeration) and the effect of *S. cerevisiae* yeast, e.g. amount of ethanol formed.

Non-*Saccharomyces* yeasts have poor tolerance to low oxygen availability, especially compared with *S. cerevisiae* (Hansen *et al*., 2001). The removal of oxygen by vigorously fermenting *S. cerevisiae* can contribute to an early death of some non-*Saccharomyces* yeasts.

For some non-*Saccharomyces* yeasts, i.e. *C. stellata, C. colliculosa* and *C. pulcherrima,* it has been shown that the pH of the medium or must does not have a large effect on growth rate (Gao & Fleet, 1988; Heard & Fleet, 1988), however, it has also been reported that higher pH increases the fermentation ability of *C. pulcherrima* (Jolly *et al*., 2003c). The effect of pH on other non-*Saccharomyces* yeasts is not well defined. An increase in sugar concentration, however, does have an effect on non-*Saccharomyces* yeasts due to their generally poor osmotolerance (Heard, 1999).

Temperature and SO_2 are possibly the two factors that have the greatest effect on non-*Saccharomyces* yeasts. Some non-*Saccharomyces* yeasts are inhibited at temperatures above 25°C (Sharf & Margalith, 1983; Fleet, 1990; Boulton *et al*., 1996).

TABLE 5

Extracellular hydrolytic enzymes found in non-*Saccharomyces* yeasts.

Species (teleomorph / anamorph)	Extracellular enzyme activity										Reference
	Pectinase	Protease	Glucanase	Lichenase	β-Glucosidase	Cellulase	Xylanase	Amylase	Sulphite reductase	Lipase	
Zygoascus hellenicus / Candida hellenica			+		+				+		S
Brettanomyces clausenii	+										F
Kluyveromyces thermotolerans	+										F
Pichia fermentans / Candida lambica			+						+		S
[-2] / *Candida oleophila*	+						+		+		S
Issatchenkia occidentalis / Candida sorbosa			+						+		S
[-2] / *Candida stellata*	+	+	+	+		+	+	+	+	+	C, F, S
Pichia membranifaciens / Candida valida	+	+			+				+		F, S
Debaryomyces hansenii / Candida famata		+			+				+		R[4], S, B
Hanseniaspora uvarum / Kloeckera apiculata	+	+	+	+	+	+	+	+	+		R[4], C, S, Ro[4]
Issatchenkia orientalis / Candida krusei										+	C
Metschnikowia pulcherrima / Candida pulcherrima	+	+	+	+	+	+	+	+	+	+	C, F, S, Ro
Pichia anomala / Candida pelliculosa		+			+				+		R[4], C, F, S, Sp
Torulaspora delbrueckii / Candida colliculosa										+	C
Pichia farinosa / [-3]			+								S
Debaryomyces castellii / [-3]					+						R[4]
Debaryomyces polymorphus / [-3]					+						R[4]
Pichia kluyveri / [-3]			+								S
Pichia guilliermondii / Candida guilliermondii					+						Ro[4]

[1] R = Rosi *et al*., 1994; C = Charoenchai, *et al*., 1997; F = Fernández *et al*., 2000; S = Strauss *et al*., 2001; Sp = Spagna *et al*., 2002 ; Ro = Rodriguez *et al*., 2004, and B = Bolumar *et al*., 2005.

[2] No teleomorph.

[3] No anamorph.

[4] Only screened for β-glucosidase activity.

Therefore, reducing the temperature can lead to greater contribution by non-*Saccharomyces* species. *K. apiculata*, for example, is able to ferment better at 10°C than at 25°C (Heard & Fleet, 1988; Bilbao *et al*., 1997). The ethanol tolerance of some non-*Saccharomyces* yeasts, e.g. *C. stellata* and *K. apiculata*, is also improved at lower temperatures (e.g. 10°C compared with 25°C) (Gao & Fleet, 1988). Other non-*Saccharomyces* yeasts, e.g. *H. guilliermondii* already have an ethanol tolerance very similar to *S. cerevisiae*, however, this is influenced by prior growth conditions (Pina *et al*., 2004). Reducing the fermentation temperature, with its synergistic effect on ethanol tolerance, will therefore lead to an increased presence of non-*Saccharomyces* yeasts. This change in the ecology of the fermentation will also result in a change in the concentration of aroma and flavour metabolites formed by the non-*Saccharomyces* yeasts, with a consequential impact on wine quality.

While it has generally been accepted that SO_2 added to wine suppresses non-*Saccharomyces* yeasts, the work of Henick-Kling *et al*. (1998), Constantí *et al*. (1998), Egli *et al.* (1998) and Rementeria *et al*. (2003) challenges this belief. Low sulphur addition (20 mg/L) does not suppress non-*Saccharomyces* yeasts, higher levels (50 mg/L) do suppress some, but others, e.g. *C. guilliermondii* and *Zygosaccharomyces* spp., can survive. The effectiveness of SO_2 also depends on the type of must and the number of non-*Saccharomyces* yeasts present in the first place. Results of Fleet (1990) and Granchi *et al*. (1998) support this. They found that SO_2 in the range 50 to 100 mg/L did not prevent growth of non-*Saccharomyces* yeasts in red wine fermentations, but these concentrations are normally effective in white wine fermentations. Suppression of sensitive yeasts by SO_2 allows more tolerant yeasts, present in lower numbers, to proliferate. The judicious use of SO_2 can therefore alter the non-*Saccharomyces* population profile in some instances. In other instances, the addition of SO_2 will decrease some populations, but not the species diversity (Rementeria *et al.*, 2003).

Other factors that can impact on the presence and growth of non-*Saccharomyces* yeasts are yeast-yeast and yeast-bacteria interactions, including neutralism, commensalism, mutualism/synergism, amensalism or antagonism and competition (Boddy & Wimpenny, 1992; Boulton *et al.*, 1996; Henick-Kling *et al*., 1998; Fleet, 2003). The utilisation of specific nutrients, e.g. amino acids and vitamins, by one species may make the environment less favourable to other species. In addition, metabolites produced, such as ethanol, inhibitory peptides, proteins and glyco-proteins, can inhibit or destroy other species by lysis of their cell walls. Specifically, zymocidal strains of *S. cerevisiae* (killer yeasts) can affect other species. Other metabolites can, in turn, lead to enhanced growth. Autolysis, with the subsequent release of cellu-

lar material, can also encourage yeast growth (Charpentier *et al*., 1986; Fleet, 2003).

Growth of non-*Saccharomyces* yeasts can also be limited by the *S. cerevisiae* starter culture (Constantí *et al*., 1998; Henick-Kling *et al.*, 1998). High concentrations of *S. cerevisiae* appear to inhibit some non-*Saccharomyces* yeasts by means of a cell-cell mediated mechanism (Nissen *et al*., 2003). Subsequently, different starter cultures will have different effects on the non-*Saccharomyces* populations. Therefore, by controlling the parameters of inoculum, i.e. *S. cerevisiae* strain and inoculum concentration, the non-*Saccharomyces* contribution can also be controlled. This is illustrated by the experiments of Egli *et al*. (1998). They compared three types of fermentation, i.e. fermentations with indigenous microflora (spontaneous), a vigorous yeast starter (*S. cerevisiae* strain EC1118) and a slowly fermenting yeast starter (*S. cerevisiae* strain Assmanshausen). The sensory profiles of the resultant wines differed from each other. The faster growing EC1118 strain limited the growth of the non-*Saccharomyces* component more strongly than the slow growing Assmanshausen. Growth could be further suppressed by the addition of SO_2.

It has also been suggested that the use of fungicides and pesticides results in a reduced yeast presence on grapes (Parish & Caroll, 1985; Regueiro *et al*., 1993; Guerra *et al*., 1999). It has been shown that yeast populations are higher when no agrochemicals have been applied for some time (Renouf *et al*., 2005). Higher levels of non-*Saccharomyces* yeasts can impact on the resultant fermentation and wine quality. In contrast, Cabras *et al*. (1999) showed that certain pesticides could stimulate yeasts (especially *K. apiculata*) to produce more ethanol. This increased vigour would, by implication, also extend to other metabolites. As pesticides are used to a greater or lesser extent in all vineyards (with a few possible exceptions where natural processes are followed), there might be instances where certain pesticides can stimulate non-*Saccharomyces* growth on the grapes. The resultant higher levels of such yeasts might also have a knock-on effect in the subsequent wine fermentation.

Non-*Saccharomyces* populations might also be affected by 'quorum sensing', the mechanism whereby microbial cells can communicate with each other and cause a population to follow a specific growth pattern (Bisson, 1999; Fleet, 2003; Parsek & Greenberg, 2005). Bicarbonate, ammonia and farnesol have been suggested as cell communication molecules for yeast. The effect of quorum sensing and its significance to wine production is not known.

THE USE OF NON-*SACCHAROMYCES* YEASTS IN WINE PRODUCTION

A number of authors have reported that the use of selected and cultured non-*Saccharomyces* yeasts, including *Candida*, *Kloeckera*, *Brettanomyces* and *Zygosaccharomyces* species, is beneficial in wine production. As these yeasts are all poor fermenters, *S. cerevisiae* (either indigenous or inoculated) is always needed to complete wine fermentation. Typically, non-*Saccharomyces* yeasts have been used in sequential fermentation where these yeasts are allowed to grow or ferment for between one hour and fifteen days before inoculation with *S. cerevisiae* (Herraiz *et al*., 1990; Zironi *et al*., 1993; Ciani & Ferraro, 1998; Ferraro *et al*., 2000; Jolly *et al*., 2003b; 2003c). Unfortunately, some authors only report on experiments conducted on laboratory-scale, utilising small volumes of grape juice. These results are not necessarily the same as what could be expected in larger commercial fermentations. Factors such as small amounts of air which can enter small-volume fermentations during e.g. sampling, and the rapid sedimentation of yeast which reduces fermentation rate, can affect the final results (Henschke, 1990).

Candida stellata

C. stellata is known as a high glycerol producer and Ciani & Picciotti (1995) suggested that it be used as a starter culture to increase glycerol levels in wine. Values of up to 11.76 g/L have been reported (Ciani & Maccarelli, 1998), which is higher than the sensory threshold level for glycerol sweetness, i.e. 5.2 g/L (Noble & Bursick, 1984). Glycerol is also thought to contribute to the mouth-feel and complexity of wine flavour, at lower levels (Scanes *et al*., 1998; Prior *et al*., 2000). This yeast is therefore a prime candidate for investigating a positive non-*Saccharomyces* contribution to wine quality.

Ciani & Ferraro (1998) used a *C. stellata* strain (strain 3827, Industrial Yeast Collection, University of Perugia) to improve the analytical profile of small-scale (500 mL) Pinot grigio wines during batch fermentations. The Pinot grigio grapes were harvested at 18.5°B and sweetened to 27°B with sucrose. Immobilised *C. stellata* cells at concentrations of 1 x 10^9 cells/mL were used in three fermentation types: a simultaneous inoculation with *S. cerevisiae*; a sequential fermentation where *S. cerevisiae* was added three days after *C. stellata*; and a substituted fermentation where *C. stellata* was replaced with *S. cerevisiae* after three days. The *S. cerevisiae* inoculum was 1 x 10^6 cells/mL for all treatments. Results showed that in comparison to the *S. cerevisiae* control fermentations, the combined fermentations occurred more rapidly with increased glycerol content. This was accompanied by a decrease in acetic acid and higher alcohols and an increase in succinic acid. Other by-products were similar to those found in the *S. cerevisiae* control fermentation. Despite the high initial sugar content, all of the sugar was consumed due to the complementary utilisation of fructose and glucose by the *C. stellata* (fructophilic yeast) and *S. cerevisiae* (glucophilic yeast). It was concluded that of all three treatments, the sequential fermentation was the best combination for improving the analytical profile of the wines. It was further noted that acetoin produced by *C. stellata* was utilised by *S. cerevisiae* to form 2,3-butanediol that can lead to off-flavour in wine. As no sensory analyses were carried out it is not clear what impact this had on the flavour of the wine.

In a subsequent study, Ferraro *et al*. (2000) confirmed the above findings in the pilot-scale (100 L) production of wine. They used Trebbiano Toscano grape juice at 19°B, pH 2.94 and 11.52 mg/L free SO_2. The inoculum of immobilised *C. stellata* (5 x 10^8 cells/mL) was followed after three days by an inoculum of *S. cerevisiae* (5 x 10^6 cells/mL). High glycerol production was evident on the fourth day of fermentation, while the alcohol was still lower than 5%. Thereafter, the glycerol production was slower and the ethanol concentration increased due to metabolism by *S. cerevisiae*. The final wine had a 70% higher glycerol concentration than the control (*S. cerevisiae* only), while the ethanol concentration was 10.6% compared with the 12.24% of the control. The acetic acid was 0.05 g/L lower than the control fermentation.

Apart from the increase in glycerol, the reduction in alcohol as reported above could be beneficial for the production of wines from grapes with high sugar content (as often found in grapes from warm regions). Reduction in acetic acid concentration will always be beneficial. However, while the analytical profile of the wine was improved, a shortcoming of the work of Ferraro *et al*. (2000) is that they did not report on the sensory profile of the wine. During the three-day lag before inoculation with *S. cerevisiae*, oxidation of the must might have occurred. However, spontaneous fermentation would have started and this might have offset the oxidation risk.

In another investigation Soden *et al*. (1998; 2000) used *C. stellata* in combination with *S. cerevisiae* for the production of Chardonnay wines. They used two different inoculation protocols, viz. co-inoculation and sequential inoculation, starting with the *C. stellata*, and these were compared with the two yeasts in separate monoculture ferments. The yeasts were inoculated at a concentration of 5 x 10^6 cells/mL, except in the co-inoculated fermentation where the *S. cerevisiae* was inoculated at a lower cell count (5 x 10^5 cells/mL). In the sequential fermentation, *S. cerevisiae* was inoculated 15 days after *C. stellata*. At this point the *C. stellata* had depleted the fructose, but not the glucose, and had stopped fermenting. All the wines except the *C. stellata* monoculture fermented dry.

The wines underwent descriptive sensory analyses and the reference *S. cerevisiae* wine was judged to have 'tropical fruit', 'floral', 'lime' and 'banana' aromas of a typical Chardonnay. The monoculture *C. stellata* wine had significantly more 'apricot', 'honey' and 'sauerkraut' aromas and significantly less 'lime', 'tropical fruit', 'banana' and 'floral' aromas. The co-inoculated wine had aromas similar to wine resulting from use of the *S. cerevisiae* monoculture, but was scored lower for 'floral' and 'banana'. The sequential fermentations produced a wine significantly different from the *S. cerevisiae* reference wine regarding 'banana', 'floral' and 'lime' aromas, but the wine was similar in the 'honey', 'apricot' and 'sauerkraut' aromas attributed to the *C. stellata* yeast. The wine also had a high 'ethyl acetate' aroma, had the highest concentration of glycerol and succinic acid, and a lower concentration of ethanol. On the negative side, 'sauerkraut' and 'ethyl acetate' nuances could be considered to detract from wine quality as they are listed under 'microbiological' and 'oxidised' according to wine evaluation terminology (Noble *et al*., 1987). The long time lapse of 15 days before the inoculation with *S. cerevisiae* in the sequential fermentation would have contributed to the 'oxidised' aroma of the wine. A shorter delay before inoculation with *S. cerevisiae* could have given a better wine. Soden *et al.* (2000) concluded that, with selection of the appropriate strains and the establishment of effective inoculation protocols, greater flavour diversity and complexity could be obtained in wine during commercial winemaking.

In another study where *C. stellata* and *S. cerevisiae* were used in sequential fermentations for the production of small-scale Chardonnay wines, the time lapse between inoculations of the two species was only one hour (Jolly *et al*., 2003b). No increases in glycerol levels were noted, as was the case when the *C. stellata* isolate was used on its own in laboratory-scale fermentations. For the small-scale Chardonnay wine, the total esters was significantly higher for the *C. stellata* sequential wine in comparison to the *S. cerevisiae* only reference wine. However, during a comparative sensory evaluation the reference wine was preferred. No descriptive sensory analysis was performed.

Candida pulcherrima

C. pulcherrima, which produces high concentrations of esters (Bisson & Kunkee, 1991), especially the pear-associated ester ethyl caprylate (Lambrechts & Pretorius, 2000; Clemente-Jimenez *et al*., 2004), can occur in high numbers in grape must (Schütz & Gafner, 1993; Jolly *et al*., 2003a). Furthermore, it was shown by Zohre & Erten (2002) that production of undesirable volatile compounds did not occur during mixed culture fermentations of this yeast and *S. cerevisiae*. *C. pulcherrima* might therefore make a positive sensory contribution to wine. In another study, a randomly selected *C. pulcherrima* isolate was used in sequential fermentations with *S. cerevisiae*, for small-scale production of Chardonnay, Sauvignon blanc and Chenin blanc wines (Jolly *et al*., 2003b). A sensory evaluation of the wines showed that Sauvignon blanc and Chenin blanc were better than a reference wine (*S. cerevisiae* only) five and 18 months after production. The Chardonnay wine was judged to be of an inferior quality. It thus appears that there are specific non-*Saccharomyces*/grape variety combinations that lead to improved quality.

In a subsequent study, the effect of winemaking practices (namely the use of diammonium phosphate [DAP] and SO_2), fermentation temperatures and must pH on the performance of *C. pulcherrima* during fermentation was investigated (Jolly *et al.*, 2003c). It was reported that DAP addition, higher pH values and increased temperatures all resulted in a slight increase in fermentation ability. The fermentation ability was not affected by SO_2 concentrations normally used in wine fermentation (0-30 mg/L). Elevated levels (60 mg/L) of SO_2 did have a negative effect, however, this is a much higher concentration than would normally be used in a commercial fermentation. A Chenin blanc wine production trial showed that a selected *C. pulcherrima* strain had a positive influence on wine quality (as measured by a sensory panel). No difference in standard chemical analyses was noted and the improvement was not linked to ester levels; the authors implicated other metabolites, but further chemical analyses and suitable methodology were required to identify them.

***Kloeckera* and *Hanseniaspora* spp.**

The apiculate yeasts *K. apiculata* and *H. uvarum*, the non-*Saccharomyces* yeasts found in the highest numbers in grape must, are in the best position to make a contribution to wine quality. These yeasts, with low fermentative power, are important in the production of volatile compounds in wine, and the chemical composition of wines made with *Kloeckera* spp./*S. cerevisiae* combinations differ from reference wines (Herraiz *et al*., 1990; Mateo *et al*., 1991; Zironi *et al*., 1993; Gil *et al*., 1996).

Caridi & Ramondino (1999) evaluated a range of 20 *Hanseniaspora* spp. (*Kloeckera* spp.) of oenological origin for their ability to ferment a must (17.9°B; pH 3.81). They found that the ethanol produced ranged from 5.02 to 8.72%, in comparison to the 11.17% of the control (*S. cerevisiae*), but volatile acidity was higher (0.75g/L to 2.25g/L) than the control (0.65g/L). These results indicate that although some strains of *Hanseniaspora* are able to produce higher levels of ethanol than other strains, the high levels of volatile acidity would be detrimental to the senso-

ry characteristics of the wine. However, Romano *et al*. (1992) and Ciani & Maccarelli (1998) showed that not all *Kloeckera* strains formed high levels of volatile acidity and that some were similar to *S. cerevisiae* in this regard. The production of other secondary metabolites, i.e. glycerol, acetaldehyde, ethyl acetate and hydrogen sulphide, also differed between strains (Romano *et al*., 1997). Selected strains of apiculate yeasts might therefore favour aroma and flavour enhancement in wines.

Most authors working with these yeasts only utilised *Kloeckera* spp. in small-scale laboratory trials and subsequently only analysed the wines chemically. Their approaches to the use of *Kloeckera* also differed. Zironi *et al*. (1993), in their sequential fermentations, allowed a *Kloeckera/Hanseniaspora* ferment to go for six days before inoculating with *S. cerevisiae*, while Herraiz *et al*. (1990) waited eight days. Differences in chemical analyses of the wines were noted. Of more concern was that the initial growth of *Kloeckera* had a retarding effect on the subsequent growth of *S. cerevisiae.* This phenomenon could have further implications as a cause for lagging or stuck fermentations. Therefore, a cautionary approach would have to be taken when considering using *Kloeckera* spp. in wine production. *K. apiculata* has also been implicated in the formation of some biogenic amines (Caruso *et al*., 2002).

Sensory evaluations on wines produced by *Kloeckera* spp. were carried out by Owuama & Saunders (1990). They used *K. apiculata/S. cerevisiae* combinations for the fermentation of cashew apple juice (25°B). Inoculation of the two species was simultaneous and 9.3% ethanol was produced with 4.2% residual sugar. Sensory evaluation (colour, aroma and taste) of the wines showed that although the *Saccharomyces* reference wine was the best, the product of the combined fermentation was also acceptable for consumption.

A *K. apiculata* isolate was also used with *S. cerevisiae* for the production of small-scale Chardonnay, Sauvignon blanc and Chenin blanc wines (Jolly *et al*., 2003b). Inoculation of the *S. cerevisiae* was one hour after that of the *K. apiculata* and *ca.* 13% ethanol was produced with less than 2 g/L residual sugar. Sensory evaluation of the wines five and 18 months after production showed that the Sauvignon blanc wine was preferred by the judges. All the other wines were judged to be inferior to a reference wine produced by *S. cerevisiae* only.

The production of 2-phenyl-ethyl acetate by the apiculate yeast *H. guilliermondii* has been investigated in laboratory fermentations (Rojas *et al*., 2003). This acetate ester contributes to 'rose', 'honey', 'fruity' and 'flowery' aroma nuances (Lambrechts & Pretorius, 2000; Swiegers & Pretorius, 2005; Swiegers *et al*., 2005), and is formed to a greater or lesser extent by most yeasts. As part of the 'fermentation bouquet', it can contribute to the overall flavour of a young wine. However, the high level of ethyl acetate produced by the strain investigated by Rojas *et al*. (2003) was a serious handicap.

Another role that has been suggested for *Kloeckera* and *Hanseniaspora* spp. is that of the production of wine destined for vinegar manufacture (Ciani & Picciotti, 1995). The high production of acetoin and ethyl acetate will favourably influence the quality of vinegar.

Zygosaccharomyces spp.

Zygosaccharomyces spp. are considered to be winery contaminants and are especially a problem in wineries producing sweet and sparkling wines (Amerine & Cruess, 1960; Loureiro & Malfeito-Ferreira, 2003). Notwithstanding, *Zygosaccharomyces* spp. have been investigated by Romano & Suzzi (1993a) as positive contributors to wine fermentation. The species studied included a *Z. fermentati* strain (strain F42), which produced low levels of acetic acid, H_2S and SO_2 and had high fermentation vigour. Another species, *Z. bailii* (strain F37), showed malic acid degradation and generally low H_2S production. In addition, both species flocculated. The authors suggested that these characteristics could benefit wine production during, for example, re-fermentation of wine.

Most of the literature recognises *Zygosaccharomyces* yeasts as spoilage organisms producing high quantities of acetic acid. However, Romano & Suzzi (1993a) suggested that this acetic acid production might be due to yeasts bearing a close resemblance to *Zygosaccharomyces.* In older literature, acetic acid-producing yeasts were wrongly identified as *Zygosaccharomyces* species.

Selected strains of *Zygosaccharomyces* spp. might be useful especially for the production of alternate beverages. *Z. bailii* is also, in contrast to many other non-*Saccharomyces* yeasts and *S. cerevisiae*, fructophilic. This could be beneficial in grape musts from riper grapes (high Balling), where the fructose concentration can exceed that of glucose at the start of fermentation (Margalith, 1981).

Schizosaccharomyces spp.

Schizosaccharomyces spp. have been used for the production of mango (*Magnifera indida* L.) wine without addition of *S. cerevisiae* (Obisanya *et al*., 1987). Two strains of *Schizosaccharomyces spp*. isolated from palm wine were found to be suitable for the production of sweet mango table wine with between 8 and 9% alcohol. Reference wines produced by *S. cerevisiae* were, however, found to be superior in flavour and taste, while fermenting dry (approximately 4 g/L sugar).

Another characteristic of *Schizosaccharomyces* spp. is their ability to degrade malic acid; it has been shown that high-density cell suspensions of *Schizosaccharomyces* yeasts could degrade 95-99% of malic acid in a buffered assay system (Gao & Fleet, 1995). However, none of the yeasts could metabolise malic acid fast enough to be of use in a reactor system for the treatment of grape juice. Increasing the cell density had no improved effect.

In another trial, a *Schizosaccharomyces malidevorans* mutant that could utilise malic acid more rapidly than the wild-type strain was used for commercial-scale (1000 to 2500 L) deacidification of grape juice (Thornton & Rodriguez, 1996). The varieties treated were Chardonnay, Semillon and Cabernet Sauvignon. The temperature and SO_2 levels were those normally applied in wine production, i.e. 15-25°C and 30 mg/L (free), respectively. No sensory defects were noted in the finished wines, which were used for blending, before being sold as varietal wines.

Torulaspora delbrueckii

T. delbrueckii (teleomorph of *C. colliculosa*), formerly classified as *Saccharomyces rosei*, was previously suggested for vinification of musts low in sugar and acid (Castelli, 1955). It was subsequently used for the commercial production of red and rosé wines in Italy (Castelli, 1955). In more recent experiments carried out by Moreno *et al*. (1991) it was found that *T. delbrueckii* in

pure culture produced lower levels of volatile acidity than the *S. cerevisiae* strains tested. Furthermore, in mixed fermentations (indigenous population plus pure cultures of *T. delbrueckii* or *S. cerevisiae*) the volatile acidity increased with the ripeness of the grapes, however, these increases were generally smaller for the *T. delbrueckii* than the *S. cerevisiae* strains.

In another investigation, the anamorphic form of this yeast (*C. colliculosa*) was used together with *S. cerevisiae* for the production of small-scale Chardonnay, Sauvignon blanc and Chenin blanc wines (Jolly *et al*., 2003b). The Sauvignon blanc and Chenin blanc wines were both judged to be better than their respective *S. cerevisiae* reference wines five and 18 months after production. However, standard wine chemical and ester analyses did not differ from the reference wines.

***Brettanomyces* spp.**

The contribution of *Brettanomyces* spp. to wine aroma has been likened to a 'Bordeaux-like' character. Apart from the previously mentioned negative aroma nuances imparted by these yeasts, positive aromas such as 'smoky', 'spicy' and 'toffee' are also cited (Eschenbruch & Wong, 1993; Arvik & Henick-Kling, 2002). *Brettanomyces* spp. have also been implicated in Belgian acidic ale production where it is found in the final 20 to 24 month stage in the fermentation casks (Martens *et al.*, 1997). While the deliberate inoculation of *Brettanomyces* in must or wine for commercial production has not been reported, some winemakers are working with the indigenous populations of *Brettanomyces* found in their cellars to make more complex wines, some of which are highly regarded because of their aromas and flavours (Arvik & Henick-Kling, 2002). This topic is being investigated by different research groups (Grbin & Henschke, 2000; Arvik & Henick-Kling, 2002) and a favourable *Brettanomyces* strain or protocol for the beneficial use of *Brettanomyces* spp. for wine production might still be found. However, the formation of 'mousy' and 'medicinal' off-flavour compounds will have to be controlled. Strain selection will also have to ensure that no biogenic amines are produced (Caruso *et al*., 2002).

Saccharomycodes ludwigii

S. ludwigii is a lesser known, lemon-shaped yeast, typically with a large cell size, frequently isolated from wine at the end of fermentation or from wine in storage (Romano *et al*., 1999). Secondary metabolites produced at high levels by this yeast include isobutyl alcohol, acetoin and ethyl acetate. It is also known to be highly resistant to SO_2 and tolerant to ethanol. A selected strain of *S. ludwigii* was used to ferment feijoa or pineapple guava (*Feijoa sellowiana*) juice. The resultant beverage was evaluated for aroma, flavour and taste by a consumer panel. Despite the high levels of acetic acid, the beverage was described as "fresh" with a "fruity flavour", akin to apple and kiwi, and similar to apple juice in taste, but with more acid. Romano *et al*. (1999) concluded that this beverage had potential as a refreshing summer drink.

Kluyveromyces thermotolerans

A commercial active dried yeast blend of *K. thermotolerans* and *S. cerevisiae* (Viniflora® SYMPHONY.nsac) has recently been released (Anon., 2004a). This combination has been developed for the enhancement of aroma and flavour in white (Chardonnay, Pinot blanc, Pinot gris and Riesling) and red (Cabernet Sauvignon, Merlot, Shiraz and Pinot noir) grape varieties. According to the product information sheet, the use of this yeast in simultaneous inoculations can lead to the enhancement of floral and tropical fruit aromas, and more complex and rounded flavours in white and red wine, respectively. Although the ratio of the *K. thermotolerans* to *S. cerevisiae* is not specified, it appears to be in the region of 1:30 (Jolly, unpublished data, 2005).

Other non-*Saccharomyces* spp.

Other non-*Saccharomyces* yeasts that have also been investigated for their potential contribution to wine include *Pichia* and *Williopsis* spp.

The production of 2-phenylethanol by *Pichia fermentans* has been investigated under optimised culture conditions by Huang *et al*. (2001). This alcohol, contributing to an aroma of rose petals, is formed to a greater or lesser extent by most yeasts, and as part of the 'fermentation bouquet' can contribute to the overall flavour of a young wine. It was found that the production of this compound increased with an increase in biomass during the initial stage of fermentation. A maximum concentration was reached after 16 hours.

The use of *P. fermentans* was also investigated by Clemente-Jimenez *et al.* (2005) in microvinifications (250 mL). It was found that mixed fermentations with *S. cerevisiae* resulted in an increase in some aromatic compounds such as acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl caprylate, 2,3-butanediol and glycerol.

Other *Pichia* spp., i.e. *P. anomala*, *P. membranifaciens* and *P. subpelliculosa*, together with *Williopsis saturnus* have also been suggested for the production of low-alcohol wines (*ca*. 3%$^{v}/_{v}$) in an aerated vessel (Erten & Camphill, 2001). In this method stirring and agitation resulted in more yeast biomass being formed and decreased ethanol production from fermentable sugars. The wines, made by *P. subpelliculosa* and *W. saturnus* in particular, were acceptable to a small tasting panel. This method of low-alcohol wine production eliminates the need for costly and complex post-production removal of alcohol from wines produced during normal fermentations. The higher levels of flavour compounds, especially esters, produced by these aerobic yeasts appeared to counteract the loss of flavour enhancing properties of alcohol. The "body" of the wine normally attributed to ethanol also appeared to have been replaced by the higher concentration of esters.

The demand by the beverage industry for new and interesting products and the well-developed flavour produced by aerobic non-*Saccharomyces* yeasts makes this technology attractive. However, problems envisioned are the need for non-standard fermentation equipment and the reluctance of wine and beverage producers to deliberately cultivate organisms normally considered as contaminants.

The early death of some non-*Saccharomyces* yeasts during fermentation can also be a source of specific nutrients for *S. cerevisiae*, enabling it to ferment optimally. These nutrients include cellular constituents such as cell wall polysaccharides (mannoproteins). For this method of nutrient supply to be effective, any killer or other inhibitory effects by the non-*Saccharomyces* yeasts against *S. cerevisiae* should be known (Herraiz *et al*., 1990; Panon, 1997; Nguyen & Panon, 1998; Fleet, 2003) so that the subsequent *S. cerevisiae* fermentation is not adversely affected.

Combinations of non-*Saccharomyces* yeasts

Eschenbruch & Wong (1993) inoculated a red grape variety (Blauburger) with a combination of the non-*Saccharomyces* yeasts *Kloeckera* sp. and *Brettanomyces* sp. This was followed after 24 hours with a *S. cerevisiae* pure culture. These authors reported that the wine developed an overall flavour and complexity that they termed a "Brettanomyces" character. This character, they maintained, was a direct result of the contribution of the *Kloeckera* sp. and *Brettanomyces* sp. They reported further that a lower cell concentration should be used as higher cell concentrations (10^6 cells/mL) resulted in off-characters. Their control fermentations showed an indigenous population of *Kloeckera* and *Candida* yeasts, but the numbers of these declined and essentially disappeared as the fermentation proceeded. No *Brettanomyces* yeasts were detected. After inoculation of the control, the *S. cerevisiae* yeast quickly became the predominant species. This was also noted in the non-*Saccharomyces* inoculated fermentations. The numbers of *Candida* sp. once again decreased as the fermentation proceeded. However, the cell densities of the *Brettanomyces* and *Kloeckera* (represented by the added yeasts and indigenous yeasts already present) decreased only slightly during the fermentation. These yeasts therefore could contribute towards the fermentation metabolism, resulting in a contribution to taste and flavour differences.

In another investigation with combinations of non-*Saccharomyces* yeasts, *T. delbrueckii* was used in sequential fermentations with *K. apiculata*. The *K. apiculata* was inoculated first, followed three days later by *T. delbrueckii*, and finally *S. cerevisiae* was inoculated after eight days. The wines produced had volatile compositions different from the *S. cerevisiae* wines, but were not subjected to sensory evaluation (Herraiz *et al*., 1990).

Another commercial active dried wine yeast culture (Viniflora® HARMONY.nsac) combines the non-*Saccharomyces* yeasts *T. delbrueckii* and *K. thermotolerans* with *S. cerevisiae* (Anon., 2004b). According to the technical data sheet this combination (simultaneous inoculation) leads to wines with a richer and rounder flavour with enhanced fruity notes. Improvements in wine quality have been observed for a number of white (Chardonnay, Pinot blanc, Pinot gris, Riesling) and red grape varieties (Cabernet Sauvignon, Pinot noir, Shiraz, Merlot). The proportion of the three different yeasts to each other is not disclosed, but the non-*Saccharomyces* to *S. cerevisiae* ratio appears to be in the region of 1:14 (Jolly, unpublished data, 2005).

RESEARCH TRENDS IN RELATION TO NON-*SACCHAROMYCES* YEASTS

The utilisation of non-*Saccharomyces* yeasts such as *C. stellata*, *T. delbrueckii* and others to improve the analytical profile and flavour of wines has already been discussed. Concurrently with the envisioned use of these new "wine yeasts", new fermentation techniques could be implemented and alternative alcoholic beverages produced (Ciani & Maccarelli, 1998). It has already been shown that non-*Saccharomyces* yeasts normally found during wine fermentation are also responsible for the fermentation of Kombucha, a traditional beverage produced by fermenting sweetened black tea (Teoh *et al*., 2004). Non-*Saccharomyces* isolates from wine could therefore be screened for improved strains for Kombucha production.

It has also been shown that *S. cerevisiae* can initiate biofilm formation (Reynolds & Fink, 2001), a characteristic previously thought to be restricted to bacteria (Parsek & Greenberg, 2005). This has implications for wine fermentation and the storage of wines. The ability of wine-related non-*Saccharomyces* to form biofilms is not well researched, but it has been suggested that yeast cells on the surfaces of grape berries may interact in a biofilm system (Renouf *et al*., 2005). It is also known that *C. albicans*, previously isolated from grapes (Parish & Caroll, 1985), forms biofilms which contribute to its pathogenesis (Douglas, 2003). It can therefore be speculated that other non-*Saccharomyces* yeasts, even if unable to initiate biofilms themselves, may become constituents of a *S. cerevisiae* or *C. albicans* biofilm and, in so doing, contribute positively or negatively to wine production.

As *S. cerevisiae* is glucophilic (a preference for glucose above fructose) (Ough & Amerine, 1963; Margalith, 1981; Berthels *et al*., 2004), the use of fructophilic non-*Saccharomyces* yeasts has also been suggested for the remediation of some types of sluggish or stuck fermentations (Gafner *et al*., 2000). Should the glucose be utilised faster than the fructose during a 'normal' fermentation, then a glucose-fructose imbalance can occur and *S. cerevisiae* is unable to ferment further. Under these circumstances fructophilic yeasts, e.g. *C. stellata* and *Z. bailii*, which have a preference for fructose, can be used to metabolise the fructose (Sütterlin *et al*., 2004). Once the glucose-fructose balance is restored, *S. cerevisiae* should start fermenting again. An active dried experimental *Z. bailii* strain has already been produced and is currently being evaluated by commercial wine cellars (P. Loubser, Lallemand, South Africa, personal communication, 2006). This technology holds great potential as sluggish and stuck fermentations are a common occurrence in all wine industries.

In another study, killer toxins from *P. anomala* and *Kluyveromyces wickerhamii* have been investigated as antimicrobial agents against the spoilage yeast *Dekkera/Brettanomyces* (Comitini *et al*., 2004). This has a potential application during wine maturation and storage.

Apart from the investigations and experimentation with non-*Saccharomyces* yeasts in wine production, other uses for non-*Saccharomyces* yeasts and their metabolites are being sought. These include the use of *Pichia pastoris* for the biomodification of citrus aroma oil to improve and add value to the essence (Goodrich *et al*., 1998). A protease enzyme isolated from *D. hansenii* has also been suggested for hydrolysis of muscle proteins during meat processing (Bolumar *et al*., 2005).

Another application for non-*Saccharomyces* yeasts is the use of Zygocin, a protein toxin produced and secreted by killer strains of *Z. bailii* (Weiler & Schmitt, 2003). Purified forms of the toxin have potential as an antimycotic for a variety of human and phytopathogenic fungi (Schmitt & Breinig, 2002). The use of non-*Saccharomyces* as bio-control alternatives to chemicals has also been investigated. A "sister" species to *M. pulcherrima* (teleomorph of *C. pulcherrima*), *Metschnikowia fructicola* isolated from grapes, shows activity against botrytis rot on stored grapes (Kurtzman & Droby, 2001), while *in vitro* experiments show that *P. membranifaciens* is antagonistic towards the causative agent, *Botrytis cinerea* (Masih *et al*., 2001). Table grape storage and post harvest damage due to botrytis is a major problem in grape-producing regions that lie far from their respective domestic and

international markets. The possible use of *Pichia guilliermondii* for copper uptake in bio-remediation of sewage sludge has also been suggested (De Silóniz *et al*., 2002). Considering the wide biodiversity of yeasts found on grapes and in must, the potential for finding yeasts benefiting mankind outside the parameters of wine production is large.

FINAL COMMENTS

It is generally accepted that the wealth of yeast biodiversity with hidden potential, especially for oenology, is largely untapped (Pretorius, 2000). However, in order to exploit the potential benefits of non-*Saccharomyces* yeasts in wine production and to minimise potential spoilage, the yeast populations on grapes and in must, as well as the effect of wine making practices on these yeasts, must be known, as must the metabolic characteristics of non-*Saccharomyces* yeasts (Romano *et al*., 2003). This knowledge will help realise the predictions of Heard (1999) concerning the use of mixed starter cultures. His vision includes the use of mixed yeast starter cultures tailored to reflect the characteristics of a given wine region and the use of indigenous yeast species with modern technology to produce novel wine-based beverages.

Strain selection will be very important, as not all strains within a species will necessarily show the same desirable characteristics. For example, significant variability is found in the formation of undesirable biogenic amines amongst strains within some non-*Saccharomyces* yeast species (Caruso *et al*., 2002).

Whatever the outcome of the search for non-*Saccharomyces* yeasts for use in wine production, the accepted list of desirable characteristics as pertaining to the wine yeast *S. cerevisiae* (Yap, 1987; Henschke, 1997; Pretorius, 2000) will not necessarily apply to non-*Saccharomyces* yeasts. High fermentation efficiency, high sulphite tolerance and zymocidal (killer) properties, for example, might not be needed in the new technology of wine production. The new non-*Saccharomyces* wine yeasts will necessarily have a different list of desired characteristics. Furthermore, the already mentioned problems envisioned by Erten & Campbell (2001) regarding the need for non-standard fermentation equipment and the reluctance of wine producers to cultivate and use, on a large-scale, microorganisms generally considered as spoilage organisms (Loureiro & Malfeito-Ferreira, 2003), should be noted. Intensive education will have to accompany any new non-*Saccharomyces* technology in wine production. However, the goals as set out by Pretorius (2000; 2003) regarding efficient sugar utilisation, enhanced production of desirable volatile esters, enhanced liberation of grape terpenoids and production of glycerol to improve wine flavour and other sensory properties can be met by selected non-*Saccharomyces* wine yeasts. This path will bypass current controversies regarding the genetic modification of the workhorse of wine production, i.e. the "wine yeast" *S. cerevisiae*. After the acceptance of genetically modified organisms (GMO) by wine consumers and industries, genetic modification of selected non-*Saccharomyces* yeasts can further enhance their performance and role in wine production.

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