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ORIGINAL PAPER

Influence of wine-related physicochemical factors on the growth and metabolism of non-*Saccharomyces* and *Saccharomyces* yeasts in mixed culture

Lucía M. Mendoza · María C. Manca de Nadra · Elena Bru · Marta E. Farías

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Abstract The influence of two physicochemical factors involved in winemaking, temperature and SO_2, on the kinetics and metabolic behavior of *Kloeckera apiculata* and *Saccharomyces cerevisiae* was examined. Highest biomass was reached at 15 and 25°C for *K. apiculata* and *S. cerevisiae*, respectively. Pure cultures of *K. apiculata* died off early with increasing temperature, but in co-culture with *S. cerevisiae* it showed higher viability and a change in the death curve from exponential to linear. Statistical analysis revealed that metabolite production was significantly different for the three cultures and also at the different fermentation temperatures. Besides, the interaction between culture type and temperature was significant. At temperatures from 15 to 30°C the mixed culture showed similar ethanol and lower acetic acid production compared with a pure culture of *K. apiculata*. SO_2 addition slightly increased survival of the non-*Saccharomyces* species in pure and mixed cultures. Statistical evaluation indicated that culture type and SO_2 addition significantly affected metabolite production, but the interaction between culture and SO_2 was not significant. These results contribute to current knowledge of enological factors and their effect on prevalence and fermentative activities of the composite yeast flora and the statistical significance emphasizes the importance of the combined influence of the culture type and physicochemical factors on the production of fermentation metabolites.

Keywords *K. apiculata* · Mixed culture · Temperature · Sulfur dioxide · Winemaking

L. M. Mendoza · M. C. M. de Nadra · E. Bru · M. E. Farías
Centro de Referencia para Lactobacillus (CERELA),
Chacabuco 145, 4000 San Miguel de Tucumán, Argentina

M. C. M. de Nadra · M. E. Farías (✉)
Facultad de Bioquímica, Química y Farmacia,
Universidad Nacional de Tucumán, Ayacucho 491,
4000 San Miguel de Tucumán, Argentina
e-mail: mfarias@cerela.org.ar

Introduction

In the production of wine, natural grape juice fermentation is carried out by a succession of different yeast populations. Recently, several groups have examined various non-*Saccharomyces* yeasts as potential adjuncts (or alternatives) to *Saccharomyces cerevisiae* in an effort to modify wine flavor and improve product quality [18, 21, 31]. Apiculate wine yeasts (*K. apiculata*/*Hanseniaspora uvarum* and *Hanseniaspora guilliermondii*) have become an object of interest as they are frequently found in grapes and are also dominators of the early stages of must fermentation [15, 17]. Their intolerance to high concentrations of ethanol, the high sugar concentration and the low available oxygen conditions during fermentation are the main reasons why *S. cerevisiae* becomes dominant and keeps its activity until the end of fermentation [8, 10, 14, 18, 23, 32]. Mendoza et al. [24] reported that non-*Saccharomyces* apiculate yeast and *Saccharomyces* strain growing together reached lower maximal biomass than its respective pure cultures, but the apiculate cell showed longer survival. Thus, the metabolites formed by this non-*Saccharomyces* species may contribute to wine quality. The persistence of non-*Saccharomyces* fermentation species during fermentation may depend, however, upon many factors. The fermentation temperature is one of the important vinification factors that affect the rate of yeast growth and the alcoholic fermentation. Also the number of different species, as well as their endurance

during alcoholic fermentation, is conditioned by both the temperature of the must and the temperature during fermentation. These changes determine the chemical and organoleptic qualities of the wine [14].

Addition of SO_2 to grapes or must to control oxidation reactions and restrict the growth of the indigenous yeast population is a well established practice in winemaking [1] and so is inoculation of the grape juice with selected yeasts. Sulfur dioxide is highly toxic to most non-*Saccharomyces* yeasts, while production strains of *Saccharomyces* in general are quite resistant to it [2, 13, 30, 36]. The total concentration of SO_2 in grape juice during fermentation consists of bound and free forms. The free SO_2 will consist mostly in bisulfite anion and a small proportion of molecular SO_2. The undissociated molecular form of free sulfur dioxide is the most important antimicrobial agent. At pH 3.0–4.0, it exists mainly as the bisulfite ion (94–98%), and only a very small proportion (2–6%) occurs in the molecular form [30]. Generally, it accepts that 0.5 to 2.0 mg/l of molecular SO_2 is necessary to obtain a good biological stability.

Several studies have reported on the influence of vinification conditions on the dynamics of yeast populations and their effects on the characteristics of finished wines [10, 11, 23]. However, few studies have carried out a statistical evaluation of the effect of enological factors on growth and physiology of the microorganisms involved [27].

The aim of the present work was to study the influence of different fermentation temperatures and sulfur dioxide concentrations on the kinetics and metabolic behavior of a mixed culture of *Saccharomyces* and non-*Saccharomyces* wine yeasts. Statistical analysis was applied to evaluate the correlation between the different factors and culture type on the fermentative activities of the microorganisms.

Materials and methods

Microorganisms

Kloeckera apiculata mc1 and *S. cerevisiae* mc2 were isolated from Argentinean wines.

Culture medium and growth conditions

The wine yeasts were inoculated in basal medium (10 g/l yeast extract, 1 ml/l tween 80 and 170 ml/l grape juice), pH was adjusted to 3.5. The medium was heated at 90°C for 10 min and then inoculated with 10^6 cells/ml of 14 h pre-cultures grown in the same medium as follows: (1) pure cultures of *K. apiculata* and *S. cerevisiae* and (2) a mixed culture of both yeast strains cultured at a 1:1 ratio.

All cultures were incubated statically at 25°C during 10 days. When the temperature effect was studied, incubation was carried out at 15, 25, 30 and 35°C.

In order to study the influence of SO_2 the basal medium was supplemented with 50, 100 and 250 mg/l of sodium metabisulfite.

Samples were taken every day during fermentation and yeast growth was monitored by successive dilutions. Differential enumeration of wine yeasts was carried out by plating them onto selective malt agar medium (20 g/l glucose, 1 g/l peptone, 20 g/l yeast extract, 3 g/l malt extract and 20 g/l agar); pH 6.8. This medium allows non-*Saccharomyces* species to be distinguished morphologically from *Saccharomyces* colonies. Plates were incubated at 25°C for 3–4 days. *S. cerevisiae* developed brilliant, white-cream colonies with spherical and protuberant forms and regular borders. *K. apiculata* colonies were of an opaque white–gray color, smoothed or flattened with slightly irregular borders, which differed in tonality of the rest of the colony [16].

Analytical determinations

Determination of the concentrations of sugars and fermentation products

Cell-free samples were obtained by centrifugation of the growth medium. Samples were stored at −20°C until analysis. The ethanol, glucose, fructose, acetic acid and glycerol concentrations were determined by HPLC (ISCO 2350, Software Peak Simple II) using a column HPX.87H (Bio-Rad, 300 × 7.8 mm). The column was eluted at 45°C with a degassed mobile phase containing 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. All the compounds were determined with a RI detector (Knauer).

Samples were analyzed in duplicate. The identification and quantification of compounds were carried out by comparing retention time and concentration with standard solution (Sigma).

Determination of the concentration of free and total sulfur dioxide

Sulfur dioxide present in natural grape juice medium was determined by the Ripper Method [37]. To determine free sulfur dioxide, 25 ml of the sample was transferred to an Erlenmeyer flask, add approximately 5 ml of starch indicator and a pinch of bicarbonate, add 5 ml diluted sulfuric acid and rapidly titrate with standard iodine solution to blue end point that is stable for approximately 20 s. Total sulfur dioxide was evaluated by first treating the sample with sodium hydroxide to release bound sulfur dioxide, add 25 ml of 1 N NaOH, swirl the solution and stopper the flask. Allow 10 min for the hydrolysis reaction to occur.

$$SO_2(mg/l) = \frac{[I_2(ml)][I_2(N)](32)(1,000)}{sample(ml)}$$

Statistical analysis

Fitting of yeast death curves

The death curves of pure and mixed cultures of both yeasts were fitted using the equations previously described by Mendoza et al. [24]. The fittings were carried out with Statistica for Windows, Release 7.0, using the Levenberg-Marquardt least-squares algorithm [34].

Analysis of sugar consumption rate

Differences in sugar utilization among the three culture types at different fermentation temperatures were determined using analysis of variance (ANOVA). Significant differences were subsequently analyzed using Tukey's test ($P < 0.05$).

Analysis of fermentation metabolite production

A multivariate analysis of variance (MANOVA) was applied to the experimental data. Afterward, the Hotelling–Bonferroni test was used to determine significant differences; data were considered different if $P < 0.05$. Principal components analysis (PCA) was used to simplify the interpretation of the results and was presented in biplot graphics.

All statistical analyses were performed using a professional version of Infostat software.

Results and discussion

The use of controlled multistarter fermentations in winemaking has been proposed to obtain a more complex aroma and improve the quality of wines [5, 7, 12, 33]. For the practical application of this biotechnological process, it is necessary to determine the influence of some of the fundamental fermentation parameters on the growth and metabolic activity of the microorganisms involved. In the current study, the influence of two environmental parameters, i.e. temperature and SO_2, on survival and physiological behavior of *S. cerevisiae* and *K. apiculata* in a mixed culture was studied.

Influence of fermentation temperature on growth of non-*Saccharomyces* and *Saccharomyces* yeasts

Table 1 represents the growth kinetics parameters of pure and mixed cultures of both wine yeasts at different incubation temperatures.

The growth rate of *K. apiculata* and *S. cerevisiae* in pure and mixed cultures increased with increasing temperature (until 30°C). At the temperatures assayed, maximum cell density of both yeasts in pure and composite cultures was obtained after 24 h, except for the elliptic yeast, which reached the maximum population after 7 days when incubated at 15°C. Regardless of the culture type (pure or mixed) the highest biomass was achieved at 15 and 25°C for *K. apiculata* and *S. cerevisiae*, respectively. These results are in agreement with findings in previous studies. Heard and Fleet [19] observed that *K. apiculata* grew and survived better in fermentations performed below 20°C and dominated fermentations at 10°C. However, *S. cerevisiae* exhibited higher cell population and kinetics at temperatures between 20 and 30°C. Similar results have been obtained in cider production [3]. Erten [12] has pointed out that *K. apiculata* dominated over *S. cerevisiae* and survived longer at low temperatures compared to fermentations conducted above 20°C.

It is important to mention that independently of the temperature, both yeasts in co-culture conditions exhibited

Table 1 Effect of fermentation temperature on growth kinetic parameters of *K. apiculata* and *S. cerevisiae* in single and composite culture

T^a (°C)	Growth kinetics															
	Rate (per h)[b]				Relative growth (%)[c]				Day of maximal cell population				Maximal cell population (cfu/ml)			
	K	Km	S	Sm	K	Km	S	Sm	K	Km	S	Sm	K	Km	S	Sm
15	0.14	0.10	0.09	0.07	16.2	17.1	18	14.9	1	1	7	7	1.1×10^8	4.7×10^7	9.4×10^7	4.2×10^7
25	0.17	0.12	0.20	0.16	3.3	7.6	24	15.1	1	1	1	1	6.5×10^7	2.8×10^7	1.9×10^8	5.4×10^7
30	0.20	0.14	0.21	0.15	−6.4	4	29	9	1	1	1	1	2.8×10^7	6.0×10^6	1.8×10^8	4.7×10^7
35	0.07	0.02	0.17	0.09	−39	−30	20.5	11	1	1	1	1	1.1×10^7	8.9×10^6	8.8×10^7	3.1×10^7

K, *K. apiculata* in pure culture; Km, *K. apiculata* in mixed culture; S, *S. cerevisiae* in pure culture; Sm, *S. cerevisiae* in mixed culture

[a] Temperature of incubation

[b] Growth rate (per h) was estimated from plots of the log of cfu/ml against time, using the straight line of the exponential growth phase

[c] Relative growth (%) = $(X_i - X_0/X_0) \times 100$, X_0 initial viable cell number; X_i viable cell number after 10 days. Negative values indicate cellular death

a lower growth rate and less final biomass than their respective pure cultures. However, the apiculate yeast remained viable during longer periods. After 10 days of incubation at 25°C the relative growth of *K. apiculata* in mixed culture was 2.3-fold higher than in a pure culture. At 30°C a loss of 6.4% in viability was observed in a pure culture of *K. apiculata*, whereas the relative growth in mixed culture showed an increase of 4%.

Table 2 shows the death kinetics of pure and composite cultures of both yeasts. Under both culture conditions the apiculate yeast showed an increase in the death rate with rising temperature, but this effect was less marked in mixed culture. This behavior is in agreement with the higher relative growth of *K. apiculata* observed in composite culture. Furthermore, a change in the curve from exponential to linear was observed when the apiculate yeast was co-cultured, which suggests that the reason for the loss of viability in both cultures would be different.

Pure and mixed cultures of the *Saccharomyces* yeast showed linear death kinetics. These results do not agree with those found by other authors who have reported early death of wine-related yeasts in mixed fermentations [18, 27–29]. Studies with different mixed inocula of *S. cerevisiae* and non-*Saccharomyces* species demonstrated that both yeasts grew simultaneously during the first days and then suddenly non-*Saccharomyces* yeasts began to die off, whereas *Saccharomyces* strains did not modify their viability.

Effect of temperature on metabolic behavior of *K. apiculata* and *S. cerevisiae*

At the end of each fermentation characteristics with an impact on the enological properties of wine, such as sugar utilization and alcohol and secondary metabolite production, were studied.

Pure cultures of *K. apiculata* showed the lowest sugar consumption rate at 15°C, whereas rates at 25, 30 and 35°C were quite similar. *S. cerevisiae* showed higher glucose and fructose consumption rates with increasing temperature, and maximum consumption was found at 35°C. In mixed culture, the sugar utilization rates at all temperatures were similar to those obtained in pure cultures of *S. cerevisiae* (Table 3). These results are in agreement with Erten [12], who has indicated that a mixed culture of *K. apiculata* and *S. cerevisiae* reached highest sugar utilization at 25°C, followed by 20 and 15°C.

To evaluate the effect of the culture type and the incubation temperature on the fermentation products, such as ethanol, glycerol and acetic acid, MANOVA analysis was applied. The metabolite concentrations showed significant differences under the different culture conditions assayed and also for several of the fermentation temperatures ($P < 0.0001$). The interaction between culture type and temperature was statistically significant, suggesting that the effect of the fermentation temperature on the metabolite concentration depended on the culture condition. Therefore, the Hotelling–Bonferroni test was applied to identify treatments with significant differences (Table 4). In addition, PCA was used to interpret the interaction between culture and temperature. Figure 1 shows that the first two principal components accounted for about 98% of the total variation (PC1 63.6% and PC2 34.8%). The horizontal component (PC1) was associated with the production of glycerol and acetic acid while PC2 was associated with the ethanol concentration. The highest concentrations of secondary metabolites were obtained in pure cultures of *K. apiculata* at 25 and 30°C, showing positive scores for PC1. The significant differences between both conditions (Table 4) were due to the higher ethanol concentration observed at 25°C. Pure cultures of *S. cerevisiae* produced the lowest amounts of glycerol and acetic acid at 15 and 35°C and the statistically significant differences were related to ethanol production. At 30°C, the elliptic yeast displayed a similar production of the three fermentation metabolites when compared to 35°C. At higher temperatures the production of ethanol decreased in cultures of the apiculate yeast, whereas an opposite behavior was observed in cultures of *S. cerevisiae*.

In composite cultures at 15, 25 and 30°C the microorganisms produced intermediate concentrations of glycerol and acetic acid, similar to amounts observed in cultures of the non-*Saccharomyces* yeast at 15 and 35°C. At 35°C mixed culture showed a different behavior compared to other temperatures, producing lower concentrations of secondary metabolites with values similar to those produced by the elliptic yeast at 25°C.

Table 2 Effect of fermentation temperature on death kinetic parameters of *K. apiculata* and *S. cerevisiae* in single and composite culture

T^a (°C)	Death kinetics[b]							
	Rate (per day)				Curve shape			
	K	Km	S	Sm	K	Km	S	Sm
15	0	0	0	0	–	–	–	–
25	0.13	0.06	0	0	Linear	Linear	–	–
30	0.51	0.12	0.07	0.11	Exponential	Linear	Linear	Linear
35	0.65	0.27	0.09	0.13	Exponential	Linear	Linear	Linear

K, *K. apiculata* in pure culture; Km, *K. apiculata* in mixed culture; S, *S. cerevisiae* in pure culture; Sm, *S. cerevisiae* in mixed culture

[a] Temperature of incubation

[b] Death kinetics were fitted using an exponential model log $(N_t/N_0) = c + \exp(a + bt)$ and a linear model log $(N_t/N_0) = a + bt$

Table 3 Influence of different temperatures of fermentation on sugar utilization by *K. apiculata* and *S. cerevisiae* in pure and mixed cultures

Consumption rate (g/l/h)		Temperature of incubation			
		15°C	25°C	30°C	35°C
Glucose	*K. apiculata*	0.34 ± 0.02^{a}	0.52 ± 0.03^{b}	0.49 ± 0.02^{b}	0.51 ± 0.02^{b}
	S. cerevisiae	0.24 ± 0.02^{a}	0.47 ± 0.03^{b}	0.50 ± 0.02^{b}	0.70 ± 0.03^{c}
	Mixed culture	0.26 ± 0.03^{a}	0.45 ± 0.02^{b}	0.59 ± 0.03^{c}	0.71 ± 0.04^{d}
Fructose	*K. apiculata*	0.22 ± 0.03^{a}	0.33 ± 0.03^{b}	0.29 ± 0.01^{b}	0.35 ± 0.02^{b}
	S. cerevisiae	0.11 ± 0.04^{a}	0.20 ± 0.03^{b}	0.26 ± 0.02^{b}	0.52 ± 0.03^{c}
	Mixed culture	0.10 ± 0.02^{a}	0.21 ± 0.02^{b}	0.34 ± 0.03^{c}	0.53 ± 0.02^{d}

Values are means ± standard deviations. Values displaying different superscript letters within the horizontal line are different according to the Tukey test

Table 4 Effect of the interaction between culture and temperature on production of fermentation metabolites (Hotelling–Bonferroni test)

Culture	Temperature (°C)	Ethanol (g/l)	Glycerol (g/l)	Acetic acid (g/l)	
S. cerevisiae	15	7.34	1.19	0.33	A
K. apiculata	30	9.59	1.68	0.85	B
K. apiculata	25	11.63	1.52	0.83	C
S. cerevisiae	25	10.19	1.28	0.52	D F
Mixed culture	35	10.43	1.25	0.50	D
S. cerevisiae	35	12.32	1.22	0.44	E
S. cerevisiae	30	12.46	1.24	0.54	E
Mixed culture	15	9.78	1.37	0.64	F G H
Mixed culture	25	10.39	1.46	0.62	F H
Mixed culture	30	9.90	1.56	0.69	G H
K. apiculata	15	10.29	1.41	0.69	G H
K. apiculata	35	9.08	1.51	0.65	G

Different letters shows significant differences ($P < 0.05$)

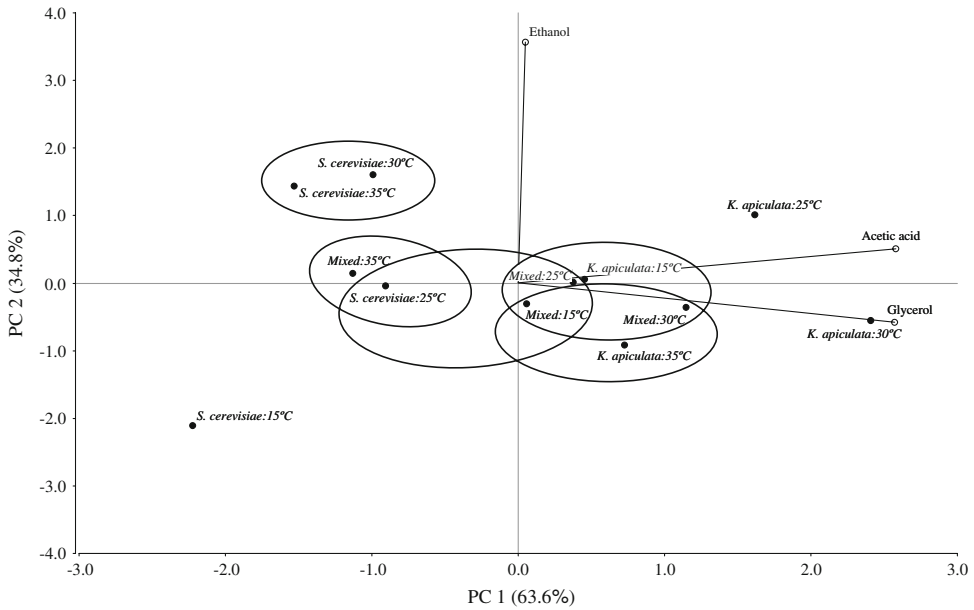

Fig. 1 Biplot graph of the first two principal components for the production of ethanol, glycerol and acetic acid by *K. apiculata* and *S. cerevisiae* in single and composite cultures at 15, 25, 30 and 35°C. Values statistically similar according to Hotelling–Bonferroni test were grouped

Independent of the temperature, ethanol production in composite culture was low and comparable with that produced by *K. apiculata* in pure cultures; maximum production was found at 25°C. This behavior in composite culture could be due to the low biomass of *S. cerevisiae*, the highest ethanol producer of both yeasts. Our results are in disagreement with Toro and Vazquez [33], who determined in a mixed culture of a *Saccharomyces* and a non-

Saccharomyces strain, that the final ethanol concentration was higher than in a pure culture of *S. cerevisiae*. Considering that the authors used *Candida cantarelli*, the difference could be related to a different metabolic behavior of this non-*Saccharomyces* genus. This assumption is supported by Moreira et al. [26], who worked with mixed starter cultures of *S. cerevisae* and *H. uvarum* (teleomorphic state of *K. apiculata*) in a commercial medium and also observed a lower ethanol yield and ethanol productivity than in pure cultures.

It is interesting to emphasize the high production of volatile acidity as acetic acid in pure cultures of *K. apiculata* at 25 and 30°C, compared to lower amounts produced by pure cultures of *S. cerevisiae*. Our results confirmed this peculiar characteristic of little acetic acid production by *Saccharomyces* yeasts under the given conditions [4, 25]. Erten [12], Bilbao et al. [3], and Heard and Fleet [19] have reported that *K. apiculata* may produce higher acetic acid concentrations than *S. cerevisiae*. However, despite the consistent production of volatile compounds in pure culture, *K. apiculata* did not produce an increase in volatile acidity in mixed culture [6, 12]. This behavior has a biotechnological importance since the increase in acetic acid to values higher than legal wine standards (1.1 g/l) could produce a sour-vinegar off odor. The low concentrations of secondary metabolites observed in mixed culture at 35°C compared to other temperatures assayed is probably due to a major contribution of *S. cerevisiae* at higher temperatures.

Effect of sulfur dioxide on growth of pure and mixed cultures of *K. apiculata* and *S. cerevisiae*

Traditionally, SO_2 has been added to the must as an antioxidant and as an antimicrobial agent to suppress the growth and dominance of non-*Saccharomyces* species and selectively encourage the growth and dominance of *S. cerevisiae* [9]. *K. apiculata* has been found to be susceptible to less than 5 mg/l free SO_2, but *Candida guilliermondii* and *Zygosaccharomyces* spp. were resistant to at least ten times that concentration [30, 35]. Henick-Kling et al. [22] have indicated that musts treated with 20 mg/l sulfite produced no effects on the yeast population or the fermentation rate, whereas, 50 mg/l SO_2 produced inhibition of non-*Saccharomyces* yeasts.

In our work, the grape juice medium was treated with sodium metabisulfite to reach concentrations of 23.68, 51.38 and 153.96 mg/l and 16.96, 36.80 and 110.27 mg/l of total and free SO_2, respectively. At the pH of the culture medium approximately 0.34, 0.74 and 2.1 mg/l would be present in its molecular SO_2 form.

Table 5 shows the effect of metabisulfite addition on the growth kinetics parameters of pure and mixed cultures of *K. apiculata* and *S. cerevisiae*. The growth rate of the apiculate yeast in single cultures decreased with increasing metabisulfite concentration. However, a slight increase in maximum cell population and viability was observed in the presence of 50 and 100 mg/l metabisulfite. In mixed culture, the addition of SO_2 produced a similar effect on both growth kinetics parameters. The growth kinetics of *S. cerevisiae* in pure or mixed cultures was not affected by SO_2 addition; only a diminution in the maximum biomass of the elliptic yeast was observed at the highest additive concentration.

The results showed little or no effectiveness of SO_2 regarding control of the non-*Saccharomyces* yeast, *K. apiculata* mc1, in pure and mixed cultures even if the theoretical concentration of molecular SO_2 (chemical form with antimicrobial activity) would be enough to produce the desired antimicrobial effect. Addition of metabisufite did not significantly modify the growth kinetics of single and mixed cultures of either yeast. Both microorganisms remained viable during the fermentation period and consequently were able to contribute to the final product.

Table 5 Effect of sulfur dioxide on growth parameters of *K. apiculata* and *S. cerevisiae* in single and composite culture

SO_2 (mg/l)	Growth rate (per h)				Maximal population (cfu/ml)				Cell viability[a]			
	Pure culture		Mixed culture		Pure culture		Mixed culture		Pure culture		Mixed culture	
	K	S	K	S	K	S	K	S	K	S	K	S
0	0.20	0.22	0.14	0.17	3.0×10^7	1.4×10^8	5.9×10^6	5.5×10^7	0.93	1.28	1.04	1.09
50	0.16	0.22	0.15	0.16	3.2×10^7	1.2×10^8	6.1×10^6	5.6×10^7	1.01	1.29	1.05	1.06
100	0.15	0.22	0.14	0.16	3.8×10^7	1.0×10^8	6.3×10^6	5.4×10^7	1.03	1.25	1.08	1.05
250	0.14	0.21	0.13	0.15	1.9×10^7	8.8×10^7	3.4×10^6	4.3×10^7	0.98	1.25	1.02	1.05

Data are the average values of results obtained from three fermentations

K, *K. apiculata*; S, *S. cerevisiae*

[a] Cell viability = log N_t/log N_0. N_0, initial viable cell number; N_t, viable cell number after 10 days

Similar results have been reported by Heard and Fleet [20] who questioned the efficacy of SO_2 in controlling the initial growth of indigenous non-*Saccharomyces* yeasts. The authors demonstrated that 100 mg/l total SO_2 did not necessarily prevent growth of indigenous non-*Saccharomyces* species, especially in red wines. The inefficacy of SO_2 to inhibit *K. apiculata* mc1 means an important finding considering that nowadays many winemakers believe that growth of non-*Saccharomyces* yeasts also contributes to desirable sensory characteristics.

Metabolism of non-*Saccharomyces* and *Saccharomyces* yeasts in presence of sulfur dioxide

The initial sugar concentration of the basal medium (31.5 ± 2 g/l) was completely consumed in both single and composite cultures after 6 days of incubation at 25°C, independently of the presence of SO_2.

MANOVA analysis of the effect of the culture conditions and SO_2 addition on metabolite production revealed that even though both variables had significant effects on the fermentation products, a lower influence was observed for SO_2 addition ($P < 0.0001$ and $P < 0.001$ for culture conditions and SO_2, respectively). The interaction between culture and SO_2 was not significant ($P > 0.01$). Application of the Hotelling–Bonferroni test (Table 6) showed that the effect of the three cultures on the concentration of ethanol, glycerol and acetic acid was significantly different. Moreover, the metabolite production was similar when wine yeasts were grown in non-supplemented medium and medium added with 250 mg/l of SO_2. However, both treatments were statistically different from those with addition of 50 and 100 mg/l of the additive.

Table 6 Influence of different culture conditions and levels of sulfur dioxide on metabolites production (Hotelling–Bonferroni test)

	Ethanol (g/l)	Glycerol (g/l)	Acetic acid (g/l)	*n*	
Culture					
S. cerevisiae	11.81	1.22	0.56	8	A
Mixed culture	10.41	1.56	0.66	8	B
K. apiculata	10.35	1.64	0.84	8	C
Sulfur dioxide (mg/l)					
100	11.42	1.49	0.70	6	A
50	10.92	1.49	0.70	6	B
0	10.46	1.47	0.68	6	C
250	10.60	1.44	0.66	6	C

Different letters shows significant differences ($P < 0.05$)

Although the interaction between culture and SO_2 was not significant, PCA was carried out to analyze the metabolic behavior of the cultures in the presence of different concentrations of metabisulfite. In Fig. 2, it can be observed that PC1 and PC2 explained 97.5% of the total data variance. PC1 was positively associated with glycerol and acetic acid production while ethanol concentration presented negative scores. PC2 explained 12.5% of the total variance and was weakly associated with production of metabolites. Glycerol production divided *K. apiculata* and *S. cerevisiae* into two opposite groups. Pure cultures of the apiculate yeast obtained higher concentrations of this metabolite and also acetic acid than pure cultures of *S. cerevisiae*. However, the ethanol concentration was higher in single cultures of *Saccharomyces* displaying negative scores for PC1. In co-culture conditions, the content of the three fermentation products showed intermediate values.

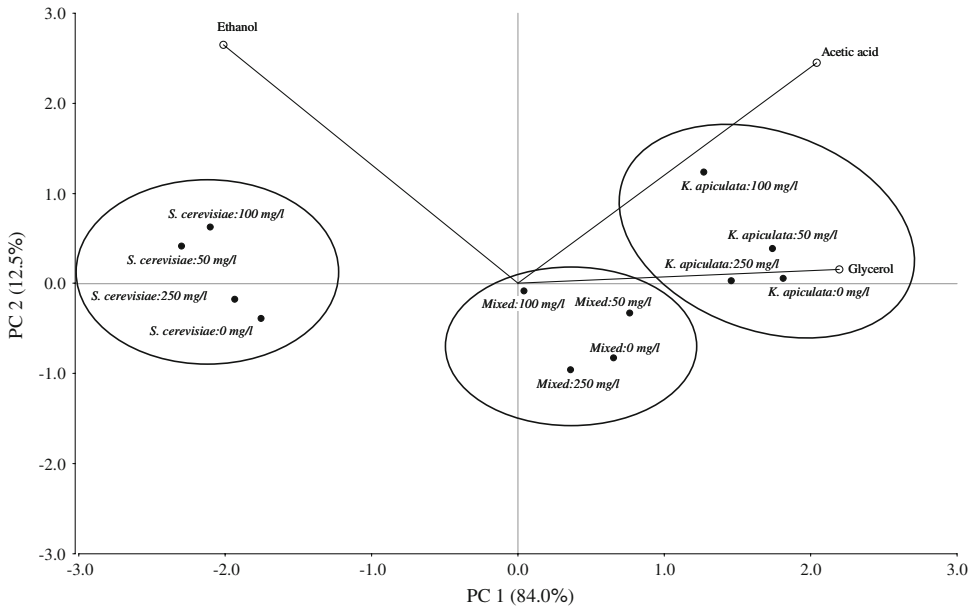

Fig. 2 Biplot graph of PC1 vs. PC2 for ethanol, glycerol and acetic acid concentrations produced by *K. apiculata*, *S. cerevisiae* and mixed culture in presence of different levels of sulfur dioxide. Values statistically similar according to Hotelling–Bonferroni test were grouped

Independent of the culture, it was observed that in the presence of 50 and 100 mg/l SO_2 wine yeasts increased production of ethanol and acetic acid compared to non-supplemented medium or in the presence of 250 mg/l SO_2. The volatile acidity, however, did not exceed the allowed maximum.

Conclusion

We have observed that fermentation at lower temperature could lead to a greater contribution of non-*Saccharomyces* populations. However, in composite culture *K. apiculata* can contribute to the final product at higher temperature, and consequently showed higher survival than in single culture. Statistical evaluation revealed that the fermentation temperature significantly affected the metabolite production and that the effect depended on the culture.

The results also showed little efficacy of SO_2 to control *K. apiculata* mc1 growth in pure and mixed cultures. This finding has an important technological impact considering the interest of winemakers to maintain viability of certain non-*Saccharomyces* strains due to their contribution to the final product. The effect of addition of SO_2 on the fermentation products was similar in pure and mixed cultures of both yeasts, but unlike temperature, no statistically significant interaction between culture and SO_2 concentration was observed.

These findings emphasize the important role of two physicochemical factors, temperature and SO_2, on the prevalence of composite yeast flora during vinification and the impact on the metabolic activities of the microorganisms that affect the end products.

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