

The Effect of Non-*Saccharomyces* Yeasts on Fermentation and Wine Quality*

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Research has shown that non-*Saccharomyces* yeast strains can be detected throughout wine fermentation. Non-*Saccharomyces* yeasts can therefore influence the course of fermentation and also the character of the resultant wine. Previously it was shown that four non-*Saccharomyces* species, i.e. *Kloeckera apiculata*, *Candida stellata*, *Candida pulcherrima* and *Candida colliculosa*, predominated in grape must at the start of fermentation. In this study these four yeasts were used singularly and in combination with an industrial wine yeast (*Saccharomyces cerevisiae* strain VIN 13) to ferment must on a laboratory scale. The resultant wine was analysed for ethanol, volatile acidity, total SO₂ and glycerol. Results show that, in comparison with the industrial wine yeast, the non-*Saccharomyces* yeast strains could not ferment all the sugar. Furthermore, while the individual non-*Saccharomyces*-fermented wines had different chemical analyses, the wines fermented by the combinations were similar to the wine produced by the industrial yeast only. In subsequent, small-scale winemaking trials some of the wines produced by combined fermentations were judged to be of better quality than those produced by the *S. cerevisiae* only. However, this quality increase could not be linked to increased ester levels.

The yeasts present in grape must at the onset of wine fermentation can be divided broadly into two groups, i.e. the wine yeast *Saccharomyces cerevisiae* and the non-*Saccharomyces* yeasts. The *Saccharomyces* yeasts are derived primarily from the cellar equipment (Vaughan-Martini & Martini, 1995; Boulton *et al.*, 1996; Martini *et al.*, 1996), but are also present on the grapes, albeit in very low numbers. They are carried over to the must during crushing (Peynaud & Domercq, 1959; Lonvaud-Funel, 1996; Török *et al.*, 1996; Mortimer & Polsinelli, 1999). A third source of *Saccharomyces* yeasts may be the industrial culture added by the winemaker.

The second group, the non-*Saccharomyces* yeasts, is found predominantly on the grapes (Martini *et al.*, 1996), but also in lesser numbers on the cellar equipment. Before inoculation with an industrial *S. cerevisiae*, they are the yeasts present in the highest numbers in the grape must. During the fermentation there is a sequence of dominance by the various non-*Saccharomyces* yeasts, followed by *S. cerevisiae*, which then completes the fermentation (Fleet *et al.*, 1984; Fleet, 1990; Jackson, 1994; Henick-Kling *et al.*, 1998). This is especially evident in spontaneously fermenting grape must, which has a low initial *S. cerevisiae* concentration.

Research has shown that non-*Saccharomyces* yeast strains can be detected throughout wine fermentation (Jolly *et al.*, 2003 and the references therein) and their dominance during the early part of fermentation can leave an imprint on the final composition of the wine (Romano *et al.*, 1997). In a previous investigation (Jolly

et al., 2003) we found four different yeast species, i.e. *Kloeckera apiculata*, *Candida stellata*, *Candida pulcherrima* and *Candida colliculosa*, predominant (>50%) before the start of fermentation in eight of 12 Chardonnay musts studied.

The aim of this study, which forms part of the comprehensive and ongoing research programme documented by Pretorius *et al.* (1999), was to evaluate a representative strain of each of the aforementioned species for their effect on wine fermentation and wine quality during laboratory and small-scale winemaking trials. This knowledge will help realise the predictions of Heard (1999) relating to the use of indigenous yeast species to improve the sensory quality of wine. 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 species with modern technology to produce novel wine-based beverages.

MATERIALS AND METHODS

Yeast strains

The five yeast strains used in this study are listed in Table 1. Stock cultures of the strains were kept in glycerol at -80°C. The non-*Saccharomyces* strains were selected randomly from a collection of natural isolates (Jolly *et al.*, 2003) and their identities were confirmed by a commercial laboratory (CBS, Delft, The Netherlands). An industrial *S. cerevisiae* wine yeast strain (strain VIN 13, Anchor Bio-Technologies, Cape Town, South Africa) was used as a reference strain and for co-fermentation.

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TABLE 1

Yeast strains used.

Species	Strain number	Origin
<i>Saccharomyces cerevisiae</i>	VIN 13	Industrial yeast from Anchor Bio-Technologies, South Africa
<i>Candida colliculosa</i>	M2/1	Natural isolate from Chardonnay (1998 vintage)
<i>Candida stellata</i>	770	Natural isolate from Chardonnay (1997 vintage)
<i>Kloeckera apiculata</i>	752	Natural isolate from Chardonnay (1997 vintage)
<i>Candida pulcherrima</i>	825	Natural isolate from Chardonnay (1997 vintage)

Laboratory-scale fermentations

Grape must

Fresh Chardonnay grape must, clarified by pectinases (0.5g/hL Ultrazym, Novazymes, Denmark) at 14°C, was stored at -20°C until needed. The thawed juice was thoroughly mixed and 500 mL aliquots were placed in 750 mL glass bottles. After sterilisation (121°C for 15 min), the bottles were closed tightly with plastic fermentation caps filled with sterile distilled water. Three musts (A, B, C) with different sugar concentrations were used. The chemical analyses were: must A - 21.0°B sugar, 0.50 g/L volatile acidity, 1 mg/L total SO₂; must B - 21.7°B sugar, 0.50 g/L volatile acidity, 1 mg/L total SO₂; and must C - 24.5°B sugar, 0.50 g/L volatile acidity, 0 mg/L total SO₂.

Yeast inoculum and fermentation procedure

Yeast starter cultures were grown for 24 h in YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose). Total cell counts were carried out in a Neubauer improved bright-lined counting chamber (1 mm depth) and all inoculations were done at 1 x 10⁶ cells/mL per yeast strain.

The four non-*Saccharomyces* yeasts were inoculated individually and in combination with the *S. cerevisiae* yeast strain. In the combined fermentations the *Saccharomyces* yeast was inoculated one hour after the non-*Saccharomyces* yeast. A reference fermentation was inoculated with *S. cerevisiae* only. All fermentations were conducted in triplicate and the fermentation vessels were placed on an orbital shaker at an ambient temperature of 20°C.

The fermentations were monitored by CO₂ weight loss and were allowed to proceed until the reference fermentation was dry (14 days). Completion of fermentation (no further weight loss) was confirmed by use of glucose test strips (Clinistix, Bayer). The progression of CO₂ weight loss was used to plot a fermentation curve.

During the course of the combined fermentations 200 µL aliquots were removed from the relevant bottles (must A only) and streaked onto lysine medium (Biolab, Merck) to check for the presence of the non-*Saccharomyces* yeast component.

Small-scale wine production

The four non-*Saccharomyces* yeasts (Table 1) were each investigated in combination with a *S. cerevisiae* (strain VIN 13) yeast for small-scale production of wine in 18 L of freshly prepared must from the 2000 vintage. The yeast cultures were propagated and inoculated in the same way as the laboratory-scale fermenta-

tions, with the exception of the *S. cerevisiae*, where an active dried VIN 13 culture was used (0.04 g/L). The fermentations were done in duplicate.

Three grape musts were used, i.e. Chardonnay (22.2°B, 7.8 g/L total acidity, pH 3.37), Sauvignon blanc (22.9°B, 6.4 g/L total acidity, pH 3.52) and Chenin blanc (21.7°B, 6.7 g/L total acidity, pH 3.71). Di-ammonium phosphate (0.50 g/L) and SO₂ (50 mg/L) were added and the fermentation was conducted at an ambient temperature of 15°C in 20 L stainless steel containers fitted with fermentation caps. After fermentation the wines were racked off the yeast lees and the free SO₂ adjusted to 35 mg/L. Bentonite (0.75 g/L) was added and the wine was cold stabilised (0°C) for one week, filtered and transferred to ten bottles according to standard practices for white-wine production. The wines were stored at 15°C until evaluated.

Chemical analyses

The wines (laboratory and small-scale) were analysed for alcohol (infralyser technique - SGS Wine & Spirit Laboratory, Stellenbosch) and for residual sugar (Rebelein), volatile acidity and SO₂ as described by Iland *et al.* (2000). Testing for glycerol (must B only) was done with enzymatic test kits (Boehringer Mannheim, Roche, Germany). Analyses for esters (volatile component analyses - Research Chemistry, Distell, Stellenbosch) were carried out at the time of the five-month sensory evaluations. The ester values were analysed by the ANOVA method.

Sensory evaluation of small-scale wines

The duplicate wines were evaluated individually, five and 18 months after production, according to the multi-wine preference tasting method (McCloskey *et al.*, 1995) by two different panels of seven trained wine tasters. The wines were given code numbers, chilled to 15°C and presented in international wine-tasting glasses. The individual scores of the duplicate wines were averaged.

RESULTS AND DISCUSSION

Yeast strains used

In a previous investigation four yeast species were found to predominate in samples of clarified Chardonnay grape must before the onset of fermentation (Jolly *et al.*, 2003). As predominant species would be in the best position to influence wine fermentation, four representative strains were randomly selected from the aforementioned isolates and used in this study. Although the teleomorphic forms may originally have been isolated, storage before identification could have led to the loss of the ability to

sporulate. For this reason they were identified as the anamorphic forms (M. Th. Smith, personal communication, 2000).

Laboratory-scale fermentations

The laboratory-scale fermentations were done to mimic commercial-scale fermentations as much as possible. However, the must had to be sterilised so that no *S. cerevisiae* inherent in the must could overgrow the slow-growing inoculated non-*Saccharomyces* yeasts. The placement of the fermentation vessels on an orbital shaker copied the natural turbulence found in large fermentations as a result of the generation of CO₂ (Henschke, 1990). The tightly sealed fermentation caps ensured that no oxygen entered the fermentation vessels. The one-hour lapse between inoculation of the non-*Saccharomyces* yeasts and the *S. cerevisiae* yeast was chosen for practical reasons. Normal winemaking practices do not call for inoculating a wine tank twice due to time constraints on the winemaker during a busy harvest. In addition, a delayed start in alcoholic fermentation can lead to oxidation of the wine and subsequent drop in quality. In order to maximise the effect of the non-*Saccharomyces* yeasts and to minimise any delay in the start of alcoholic fermentation by *S. cerevisiae*, a high inoculum of non-*Saccharomyces* and the short time delay before the inoculation of the *S. cerevisiae* wine yeast, was chosen.

Individual yeast fermentations

The non-*Saccharomyces* yeasts were slower fermenters than the reference yeast (*S. cerevisiae* strain VIN 13) in each of the three musts investigated (data for must B only is presented in Fig. 1). Furthermore, none of them could complete the fermentation within 14 days, as also indicated by high values of residual sugar (Table 2). In decreasing order of fermentation ability, the yeasts were *C. colliculosa*, *C. stellata*, *K. apiculata* and *C. pulcherrima*. *C. colliculosa* can tolerate 10 to 12% (v/v) ethanol (Fleet *et al.*, 1984) and, as the most fermentative species in this investigation, it was able to produce between 9.7 and 12.6% (v/v) alcohol (Table 2). It has been reported that the teleomorphic form of *C. colliculosa* (*Torulasporea delbrueckii*) can produce high levels of acetic acid (Fleet *et al.*, 1984), but the strain used in this study had a volatile acidity production comparable to that of *S. cerevisiae*.

Elevated levels of SO₂ (47 to 60 mg/L) were formed by the *C. colliculosa* strain. They were nearly double those of the reference and other yeasts investigated. This could be detrimental to wine fermentation and quality by inhibiting sensitive wine yeasts and/or malolactic bacteria. The final SO₂ levels in the wine may also be close to or exceed the legal limits, especially as SO₂ is

TABLE 2

Standard wine chemical analyses of single and combined yeast laboratory-scale fermentations in three different Chardonnay musts.

Yeast strain	Chemical analyses ¹						
	Residual sugar (g/L)			Ethanol (% v/v)			Glycerol ² (g/L)
	Must A	Must B	Must C	Must A	Must B	Must C	Must B
<i>S. cerevisiae</i> (reference)	1.9 ± 0.2	2.5 ± 1.4	1.9 ± 0	12.5 ± 0.1	12.6 ± 0.1	14.5 ± 0.1	8.53 ± 0.76
<i>C. colliculosa</i>	24.8 ± 2.8	47.6 ± 5.0	39.8 ± 7.5	11.2 ± 0.1	9.7 ± 0.9	12.6 ± 0.4	7.76 ± 0.99
<i>C. stellata</i>	85.5 ± 0	108.8 ± 4.0	107.6 ± 2.2	7.7 ± 0	5.9 ± 0.2	8.4 ± 0.1	11.11 ± 1.11
<i>K. apiculata</i>	135.0 ± 9.3	134.9 ± 4.1	141.0 ± 1.0	5.4 ± 0.5	4.3 ± 0.1	6.5 ± 0.2	8.33 ± 1.24
<i>C. pulcherrima</i>	158.9 ± 4.4	159.0 ± 1.4	166.7 ± 1.7	3.5 ± 0.1	2.7 ± 0.1	4.6 ± 0.1	5.79 ± 2.04
<i>C. colliculosa</i> / <i>S. cerevisiae</i>	1.9 ± 0.1	2.5 ± 1.1	2.2 ± 0.3	12.4 ± 0.1	12.6 ± 0	14.6 ± 0	6.89 ± 0.08
<i>C. stellata</i> / <i>S. cerevisiae</i>	1.7 ± 0.1	3.1 ± 0.9	2.1 ± 0.2	12.4 ± 0.1	12.6 ± 0.1	14.6 ± 0.1	7.52 ± 0.53
<i>K. apiculata</i> / <i>S. cerevisiae</i>	1.9 ± 0.2	4.4 ± 0.6	2.1 ± 0	12.5 ± 0.1	12.7 ± 0.1	14.6 ± 0	7.94 ± 0.10
<i>C. pulcherrima</i> / <i>S. cerevisiae</i>	1.9 ± 0.1	3.1 ± 0.9	2.6 ± 0.2	12.4 ± 0	12.7 ± 0	14.6 ± 0.1	7.47 ± 1.20
Yeast strain	Volatile acidity (g/L)			Total SO ₂ (mg/L)			
	Must A	Must B	Must C	Must A	Must B	Must C	
	Must A	Must B	Must C	Must A	Must B	Must C	
<i>S. cerevisiae</i> (reference)	0.22 ± 0.05	0.12 ± 0.04	0.36 ± 0.03	26 ± 1	35 ± 6	34 ± 2	
<i>C. colliculosa</i>	0.24 ± 0.01	0.10 ± 0.02	0.30 ± 0.02	50 ± 2	60 ± 1	47 ± 1	
<i>C. stellata</i>	0.80 ± 0	0.61 ± 0.04	1.10 ± 0.03	20 ± 0	25 ± 3	23 ± 1	
<i>K. apiculata</i>	0.89 ± 0.04	0.71 ± 0.02	1.06 ± 0.04	23 ± 1	24 ± 1	22 ± 2	
<i>C. pulcherrima</i>	0.24 ± 0.01	0.20 ± 0.03	0.27 ± 0.02	21 ± 1	21 ± 1	21 ± 3	
<i>C. colliculosa</i> / <i>S. cerevisiae</i>	0.14 ± 0.01	0.13 ± 0.03	0.22 ± 0.03	28 ± 2	38 ± 2	32 ± 1	
<i>C. stellata</i> / <i>S. cerevisiae</i>	0.19 ± 0.03	0.17 ± 0.05	0.35 ± 0.03	27 ± 1	39 ± 2	34 ± 1	
<i>K. apiculata</i> / <i>S. cerevisiae</i>	0.18 ± 0.02	0.10 ± 0.04	0.31 ± 0.02	27 ± 1	39 ± 3	33 ± 1	
<i>C. pulcherrima</i> / <i>S. cerevisiae</i>	0.21 ± 0.01	0.33 ± 0.17	0.34 ± 0.02	28 ± 2	37 ± 0	37 ± 3	

¹ Average value of three fermentations ± standard deviation. Original sugar: must A=21.0°B; must B=21.7°B; and must C=24.5°B.

² Glycerol analyses done on must B only.

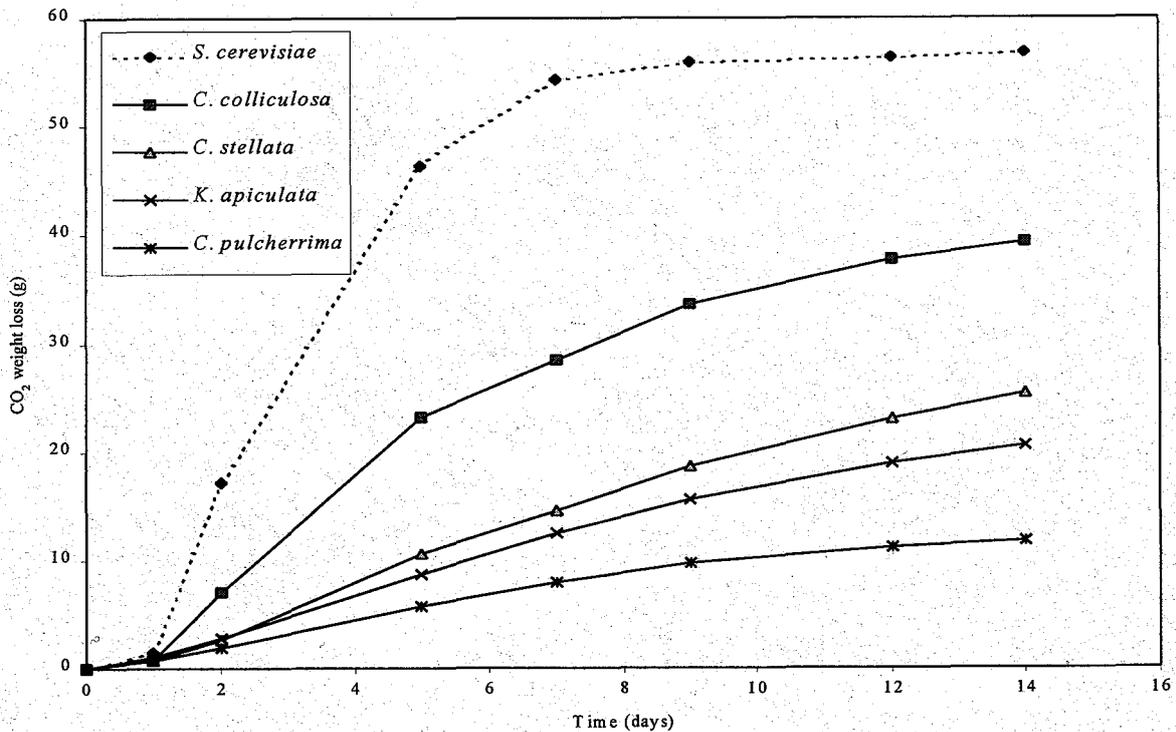


FIGURE 1

Fermentation curves of non-*Saccharomyces* yeasts in a Chardonnay must (must B) compared to a reference yeast (*S. cerevisiae* strain VIN 13).

normally added during the winemaking process. The glycerol levels were very similar to that of *S. cerevisiae* (Table 2).

In contrast, *C. stellata* did not form excessive SO₂, while producing between 5.9 and 8.4% (v/v) alcohol (Table 2). This is lower than the 10% (v/v) alcohol that is accepted as the maximum produced by *C. stellata* (Gao & Fleet, 1998; Jackson, 1994). High levels of glycerol (11.11 g/L) were produced by this strain of *C. stellata*, compared to *S. cerevisiae* (8.53 g/L) (Table 2). This could positively benefit the mouth-feel (smoothness) and complexity of a wine (Scanes *et al.*, 1998; Prior *et al.*, 2000). However, increased glycerol production is usually paired with higher acetic acid production due to the cell having to maintain its redox balances (Prior *et al.*, 2000). Production of volatile acidity by *C. stellata* is well known (Jackson, 1994). In this study volatile acidity levels were close to and, for must C, exceeded the sensory threshold (approximately 0.8 g/L) and would obviously have a negative effect on the sensory character of a wine.

K. apiculata is generally known as a high producer of volatile acidity (Van Zyl *et al.*, 1963) and can have a significant negative effect on the chemical composition and therefore the quality of wine (Gil *et al.*, 1996). However, this is strain dependent and Romano *et al.* (1992) showed that some strains produced less than 1 g/L volatile acidity, which is comparable to the production by *S. cerevisiae*. As was expected, the strain used in this study produced high levels of volatile acidity (0.71 to 1.06 g/L) (Table 2). Ethanol production was between 4.3 and 6.5% (v/v). This is similar to that documented by Jackson (1994). It has also been reported that *K. apiculata* differs from *S. cerevisiae* in the levels of higher alcohols (n-propanol, iso-butanol, iso-amyl alcohol, and active amyl alcohol) produced (Romano *et al.*, 1992). *K. apicula-*

ta can also produce high concentrations of esters (Bisson & Kunkee, 1991). These all have potential importance for imparting flavour to the wine.

C. pulcherrima was the yeast that was the least able to ferment the grape must (Fig. 1 and Table 2). This was to be expected from a yeast known to have an oxidative metabolism (Longo *et al.*, 1991). During its limited growth, only low levels of ethanol were produced, while high levels of volatile acidity or SO₂ were not noted. This species can form relatively high concentrations of esters and some fusel oils (Bisson & Kunkee, 1991), which may make a positive contribution to wine flavour.

Combined yeast fermentations

The combined yeast fermentation curves (data for must B only are shown in Fig. 2) were very similar to that of the *S. cerevisiae* reference and all the fermentations proceeded to dryness (< 4 g/L residual sugar) (Table 2). The other chemical analyses, i.e. alcohol and volatile acidity (Table 2), for the combined fermentations are similar to the reference fermentation, with the exception of the glycerol concentrations, which were lower. However, the characteristic chemical parameters due to the growth of the individual non-*Saccharomyces* yeasts were absent. This scenario was also reported by Moreno *et al.* (1991), when *S. cerevisiae* and *T. delbrueckii* (teleomorph of *C. colliculosa*) yeasts were utilised in single and mixed-culture fermentations. This can be due to the suppression of the non-*Saccharomyces* yeasts by the *Saccharomyces* yeasts during the fermentation, or the utilisation of the non-*Saccharomyces* metabolites by *S. cerevisiae*. In the combined fermentations in this study the non-*Saccharomyces* yeasts could be detected until the fifth day of fermentation (data not shown). The exception was *C. pulcherrima*, the least fermentative

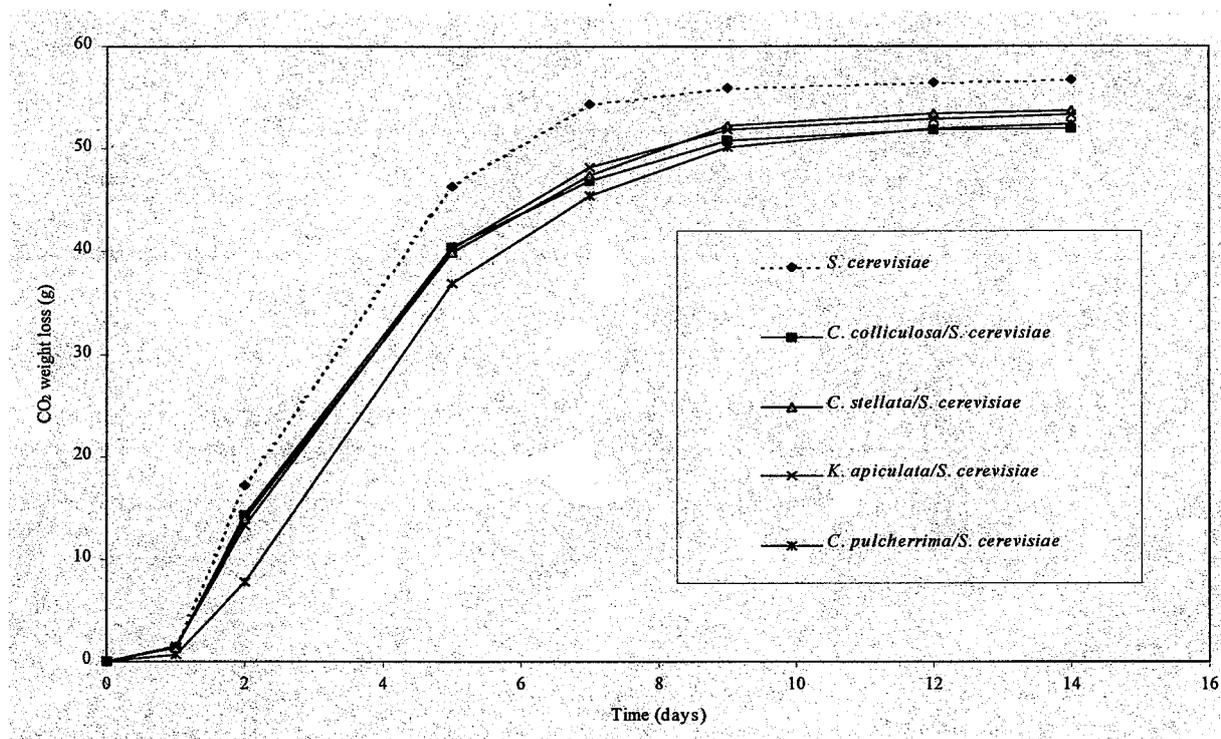


FIGURE 2

Fermentation curves of combined non-*Saccharomyces/S. cerevisiae* yeasts in a Chardonnay must (must B) compared to a reference yeast (*S. cerevisiae* strain VIN 13 only).

of the four species investigated, which could be detected until day 9. This yeast, with preferred aerobic growth, would have been expected to die off earlier in the fermentation.

In contrast, Ciani & Ferraro (1998) and Ferraro *et al.* (2000) reported an improvement in the analytical chemical profile of small-scale Pinot grigio and pilot-scale Trebbiano Toscano wines, respectively. They used immobilised *C. stellata* cells in conjunction with *S. cerevisiae* in a sequential fermentation and found that the interaction of the *C. stellata* and *S. cerevisiae* metabolisms led to wines having not only higher glycerol levels, but also lower acetic acid levels. Their inoculation and fermentation strategy, however, differed in that a higher inoculum of *C. stellata* (5×10^8 to 1×10^9 cells/mL) was allowed to grow for three days before inoculation with *S. cerevisiae* at 1 to 5×10^6 cells/mL. The growth of indigenous non-*Saccharomyces* yeasts, but not indigenous *S. cerevisiae*, was controlled by the use of SO₂ during the three-day period. The authors did not report on the sensory characteristics of the wines.

Small-scale wine production

Chemical analyses

Similar to the laboratory-scale fermentations, the chemical analyses of the wines (Table 3) showed basically no difference between the reference fermentation and the non-*Saccharomyces* combinations. The higher total SO₂ of the wines is a reflection of the SO₂ added during the winemaking process. All the wine fermentations proceeded to dryness.

As some non-*Saccharomyces* yeasts are reported to be high ester producers, the wines were all analysed for total esters at the time of the five-month sensory evaluation. In the Chardonnay wines (Table 4), the combined fermentations showed a higher

concentration of total esters than the reference wine; however, it was only significant for the *K. apiculata* and *C. stellata* combinations. The values remain higher even after the ethyl acetate and ethyl lactate values are subtracted. In contrast, the total esters of the Sauvignon blanc wines (Table 5) were generally lower for the combined fermentations, but only of significance for the *K. apiculata* combination. The *C. colliculosa* combination produced a similar amount of total esters as the reference wine. The total esters in the Chenin blanc wine showed no significant differences; however, the *K. apiculata* combination was higher due to the ethyl acetate component (Table 6).

Sensory analyses

Very little is reported in the literature on the sensory results of wines produced with the use of non-*Saccharomyces* yeasts. However, in a report by Soden *et al.* (2000) *C. stellata* was used sequentially with *S. cerevisiae* to produce Chardonnay wines with an aroma profile derived from the metabolism of both yeasts. Their strategy involved fermentation by *C. stellata* for fifteen days before inoculation with *S. cerevisiae*. The resultant wine was not judged for overall quality, but a descriptive sensory analyses shows that in comparison to a *S. cerevisiae* reference wine, the intensities for "floral", "banana", "lime" and "tropical fruit" aroma nuances were lower and "honey", "sauerkraut" and "ethyl acetate" were higher. The last two aroma nuances mentioned are grouped under "microbiological" and "oxidised" according to the Wine Aroma Wheel (Noble *et al.*, 1987) and, as negative aroma nuances, they would detract from an increased quality rating. A co-inoculated (5×10^6 cells/mL) wine produced with the same two yeasts appeared to produce a wine with less of the negative aroma nuances. Based on these results, Soden *et al.* (2000) sug-

TABLE 3

Chemical analyses of small-scale wines produced by non-Saccharomyces/S. cerevisiae combinations.

Yeast species combination	Analyses ¹				
	Chardonnay				
	Ethanol (% v/v)	Volatile acidity (g/L)	Total SO ₂ (mg/L)	Residual sugar (g/L)	Glycerol (g/L)
<i>S. cerevisiae</i> (reference)	13.3 (13.2-13.3)	0.26 (0.26-0.26)	61 (57-65)	1.6 (1.4-1.8)	5.55 (5.45-5.65)
<i>C. colliculosa/S. cerevisiae</i>	13.3 (13.2-13.3)	0.26 (0.25-0.27)	61 (59-62)	1.9 (1.8-1.9)	5.65 (5.48-5.82)
<i>C. stellata/S. cerevisiae</i>	13.6 (13.5-13.6)	0.25 (0.24-0.26)	61 (53-69)	1.7 (1.5-1.8)	5.88 (5.73-6.03)
<i>K. apiculata/S. cerevisiae</i>	13.4 (13.4-13.4)	0.28 (0.28-0.28)	57 (55-58)	1.8 (1.8-1.8)	5.65 (5.57-5.73)
<i>C. pulcherrima/S. cerevisiae</i>	13.4 (13.4-13.4)	0.26 (0.25-0.26)	64 (61-66)	1.8 (1.4-2.1)	5.89 (5.65-6.14)
Yeast species combination	Sauvignon blanc				
	Ethanol (% v/v)	Volatile acidity (g/L)	Total SO ₂ (mg/L)	Residual sugar (g/L)	Glycerol (g/L)
	<i>S. cerevisiae</i> (reference)	13.7 (13.7-13.7)	0.23 (0.23-0.23)	82 (80-83)	0.5 (0.4-0.6)
<i>C. colliculosa/S. cerevisiae</i>	13.7 (13.7-13.7)	0.21 (0.20-0.22)	74 (72-75)	1.2 (0.2-2.2)	6.08 (6.02-6.14)
<i>C. stellata/S. cerevisiae</i>	13.3 (13.0-13.6)	0.25 (0.23-0.26)	82 (75-88)	1.0 (0.9-1.1)	6.10 (5.94-6.25)
<i>K. apiculata/S. cerevisiae</i>	13.7 (13.7-13.7)	0.26 (0.25-0.26)	79 (70-88)	1.5 (1.5-1.5)	5.95 (5.86-6.04)
<i>C. pulcherrima/S. cerevisiae</i>	13.7 (13.6-13.7)	0.24 (0.23-0.24)	78 (77-78)	1.3 (1.2-1.3)	6.15 (6.07-6.23)
Yeast species combination	Chenin blanc				
	Ethanol (% v/v)	Volatile acidity (g/L)	Total SO ₂ (mg/L)	Residual sugar (g/L)	Glycerol (g/L)
	<i>S. cerevisiae</i> (reference)	13.2 (13.1-13.2)	0.19 (0.18-0.19)	104 (104-104)	1.2 (1.0-1.4)
<i>C. colliculosa/S. cerevisiae</i>	13.0 (13.0-13.1)	0.18 (0.18-0.18)	98 (96-99)	1.3 (1.2-1.4)	5.51 (5.42-5.59)
<i>C. stellata/S. cerevisiae</i>	13.0 (13.0-13.0)	0.20 (0.19-0.20)	95 (91-99)	1.3 (1.1-1.5)	5.82 (5.75-5.88)
<i>K. apiculata/S. cerevisiae</i>	13.1 (13.1-13.1)	0.21 (0.20-0.21)	95 (94-96)	1.6 (1.5-1.6)	6.10 (6.10-6.10)
<i>C. pulcherrima/S. cerevisiae</i>	13.0 (12.9-13.0)	0.19 (0.18-0.19)	98 (94-101)	0.9 (0.2-1.6)	5.53 (5.50-5.56)

¹ Average value of two fermentations. Range indicated in brackets.

TABLE 4

GC analyses of small-scale Chardonnay wines of the 2000 vintage produced by non-Saccharomyces/S. cerevisiae combinations.

Yeast combination	Total esters (mg/L)	Ethyl acetate (mg/L)	Total esters - ethyl acetate (mg/L)	Ethyl lactate (mg/L)	Total esters - ethyl acetate - ethyl lactate (mg/L)
<i>S. cerevisiae</i> (reference)	346.69b ¹	299.14b	47.56b	12.99a	34.57b
<i>C. colliculosa/S. cerevisiae</i>	376.40ab	324.04ab	52.36ab	13.77a	38.59ab
<i>C. stellata/S. cerevisiae</i>	402.42a	346.02a	56.41a	14.83a	41.58a
<i>K. apiculata/S. cerevisiae</i>	398.70a	341.08a	57.62a	14.00a	43.62a
<i>C. pulcherrima/S. cerevisiae</i>	382.67ab	324.45ab	58.22a	14.86a	43.37a

¹ Values within columns followed by the same letter do not differ significantly (p < 0.05).

gested that the controlled use of non-Saccharomyces yeasts, such as *C. stellata*, could lead to wines of greater complexity and flavour diversity. Strain selection obviously would be very important. No sensory data were given on the wines produced by Ferraro *et al.* (2000).

In this study the wines were judged for quality according to the

method of McCloskey *et al.* (1995). Overall, the different combinations of yeasts produced wines with different average relative qualities (Table 7). For the Chardonnay all the non-Saccharomyces combinations produced wines with a lesser quality than the reference wine when judged at five and 18 months, even though the wines differed from each other. The high levels of

TABLE 5

GC analyses of small-scale Sauvignon blanc wines of the 2000 vintage produced by non-*Saccharomyces/S. cerevisiae* combinations.

Yeast combination	Total esters (mg/L)	Ethyl acetate (mg/L)	Total esters - ethyl acetate (mg/L)	Ethyl lactate (mg/L)	Total esters - ethyl acetate - ethyl lactate (mg/L)
<i>S. cerevisiae</i> (reference)	384.27a ¹	331.60a	52.67a	14.10a	38.56a
<i>C. colliculosa/S. cerevisiae</i>	384.00a	331.82a	52.18ab	14.06a	38.12a
<i>C. stellata/S. cerevisiae</i>	371.42ab	320.95a	50.47b	13.51a	36.97b
<i>K. apiculata/S. cerevisiae</i>	349.92b	299.08a	50.85ab	12.71a	38.13a
<i>C. pulcherrima/S. cerevisiae</i>	373.26ab	321.24a	52.02ab	14.73a	37.30b

¹ Values within columns followed by the same letter do not differ significantly (p< 0.05).

TABLE 6

GC analyses of small-scale Chenin blanc wines of the 2000 vintage produced by non-*Saccharomyces/S. cerevisiae* combinations.

Yeast combination	Total esters (mg/L)	Ethyl acetate (mg/L)	Total esters - ethyl acetate (mg/L)	Ethyl lactate (mg/L)	Total esters - ethyl acetate - ethyl lactate (mg/L)
<i>S. cerevisiae</i> (reference)	216.06a ¹	176.35a	39.71a	11.41a	28.30a
<i>C. colliculosa/S. cerevisiae</i>	213.17a	174.27a	38.90a	10.32a	28.58a
<i>C. stellata/S. cerevisiae</i>	205.65a	168.65a	36.99a	9.66a	27.33a
<i>K. apiculata/S. cerevisiae</i>	250.74a	209.42a	41.32a	13.23a	28.09a
<i>C. pulcherrima/S. cerevisiae</i>	231.62a	188.32a	43.31a	13.97a	29.64a

¹ Values within columns followed by the same letter do not differ significantly (p<0.05).

TABLE 7

Relative score of small-scale wines produced by non-*Saccharomyces/S. cerevisiae* combinations and evaluated by the multi-wine preference method¹.

Yeast combination	Cultivar / Time of evaluation / Relative score ²					
	Chardonnay		Sauvignon blanc		Chenin blanc	
	5 months	18 months	5 months	18 months	5 months	18 months
<i>S. cerevisiae</i> (reference)	3 (3, 3)	2 (1, 3)	-1 (-1, -1)	-3 (-3, -3)	-2 (-5, 1)	-1 (-5, 3)
<i>C. colliculosa/S. cerevisiae</i>	2 (1, 3)	0 (-1, 1)	3 (1, 5)	1 (-1, 3)	-1 (-1, -1)	3 (3, 3)
<i>C. stellata/S. cerevisiae</i>	-1 (-2, 0)	-1 (-5, 3)	-3 (-5, -1)	-1 (-1, -1)	1 (-1, 3)	-4 (-5, -3)
<i>K. apiculata/S. cerevisiae</i>	1 (0, 2)	0 (-3, 3)	0 (-3, 3)	3 (3, 3)	0 (-3, 3)	-3 (-5, -1)
<i>C. pulcherrima/S. cerevisiae</i>	0 (-1, 1)	0 (-1, 1)	1 (-1, 3)	0 (-1, 1)	2 (1, 3)	5 (5, 5)

¹ McCloskey *et al.*, 1995.² Average values of two wines (seven judges). Range indicated in brackets. Only values within a column are related to each other. Highest score within a column is indicated in bold type.

ethyl acetate formed by the non-*Saccharomyces* yeasts (Table 4) would have played a role in this regard.

For the Sauvignon blanc wines (Table 7) judged at five months, the *K. apiculata*, *C. pulcherrima* and *C. colliculosa* combinations were all judged to be of better quality than the reference wine. At 18 months all four of the non-*Saccharomyces* combination Sauvignon blanc wines were judged better, showing that the wine's flavour was still developing.

In the Chenin blanc wine (Table 7) all the non-*Saccharomyces* combinations were judged to be better at five months. However, this could not be supported by the ester analyses (Table 6). At 18

months only the *C. pulcherrima* and *C. colliculosa* combinations were judged to be of better quality than the reference.

It thus would appear that specific non-*Saccharomyces/S. cerevisiae* combinations produce wines with increased quality from different grape varieties. Obviously, a large number of winemaking factors, including temperature, SO₂ levels, *S. cerevisiae* strain, time of inoculation and inoculum concentration, can all play a role in the contribution made by non-*Saccharomyces* yeasts. Lower fermentation temperatures (below 25°C) can enhance the ethanol tolerance of yeasts such as *K. apiculata* and *C. stellata* (Fleet, 1990; Bisson & Kunkee, 1991), and their sus-

tained growth may allow sensorially positive metabolites to become evident. The opposite can also be true and sensorially negative metabolites can detract from wine quality as can be seen in the sensory results of Soden *et al.* (2000). This underlines the importance of a thorough screening and selection programme to choose the most appropriate non-*Saccharomyces* strains.

The random selection of four isolates in this study did result in some incidences of increased wine quality. While ester levels could not be linked to increased quality, numerous other compounds also play a role in wine flavour. More in-depth analyses of the wines could in future elucidate the compounds that may have a subtle impact on wine flavour. Subsequently, the desired indigenous yeast species could be screened for the ability to produce the compounds linked to increased wine quality.

The growth rate of individual species will also determine the extent of their contribution to flavour development (Heard, 1999). Slower-growing yeasts would need a longer period of unhindered growth before inoculation with *S. cerevisiae*. The nutrient composition of individual musts, which can vary over vintages and geographical areas and be influenced by viticultural practices, can also impact on the ability of non-*Saccharomyces* yeasts to contribute to the fermentation. Furthermore, the interactions of the metabolism of the different yeasts with each other should also not be overlooked.

CONCLUSIONS

An improvement in wine quality was achieved by the use of four non-*Saccharomyces* yeasts, even though they were not specifically selected. These improvements were coupled to grape cultivar. Furthermore, the increase in quality was achieved despite the inoculation protocol that primarily addressed cellar practices and not optimal contribution by the non-*Saccharomyces* yeasts. Improved quality could not be linked to standard chemical analyses of the wine. Further research is now necessary to identify marker components that can be used in turn for selection of non-*Saccharomyces* yeasts. This selection, comprising grape cultivar-specific non-*Saccharomyces/S. cerevisiae*-combinations, coupled to a specific inoculation protocol (i.e. time between inoculation of the non-*Saccharomyces* and *S. cerevisiae*), could be successfully used to develop new wine styles, improve aroma of wines with a history of mediocrity, or enhance specific 'terroir'-related characteristics.

LITERATURE CITED

- Boulton, R.B., Singleton, V.L., Bisson, L.F. & Künkee, R.E., 1996. Principles and practices of winemaking. Chapman & Hall, New York.
- Bisson, L.F. & Künkee, R.E., 1991. Microbial interactions during wine production. In: Zeikus, J.G. & Johnson, E.A. (eds). Mixed cultures in biotechnology, McGraw-Hill, Inc., New York. pp. 39-68.
- Ciani, M. & Ferraro, L., 1998. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. J. Appl. Microbiol. 85, 247-54.
- Ferraro, L., Faticenti, F. & Ciani, M., 2000. Pilot scale vinification process using immobilized *Candida stellata* cells and *Saccharomyces cerevisiae*. Process Biochem. 35, 1125-1129.
- Fleet, G.H., 1990. Which yeast species really conducts the fermentation? In: Williams, P.J., Davidson, D.M. & Lee, T.H. (eds). Proc. 7th Aust. Wine Ind. Tech. Conf., 13-17 August 1989, Adelaide, Australia. pp. 153-156.
- Fleet, G.H., Lafon-Lafourcade, S. & Ribéreau-Gayon, P., 1984. Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. Appl. Environ. Microbiol. 48, 1034-1038.
- Gao, C. & Fleet, G.H., 1988. The effects of temperature and pH on the ethanol tolerance of wine yeasts, *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata*. J. Appl. Bacteriol. 65, 405-409.
- Gil, J.V., Mateo, J.J., Jimenez, M., Pastor, A. & Huerta, T., 1996. Aroma compounds in wines as influenced by apiculate yeasts. J. Food Sci. 61, 1247-1249, 1266.
- Heard, G., 1999. Novel yeasts in winemaking – looking to the future. Food Aus. 51, 347-352.
- Henick-Kling, T., Edinger, W., Daniel, P. & Monk, P., 1998. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. J. Appl. Microbiol. 84, 865-876.
- Henschke, P.A., 1990. Evaluating wine yeasts for improved wine quality. In: Williams, P.J., Davidson, D.M. & Lee, T.H. (eds). Proc. 7th Aust. Wine Ind. Tech. Conf., 13-17 August 1989, Adelaide, Australia. pp. 157-165.
- Iland, P., Ewart, A., Sitter, J., Maskides, A. & Bruer, N., 2000. Techniques for chemical analysis and quality monitoring during winemaking. Patrick Iland Promotions, Cambelltown, Australia.
- Jackson, R.S., 1994. Wine science – principles and applications. Academic Press, San Diego.
- Jolly, N.P., Augustyn, O.P.H. & Pretorius, I.S., 2003. The occurrence of non-*Saccharomyces* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. S. Afr. J. Enol. Vitic. 24, 35-42.
- Longo, E., Cansado, J., Agrelo, D. & Villa, T.G., 1991. Effect of climatic conditions on yeast diversity in grape musts from Northwest Spain. Am. J. Enol. Vitic. 42, 141-144.
- Lonvaud-Funel, A., 1996. Microorganisms of winemaking. Cerevisia: Belgian J. Brew. Biotechnol. 21, 55-58.
- Martini, A., Ciani, M. & Scorzetti, G., 1996. Direct enumeration and isolation of wine yeasts from grape surfaces. Am. J. Enol. Vitic. 47, 435-440.
- McCloskey, L., Arrhenius, S.P. & Sylvan, M., 1995. New method to assess wine quality. Practical Winery Vineyard 16, 30-33.
- Moreno, J.J., Millán, C., Ortega, J.M. & Medina, M., 1991. Analytical differentiation of wine fermentations using pure and mixed yeast cultures. J. Ind. Microbiol. 7, 181-190.
- Mortimer, R. & Polsinelli, M., 1999. On the origins of wine yeast. Res. Microbiol. 150, 199-204.
- Noble, A.C., Arnold, R.A., Buechsenstein, J., Leach, E.J., Schmidt, J.O. & Stern, P.M., 1987. Modification of a standardized system of wine aroma terminology. Am. J. Enol. Vitic. 38, 134-146.
- Peynaud, E. & Domercq, S., 1959. A review of microbiological problems in winemaking in France. Am. J. Enol. Vitic. 10, 69-77.
- Pretorius, I.S., Van der Westhuizen, T.J. & Augustyn, O.P.H., 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry – a review. S. Afr. J. Enol. Vitic. 20, 61-74.
- Prior, B.A., Toh, T.H., Jolly, N., Baccari, C.L. & Mortimer, R.K., 2000. Impact of yeast breeding for elevated glycerol production on fermentation activity and metabolite formation in Chardonnay. S. Afr. J. Enol. Vitic. 21, 92-99.
- Romano, P., Suzzi, G., Comi, G. & Zironi, R., 1992. Higher alcohol and acetic acid production by apiculate wine yeasts. J. Appl. Bacteriol. 73, 126-130.
- Romano, P., Suzzi, G., Comi, G., Zironi, R. & Maifreni, M., 1997. Glycerol and other fermentation products of apiculate wine yeasts. J. Appl. Microbiol. 82, 625-618.
- Scanes, K.T., Hohmann, S. & Prior, B.A., 1998. Glycerol production by the yeast *Saccharomyces cerevisiae* and its relevance to wine: a review. S. Afr. J. Enol. Vitic. 19, 15-21.
- Soden, A., Francis, I.L., Oakey, H. & Henschke, P.A., 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Aust. J. Grape Wine Res. 6, 21-30.
- Török, T., Mortimer, R.K., Romano, P., Suzzi, G. & Polsinelli, M., 1996. Quest for wine yeasts – an old story revisited. J. Indust. Microbiol. 17, 303-313.
- Van Zyl, J.A., de Vries, M.J. & Zeeman, A.S., 1963. The microbiology of South African winemaking III. The effect of different yeasts on the composition of fermented musts. S. Afr. Agric. Sci. 6, 165-179.
- Vaughan-Martini, A. & Martini, A., 1995. Facts, myths and legends on the prime industrial microorganism. J. Indust. Microbiol. 14, 514-522.