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Enzymatic activities produced by mixed *Saccharomyces* and non-*Saccharomyces* cultures: relationship with wine volatile composition

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Abstract During certain wine fermentation processes, yeasts, and mainly non-*Saccharomyces* strains, produce and secrete enzymes such as β-glucosidases, proteases, pectinases, xylanases and amylases. The effects of enzyme activity on the aromatic quality of wines during grape juice fermentation, using different co-inoculation strategies of non-*Saccharomyces* and *Saccharomyces cerevisiae* yeasts, were assessed in the current study. Three strains with appropriate enological performance and high enzymatic activities, BSc562 (*S. cerevisiae*), BDv566 (*Debaryomyces vanrijiae*) and BCs403 (*Candida sake*), were assayed in pure and mixed *Saccharomyces*/non-*Saccharomyces* cultures. β-Glucosidase, pectinase, protease, xylanase and amylase activities were quantified during fermentations. The aromatic profile of pure and mixed cultures was determined at the end of each fermentation. In mixed cultures, non-*Saccharomyces* species were detected until day 4–5 of the fermentation process, and highest populations were observed in MSD2 (10 % *S. cerevisiae*/90 % *D. vanrijiae*) and MSC1 (1 % *S. cerevisiae*/99 % *C. sake*). According to correlation and multivariate analysis, MSD2 presented the highest concentrations of terpenes and higher alcohols which were associated with pectinase, amylase and xylanase activities. On the other hand, MSC1 high levels of β-glucosidase, proteolytic and xylanolytic activities were correlated to esters and fatty acids. Our study contributes to a better understanding of the effect of enzymatic activities by yeasts on compound transformations that occur during wine fermentation.

Keywords Mixed cultures · Enzymatic activities · Non-*Saccharomyces* yeasts · Aromatic profile of wines

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Introduction

Saccharomyces cerevisiae has been commonly used in wine fermentation because of its ability to induce a reliable and rapid fermentation, ease of control and consistency of fermentations (Lee et al. 2012). However, diverse non-*Saccharomyces* species are found on

grapes and musts and they can dominate the early stage of the winemaking process and persist until the end of the fermentation. These yeasts are also responsible for the alcoholic fermentation and can affect sensorial characteristics of the final product (Sadoudi et al. 2012; Sun et al. 2014).

During the fermentation process, yeasts, and mainly non-*Saccharomyces* strains, produce and secrete a variety of enzymes such as esterases, β-glucosidases, proteases, pectinases, xylanases and amylases (Comitini et al. 2011; Maturano et al. 2012). These enzymes can interact with odorless grape must precursors to produce aromatic compounds that enhance the wine aroma (Charoenchai et al. 1997; Jolly et al. 2006; Hernández-Orte et al. 2008). Since these non-*Saccharomyces* yeasts are not vigorous or competitive fermenting organisms under enological conditions, and to guarantee complete fermentation, they can only be used as starter cultures in combination with *S. cerevisiae*, a powerful fermentative species (Lee et al. 2012).

Diverse studies on the growth and metabolic interactions between non-*Saccharomyces* and *Saccharomyces* yeasts in mixed cultures have shown that their impact on wine flavor, aromatic profile and quality depends on the strains and the inoculation strategies (Moreno et al. 1991; Zohre and Erten 2002; Jolly et al. 2003; Povhe Jemec and Raspor 2005; Ciani et al. 2006; Moreira et al. 2008; Anfang et al. 2009; Sadoudi et al. 2012; Sun et al. 2014). In addition, a great number of studies inform about enzyme activities in winemaking and fermentations (Zamuz et al. 2004; Fia et al. 2005; Blasco et al. 2006; Comitini et al. 2011; Maturano et al. 2012). However, there are no known reports that associate the production of enzymatic activities in mixed cultures of *Saccharomyces* and non-*Saccharomyces* during the fermentation with the final aromatic profile of wines.

In the present work, three autochthonous yeast isolates, *Debaryomyces vanrijiae, Candida sake* and *S. cerevisiae*, were selected based on their enological characteristics and high levels of enzymatic activities in synthetic media as confirmed in previous assays (Maturano et al. 2008, 2009a, b). The aim of the current study was to assess the effects of enzymatic activities on the aromatic quality of wine during grape juice fermentation, using different co-inoculation strategies of non-*Saccharomyces* and *S. cerevisiae* yeasts.

Materials and methods

Microorganisms and media

Three yeast strains, *S. cerevisiae* BSc562, *Candida sake* BCs403 and *D. vanrijiae* BDv566, were previously isolated from musts at different stages of spontaneous fermentations. All strains belong to the Culture Collection of Autochthonous Microorganisms at the Biotechnology Institute (IBT) of the Faculty of Engineering, National University of San Juan, Argentina. The microorganisms had previously been identified by conventional biochemical, morphological and physiological procedures according to Kurtzman and Fell (1998). The yeast species were also identified by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of internal transcribed spacers (ITS), as described by Esteve-Zarzoso et al. (1999).

Propagation, total biomass determination and maintenance of the yeast strains were performed on YEPD, containing (g/l): yeast extract 10; peptone 20; glucose 20. Agar was added when necessary. Determination of non-*Saccharomyces* populations was carried out on Lysine agar, containing (g/l): L-lysine 5.6; glucose 10; KH_2PO_4 0.85; $MgSO_4$ 0.5; Agar 20. This medium is unable to support *S. cerevisiae* growth.

Grape must

Grape must from *Vitis vinifera* L. c.v. Pedro Giménez, an autochthonous variety from the San Martín district, San Juan, Argentina (Gil and Pszczòlkowski 2007), was heated at 70 °C for 20 min. After cooling down to room temperature, the same procedures were repeated for three consecutive days to eliminate natural microbiota (Toro and Vazquez 2002). The effectiveness of this treatment was verified by plate counting. As a negative control, must was tyndallized as described above. No enzymatic activities were detected at negative control; therefore, we assumed that enzymatic activities were produced by inoculated yeasts. Characteristics of grape must were 24 °Bx, density 1.08 g/m^3, total acidity 5.5 g/l and pH 3.6.

Fermentations

Microvinifications were carried out at 20 °C in 5 l flasks with 3 l of fresh must. Pure cultures of *S.*

cerevisiae BSc562, *D. vanrijiae* BTd566 and *C. sake* BCs403, and mixed cultures (MSD1: 1 % *S. cerevisiae*/99 % *D. vanrijiae*; MSC1: 1 % *S. cerevisiae*/99 % *C. sake*; MSD2:10 % *S. cerevisiae*/90 % *D. vanrijiae*; MSC2: 10 % *S. cerevisiae*/90 % *C. sake*) were inoculated in order to obtain an initial cell concentration of 3×10^6 cfu/ml from 24 h pre-cultures grown in the same must. Uninoculated must was used as negative control under the same assay conditions. The biomass relation used in this study is based on the ratio between *Saccharomyces* and non-*Saccharomyces* strains found in natural grape microbiota (Toro and Vazquez 2002).

Ten mililiters samples were periodically withdrawn from all fermentations. They were filtered by Whatman paper filter, pore size: 8–12 μm, which allows cells to pass through. Then, samples were centrifuged at 11,000*g* (10 min, 4 °C). Cell pellets were washed twice with distilled water and used to determine dry weight. Cell-free supernatants were used to determine enzymatic activities (fractions were kept at −20 °C until determinations) (Díaz et al. 2007).

Enological variables

Microvinifications were aseptically closed with Müller valves (a glass device which contains 50 % sulfuric acid that allows only CO_2 to escape from the system). Fermentation kinetics was monitored by measuring the weight loss as a result of CO_2 escaping from the system until the end of each fermentation (constant weight). Reducing sugars were determined colorimetrically using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959).

Enzymatic determinations

Exo β-glucosidase, pectinase, protease, amylase and xylanase activities were quantified according to Maturano et al. (2012). One unit (U) of enzymatic activity was defined as the amount of enzyme that released 1 μmol of product/time (h or min depending of the enzymatic activity), under the given assay conditions.

Appropriate enzyme and substrate controls, as well as calibration curves were included in all assays. Tyndallized must was used as blank for enzymatic determinations in all cases. Enzyme activity is expressed as enzymatic units per gram of dry weight (in 1 ml of the samples assayed): U/g DW. In order to estimate the total enzyme production, the area under the enzyme curve (AUEC) was calculated (i.e. the area under the plotted graph of the measured enzyme level against time, from the first to the last day of the assay (MATLAB 7.01)).

Gas chromatography and gas chromatography–mass spectrometry analysis of volatiles

Samples were obtained by extraction of 15 ml wine from each treatment, which were filtered immediately and kept at −18 °C until analysis. Before analysis, they were thawed at room temperature and centrifuged (Rolco, Argentina) at 2133*g* during 5 min.

Headspace-solid-phase microextraction (HS-SPME) conditions

Samples (5000 μl) and 4975 μl pure water (Millipore, Brazil) were poured into 20 ml glass sample vials. NaCl (3 g) and 25 μl of a 25 ng μl^{-1} methanolic (R)-2-octanol solution, internal standard, were added to each sample. Vials were sealed with a Teflon-faced septum cap and mixed on a magnetic stirrer (IKA, USA) at 1100 rpm. Samples were pre-conditioned at the extraction temperature (40 °C) for 15 min. Polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers (65 μm; Supelco, USA) were used for HS-SPME. Before use, the fibers were conditioned according to the manufacturer's instructions. After pre-conditioning of the sample, SPME fibers (2 cm) were exposed to the headspace for 15 min at controlled temperature (40 °C) during the extraction process, and the fibers were inserted immediately into the GC injector port (230 °C) for 20 min for thermal desorption of the volatile compounds (Massera et al. 2012).

Chromatographic conditions

Aroma compounds were identified on a Varian CP-3800 gas chromatograph equipped with a splitless injector and a Saturn 2200 Ion Trap Mass Spectrometric detector (Varian, CA, USA). The system was operated with Saturn GC–MS Workstation software Version 6.41. The GC was equipped with a Factor Four VF5 column (30 m × 0.25 mm; 0.25 μm film thickness, Varian, CA, USA). The column temperature was programmed at an initial temperature of

40 °C (5 min), followed by a gradual increase until 100 °C at a rate of 1.5 °C min^{-1}. Then the temperature was raised to 215 °C (5 min) at a rate of 3 °C min^{-1}. Helium was used as carrier gas at a constant flow rate of 1.0 ml min^{-1}. The injection port temperature was 230 °C. An electron impact (EI) of 70 eV was used for ionization, and the temperature of the transfer line and the ion trap was 200 °C. Mass spectra of the compounds were compared with the Nist Mass Spectral Library (Nist Mass Spectral Search Program Version 2.0), considering a forward match (FM) equal or greater than 700. Identification of volatile compounds was carried out by comparing them with the retention times and with the mass spectra from the Nist 2.0 library. They were quantified using relative areas related to the internal standard (Massera et al. 2012).

Statistical analyses

Experimental data were analyzed by one-way ANOVA and significant differences between mean values were determined by Tukey's test ($p < 0.05$) using InfoStat statistical software (2002). Pearson's correlation coefficients were calculated with SPSS (version 19.0) to determine statistical significance. Based on previous experiences (Baroni et al. 2006; Di Paola-Naranjo et al. 2011; Penci et al. 2012), multivariate statistics was used to associate two groups of variables (enzymatic activities and aroma groups) related to yeasts in the mixed fermentations. Therefore, cluster analysis (CA), principal components (PC), and factor analysis (FA) were applied to the dataset to detect differences between mixed fermentations, using STATISTICA 7 by StatSoft (2005).

Results

Biomass and sugar consumption in single and co-cultures

Development of yeast populations and decline in reducing sugars were monitored during the fermentation processes. Viable populations of pure cultures barely exceeded 7 log (cfu/ml) after 24 h (Table 1). In mixed cultures, *Saccharomyces* reached a concentration of 7 log (cfu/ml) after 48 h of fermentation (biomass varied between 7.24 ± 0.3 and 7.46 ± 0.1 log (cfu/ml)), whereas the biomass of the two non-*Saccharomyces* yeasts was lower than 7 log (cfu/ml), except for *C. sake* BCs403 in MSC1 (7 ± 0.1 log (cfu/ml)) (Table 1). In mixed cultures, *S. cerevisiae* outgrew non-*Saccharomyces* strains and completed the fermentation. *D. vanrijiae* and *C. sake* (Table 1) populations were not detected after 4 days (MSD1 and MSC2) and 5 days (MSC1 and MSD2) of fermentation. Non-*Saccharomyces* yeasts in MSC1 and MSD2 were detected one more day and also registered higher population levels (Table 1).

With regard to consumption of reducing sugars, wines produced by mono-cultures of *D. vanrijiae* and *C. sake* showed higher levels of residual reducing sugars than *S. cerevisiae* wines. Residual sugar content varied greatly: from 114.5 ± 2.1 g/l in wines fermented by *D. vanrijiae* to less than 1.8 ± 0.2 g/l in *S. cerevisiae* wines. Sugar consumption in mixed fermentations after 7 days was between 93.3 and 95 %, similar to pure cultures of *S. cerevisiae* (Table 1).

Enzyme activities during fermentation

Exo-β-glucosidase, pectinase, protease, amylase and xylanase activities were quantified throughout the fermentative process (18 days). It is important to study the evolution of enzymatic activities during winemaking, since their levels are not necessarily constant throughout the process (Zamuz et al. 2004). In this study, fluctuations in their levels were detected during alcoholic fermentation. This almost certainly means that a number of biochemical reactions took place. The grape must used in this research is not an homogeneous medium; it contains different sizes of particles of vegetal-aggregate that constitute a particular substrate for enzymatic activity and yeast colonization. Comparison of AUEC values for each enzymatic activity revealed that fermentations conducted by non-*Saccharomyces* yeasts (BDv566 and BCs403) yielded highest activities (Table 2).

With regard to fermentations by mixed cultures, it was found that MSD2 (10 % *S. cerevisiae*/90 % *D. vanrijiae*) presented significantly higher AUEC values than MSD1 (1 % *S. cerevisiae*/99 % *D. vanrijiae*), except for protease activity (Table 2). In contrast, MSC1 (1/99 %) showed significantly higher AUEC values than MSC2 (10/90 %).

Table 1 Consumption of reducing sugars and production of biomass in: (a) pure cultures of BSc562 (*S. cerevisiae*), BDv566 (*Debaryomyces vanrijiae*) BCS403 (*Candida sake*) and mixed cultures, (b) MSD1 (1 % BSc562/99 % BDv566) and MSD2 (10 % BSc562/90 % BDv566); (c) MSC1 (1 % BSc562/99 % BCs403) and MSC2 (10 % BSc562/90 % BCs403)

	Day 1		Day 2		Day 4		Day 5		Day 18 (ff)	
	Res Red S.	Biomasa	Res Red S.	Biomasa	Res Red S.	Biomasa	Res Red S.	Biomasa	Res Red S.	Biomasa
Pure cultures										
S. c.	129.45 ± 3.9	7.05 ± 0.3	56.45 ± 2.8	7.37 ± 0.4	12.00 ± 0.6	7.84 ± 0.4	6.0 ± 0.3	7.73 ± 0.4	1.28 ± 0.0	6.63 ± 0.3
D. v.	233.1 ± 9.3	6.99 ± 0.3	230.00 ± 11.5	7.24 ± 0.2	215.0 ± 10.7	7.85 ± 0.3	205.0 ± 9.9	7.79 ± 0.3	114.5 ± 5.7*	6.25 ± 0.3
C. s.	241.60 ± 3.3	7.21 ± 0.2	241.60 ± 3.3	7.42 ± 0.4	225.34 ± 6	7.78 ± 0.4	212.2 ± 11.8	7.75 ± 0.4	74.70 ± 2.5*	5.47 ± 0.2
MSD1										
S. c. (1 %)	200.19 ± 4.1	6.49 ± 0.1	126.12 ± 6.3	7.24 ± 0.3	50.0 ± 2.6	7.79 ± 0.1	26.5 ± 1.3	7.56 ± 0.4	1.96 ± 0.05	6.03 ± 0.3
D. v. (99 %)		6.78 ± 0.3		6.75 ± 0.1		6.26 ± 0.1		5.11 ± 0.2		0
MSD2										
S. c. (10 %)	211.90 ± 4	6.83 ± 0.3	109.09 ± 5.8	7.46 ± 0.1	33.30 ± 2.8	7.7 ± 0.4	20.7 ± 1.9	7.68 ± 0.2	1.99 ± 0.2	6.43 ± 0.3
D. v. (90 %)		6.96 ± 0.1		6.89 ± 0.3		5.3 ± 0.3		0		0
MSC1										
S. c. (1 %)	206.33 ± 5	6.79 ± 0.1	101.10 ± 5.3	7.45 ± 0.3	36.91 ± 2.4	7.68 ± 0.1	18.1 ± 1.7	7.69 ± 0.4	2.0 ± 0.05	5.84 ± 0.2
C. s. (99 %)		7.08 ± 0.3		7.00 ± 0.1		6.0 ± 0.3		0		0
MSC2										
S. c. (10 %)	221.90 ± 5.1	6.73 ± 0.3	195.60 ± 9	7.44 ± 0.4	71.08 ± 4.3	7.75 ± 0.4	35.0 ± 2.2	7.73 ± 0.3	1.9 ± 0.03	6.07 ± 0.1
C. s. (90 %)		6.89 ± 0.1		6.82 ± 0.3		6.13 ± 0.3		5.17 ± 0.2		0

Days in which main changes occurred and last day of fermentation are shown

MSD1: 1 % BSc562/99 % BDv566, MSD2: 10 % BSc562/90 % BDv566, MSC1: 1 % BSc562/99 % BCS403 and MSC2: 10 % BSc562/90 % BCs403, *ff* final fermentation, *Res, Red S.* residual reducing sugars, * stuck fermentation

Table 2 Area under the enzyme curve (AUEC) of hydrolytic enzymes produced by pure and mixed cultures of *Saccharomyces cerevisiae* BSc562, *Debaryomyces vanrijiae* BDv566 and *Candida sake* BCS403

Enzymatic activity	β-Glucosidases	Pectinases	Proteases	Xylanases	Amylases
BSc562	2569 ± 21^{f}	6932 ± 83^{b}	809 ± 78^{b}	n.d.	n.d.
BDv566	24594 ± 196^{b}	20580 ± 623^{a}	1576 ± 165^{a}	41086 ± 133^{a}	5336 ± 39^{a}
BCs403	37045 ± 145^{a}	19497 ± 409^{a}	1678 ± 56^{a}	37915 ± 205^{b}	4108 ± 21^{b}
MSD1	4614 ± 134^{d}	4343 ± 98^{d}	509 ± 34^{c}	7043 ± 87^{e}	972 ± 19^{d}
MSD2	4818 ± 111^{d}	5173 ± 43^{c}	483 ± 23^{c}	14714 ± 54^{c}	1830 ± 92^{c}
MSC1	5629 ± 89^{c}	4243 ± 99d	766 ± 33^{b}	10240 ± 101^{d}	1011 ± 26^{d}
MSC2	$4924 \pm 103^{d,e}$	$4118 \pm 96^{d,e}$	585 ± 76^{d}	1953 ± 68^{f}	$925 \pm 61^{d,e}$

Different letters within the same row indicate significant differences ($p < 0.05$) between fermentations for the same enzymatic activity according to Tukey's honestly significant difference (HSD) test

BSc562: *S. cerevisiae*, BDv566: *D. vanrijiae*, BCs403: *C. sake*, MSD1: 1 % BSc562/99 % BDv566, MSD2: 10 % BSc562/90 % BDv566, MSC1: 1 % BSc562/99 % BCs403, MSC2: 10 % BSc562/90 % BCs403

n.d. not detected

Aromatic profile of pure and mixed cultures

Samples of grape must without inoculation, single culture fermentations and mixed culture fermentations were analyzed by HS–SPME–GC–MS.

The average concentration and relative standard deviation (RSD) of volatile compounds during the different fermentations are shown in Table 3. A total of 75 aromatic compounds were identified in this study, including esters, higher alcohols, fatty acids, terpenes, C_{13}-norisoprenoids, aldehydes and ketones. Their origin and production are considered to be mostly affected by microbial activity during the fermentation process (Bisson and Karpel 2010). In most cases, differences in concentrations between pure and mixed cultures were statistically significant ($p \leq 0.05$) (Table 3).

Grape must showed the lowest values for most of the aromatic compounds in the vinifications assayed, with the exception of C_6 alcohols (*cis*-3-hexen-1-ol, *trans*-3-hexen-1-ol, 1-hexanol), aldehydes and ketones (Table 3).

S. cerevisiae showed highest total ester content of the single fermentation cultures (Table 3). Mixed cultures showed even greater amounts of ester compounds and in some cases, concentrations were significantly higher than their respective single cultures (Table 3).

Fermentations only conducted by *S. cerevisiae* showed lowest values of higher alcohols (Table 3), whereas mixed cultures showed significantly higher concentrations than in pure cultures.

All fermentations in the current study showed statistically different concentrations of fatty acids, which were not detected in pure *C. sake* cultures. On the other hand, vinifications conducted by *D. vanrijiae* showed the highest total acid concentration (Table 3), whereas MSC1 presented the highest total acid concentration in mixed cultures (Table 3).

Total content of terpenes in *D. vanrijiae* pure cultures was higher than in *S. cerevisiae* and *C. sake* monocultures (Table 3). Additionally, mixed cultures showed a significantly higher total terpene content than *S. cerevisiae* pure cultures, especially MSD2 and MSC1.

Fermentation by *D. vanrijiae* showed the highest values of total C_{13}-norisoprenoids (Table 3). MSD1 and MSD2, co-cultures of *S. cerevisiae*/*D. vanrijiae*, presented the highest concentrations among the mixed treatments.

Correlation between enzymatic activities and the volatile profile during mixed fermentations

Simple correlation analysis was used to assess the correlation between enzymatic activities (AUEC values) and the volatile compounds present in mixed fermentations (Tables 4, 5). Two independent correlation analyses were carried out: the first analysis included values from the aromatic profile and enzymatic activities of *Saccharomyces–Debaryomyces*

Table 3 Volatile compounds in control (grape must prior inoculation) pure and mixed cultures of *Saccharomyces cerevisiae* BSc562, *Debaryomyces vanrijiae* BDv566 and *Candida sake* BCs403

	Control	BSc562	BDv566	BCs403	MSD1	MSD2	MSC1	MSC2
Esters								
Ethyl butanoate	9.23 (0.8)^{a}	20.56 (9.3)^{b}	6.63 (0.2)^{a}	10.14 (7)^{a}	20.48 (0.4)^{b}	18.37 (11.9)^{b}	18.17 (0.6)^{b}	20.16 (5.8)^{b}
Ethyl 2-butenoate	n.d.	5.48 (0.5)^{b}	n.d.	n.d.	5.42 (0.9)^{a}	n.d.	5.42 (0.4)a.b	5.43 (0.6)^{b}
Ethyl-3-methylbutanoate	n.d.	6.06 (4.5)^{b}	n.d.	n.d.	5.63 (0.4)^{a}	n.d.	5.45 (3.5)^{a}	n.d.
3-Methyl-1-butanol acetate	n.d.	45.68 (12.9)^{c}	56.75 (2.2)^{d}	n.d.	23.66 (20.2)^{a}	41.33 (14.5)^{b,c}	41.92 (1.7)^{b,c}	34.98 (3.9)^{b}
Ethyl pentanoate	n.d.	n.d.	n.d.	n.d.	5.44 (0)^{a}	5.35 (0.2)^{b}	5.45 (0.4)^{a}	n.d.
Ethyl hexanoate	8.61 (15.5)^{a}	195.82 (2.4)^{d,e}	37.28 (0.4)^{a}	108.98 (2.9)^{b}	146.98 (12.3)^{c}	186.8 (1.3)^{d}	230.22 (12.9)e.f	238.04 (3.7)^{f}
Hexyl acetate	n.d.	41.14 (0.6)^{e}	8.63 (6.8)^{a,b}	13.3 (6.5)^{b,c}	17.61 (4.1)^{c}	25.92 (1.2)^{d}	27.37 (10.4)^{d}	5.66 (0.2)^{a}
Ethyl 2-hexenoate	n.d.	5.50 (1.3)^{a}	5.91 (2)^{c}	6.21 (2.4)^{d}	5.81 (0.2)^{b,c}	5.58 (1.1)^{a,b}	5.61 (0.4)^{a,b}	5.65 (1.8)^{a,b}
Methyl octanoate	n.d.	6.88 (0.9)^{a}	n.d.	n.d.	7.05 (1)^{a}	7.07 (6.5)^{a}	6.8 (1.5)^{a}	7.23 (4.7)^{a}
Butyl hexanoate	n.d.	6.06 (1.7)^{a}	n.d.	n.d.	6.19 (2.7)^{a}	6.53 (9.5)^{a}	6.15 (2.8)^{a}	6.18 (2.6)^{a}
Isopentyl hexanoate	n.d.	n.d.	n.d.	n.d.	14.77 (5)a.b	n.d.	n.d.	11.55 (6.1)^{a}
2-Phenylethyl acetate	5.85 (9.7)^{a}	143.24 (0.4)^{e}	41.09 (0.6)^{b}	40.72(5.7)^{b}	61.24 (13.6)^{c}	122.93 (3.1)^{d}	124.55 (1.6)^{d}	121.39 (6.8)^{d}
Isobornyl acetate	n.d.	6.13 (2.6)^{a}	6 (2.8)^{a}	8.11 (17.4)^{b}	6.11 (8)^{a}	5.44 (1.5)^{a}	5.42 (0.4)^{a}	5.36 (0.9)^{a}
Propyl octanoate	n.d.	8.06 (2.5)^{a}	n.d.	n.d.	7.75 (0.5)^{a}	7.76 (6.6)^{a}	7.42 (3.8)^{a}	8.14 (8.2)^{a}
Ethyl nonanoate	n.d.	21.96 (9.8)^{a}	22.36 (3)^{a}	27.33 (16.6)^{c}	30.25 (2.6)^{b,c}	23.83 (5.3)^{a,b}	23-01 (8.5)^{a}	24.16 (9.6)^{a,b}
Methyl decanoate	n.d.	6.06 (1.5)^{a}	n.d.	n.d.	6.29 (0.8)^{a}	6.11 (1.6)^{a}	6.2 (1.9)^{a}	6.28 (2.2)^{a}
Butyl octanoate	n.d.	9.29 (1.1)^{b,c}	5.47 (2.7)^{a}	n.d.	9.56 (3.1)^{c}	9.30 (5.5)b.c	8.49 (6)^{b}	9.65 (7.5)^{c}
Ethyl decanoate	n.d.	374.06 (4.3)^{d}	57.16 (4.4)^{a}	462.61 (1.2)^{e}	304.58 (3.6)^{c}	176.89 (0.6)^{b}	937.63 (1)^{f}	1315.19 (2.6)^{g}
β-Phenyl butanoate	n.d.	6.72 (0.9)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-Methylbutyl octanoate	n.d.	30.36 (1.4)^{b}	n.d.	n.d.	28.98 (0.7)^{b}	32.18 (9.1)^{b}	5.97 (0.8)^{a}	29.76 (8.2)^{b}
Pentyl octanoate	n.d.	38.84 (0.4)^{b}	n.d.	n.d.	11.55 (0.6)^{a}	n.d.	n.d.	n.d.
Propyl decanoate	n.d.	6.43 (3.7)^{a,b}	n.d.	n.d.	6.69 (3.9)^{a,b}	6.49 (3.1)^{a,b}	6.17 (3.12)^{a}	6.76 (4.1)^{b}
Ethyl undecanoate	n.d.	n.d.	6.14 (4.2)^{a}	6.14 (8)^{a}	6.18 (2.4)^{a}	6.05 (2.5)^{a}	6.03 (5.8)^{a}	n.d.
Butyl decanoate	n.d.	6.70 (0.3)^{a}	n.d.	n.d.	7.42 (3)^{b}	7.47 (3.5)^{b}	6.69 (2.5)^{a}	7.03 (3.8)^{a,b}
Ethyl docecanoate	n.d.	70.03 (2.1)a.b	n.d.	60.98 (14.6)^{a}	83.5 (5.2)b.c	108.63 (8.8)^{d}	81.01 (6.7)^{b}	98.62 (7.4)c.d
3-Methylbutyl pentadecanoate	n.d.	11.8 (2.1)^{a}	15.94 (0.5)^{b}	n.d.	n.d.	15.11 (9.6)^{b}	11.51 (0.3)^{a}	12.81 (3.4)^{a}
Ethyl tetradecanoate	n.d.	8.91 (2.9)^{a}	n.d.	12.47 (6.3)^{c}	8.47 (5.1)^{a}	8.89 (0.1)^{a}	9.71 (5.1)^{a,b}	10 (2.7)^{b}
Isopropyl myristate	5.59 (1.16)^{a}	5.83 (2.8)^{a}	8.01 (8)^{b}	5.71 (2.5)^{a}	5.73 (4.5)^{a}	5.64 (2.1)^{a}	5.46 (0.2)^{a}	5.76 (1)^{a}
Ethyl pentadecanoate	n.d.	n.d.	n.d.	5.95 (1.1)^{a}	5.42 (0.4)^{a}	5.54 (3.2)^{a}	5.43 (0.9)^{a}	n.d.
Ethyl hexadecanoate	n.d.	n.d.	n.d.	n.d.	n.d.	9.42 (14.3)^{a}	n.d.	12.45 (6.9)^{b}
Isopropyl palmitate	n.d.	n.d.	15.54 (9.2)^{b}	6.5 (2.9)^{a}	n.d.	n.d.	n.d.	n.d.

Table 3 continued

	Control	BSc562	BDv566	BCs403	MSD1	MSD2	MSC1	MSC2
Ethyl linoleate	n.d.	n.d.	n.d.	8 (1.9)^{b}	6.57 (7.6)^{a}	6.59 (6.4)^{a}	n.d.	7.15 (9.2)a.b
Ethyl octanoate	18.04 (11.4)^{a}	n.d.	228.7 (14.9)^{b}	225.72 (7.6) ^{b}	408.41 (5)^{c}	595.14 (0.3)^{d}	1046.42 (4.5)^{e}	1008.76 (4.3)^{e}
Diethyl succinate	n.d.	8.46 (2.5)^{a,b}	n.d.	n.d.	8.92 (9.4)^{b,c}	7.99 (2.1)^{a,b}	7.74 (6.7)^{a}	9.51 (5)^{c}
Total	47.33	1096.04	521.62	1008.53	1272.65	1449.24	2657.44	3033.66
Alcohols								
4-Methyl-1-pentanol	n.d.	7.38 (1.1)^{e}	5.63 (0.5)^{a,b}	5.78 (0.2)^{c}	5.8 (2.3)^{c}	5.76 (0.3)^{b,c}	5.59 (1.1)^{a}	5.92 (1.5)^{d}
3-Methyl-1-pentanol	n.d.	14.07 (6.5)^{b}	n.d.	n.d.	n.d.	9.13 (11.9)^{a}	n.d.	n.d.
Trans-3-Hexen-1-ol	19 (1.9)^{b}	n.d.	2.49 (9.8)^{a}	n.d.	n.d.	n.d.	1.39 (17.4)^{a}	n.d.
Cis-3-Hexen-1-ol	15.63 (16.3)^{c}	0.35 (5.7)^{a}	4.33 (15)^{a,b}	n.d.	0.63 (3.3)^{a}	n.d.	n.d.	0.96 (3.9)^{a}
(*S*)-3,4-Dimethylpentanol	n.d.	n.d.	n.d.	n.d.	163.61 (5.2)^{b}	n.d.	9.27 (7.7)^{a}	10.6 (3.9)^{a}
1-Octen-3-ol	41.07 (3.8)^{c}	n.d.	15.5 (9.6)^{b}	11.94 (17.7)^{a}	n.d.	n.d.	n.d.	n.d.
2-Ethyl-1-hexanol	104.59 (3.4)^{d}	3.71 (3.9)^{a}	n.d.	10.62 (12.7)^{b,c}	6.43 (21.6)^{a,b}	16.42 (22.5)^{c}	9.42 (15.2)^{b,c}	14.07 (12.9)^{c}
2-Nonen-1-ol	n.d.	n.d.	n.d.	n.d.	n.d.	9.99 (5.3)	n.d.	n.d.
Ethylphenyl alcohol	101.8 (11.15)^{a}	478.9 (12.5)^{b}	618.13 (3.3)^{c}	501.1 (5.7)^{b}	850.48 (2.3)^{d}	1442.66 (3.5)^{f}	1212.36 (6.1)^{e}	833.6 (3.5)^{d}
1-Nonanol	10.18 (3.5)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Hexyl-1-octanol	19.20 (3.5)^{b}	n.d.	10.12 (6.7)^{a}	n.d.	n.d.	n.d.	n.d.	n.d.
Total	311.47	504.41	656.20	524.70	1026.95	1483.96	1238.04	865.15
Acids								
3-Methylbutanoic acid	n.d.	n.d.	659.65 (2.2)^{b}	n.d.	5.10 (10.8)^{a}	6.11 (14.7)^{a}	3.94 (8.9)^{a}	10.08 (7.8)^{a}
2-Methylbutanoic acid	n.d.	n.d.	n.d.	4.51 (9.9)^{b}	0.64 (4.4)^{a}	n.d.	n.d.	n.d.
n-Decanoic acid	n.d.	178.67 (4.7)^{b}	n.d.	n.d.	n.d.	105.15 (4)^{a}	265.45 (7.7)^{c}	n.d.
Total	0.00	178.67	659.65	4.51	5.74	111.26	269.39	10.08
Terpenes								
Limonene	0.75 (17.9)^{a}	n.d.	1.08 (7.8)^{a}	1.06 (5)^{a}	n.d.	n.d.	n.d.	n.d.
Eucalyptol	8.97 (8)^{c}	1.16 (7.3)^{a}	4.83 (14.7)^{b}	4.6 (17.8)^{b}	1.15 (3.5)^{a}	0.87 (9.2)^{a}	1.09 (19.3)^{a}	n.d.
Cis-linalool oxide	8.48 (1.3)^{d}	1.29 (2.9)^{a}	6.24 (12.9)^{c}	n.d.	3.03 (14.4)^{b}	n.d.	n.d.	n.d.
α-Linalool	n.d.	n.d.	39.63 (0.1)^{b}	n.d.	n.d.	n.d.	1.37 (10.2)^{a}	n.d.
β-Linalool	178.45 (4.5)b.c	150.71 (8.1)b.c	203.86 (0.0)^{c}	157.15 (5.9)b.c	179.75 (4.5)b.c	158.02 (2.7)b.c	173 (11.3) a.b	123.72 (12.7)^{a}
Hotrienol	n.d.	9.65 (5.9)^{a}	n.d.	n.d.	71.11 (2.3)^{c}	51.67 (4)^{b}	n.d.	n.d.
Trans-rose oxide	n.d.	0.77 (7.3)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Menthol	10.10 (17.8)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α-Terpineol	34.9 (10.9)^{b}	n.d.	37.58 (4.5)^{b}	18.39 (23.3)^{a}	n.d.	n.d.	n.d.	n.d.
Citronellol	n.d.	90.08 (1.7)a.b	39.33 (8.5)^{a}	n.d.	93.97 (3.5)a.b	58.48 (5.4)^{a}	78.77 (2.3)^{a}	154.2 (13.2)^{a}

Table 3 continued

	Control	BSc562	BDv566	BCs403	MSD1	MSD2	MSC1	MSC2
Trans-geraniol	n.d.	n.d.	27.27 (11.8)[a]	33.91 (16.4)[b]	n.d.	n.d.	n.d.	n.d.
Cis-myrtanol	n.d.	n.d.	3.55 (20.5)	n.d.	n.d.	n.d.	n.d.	n.d.
Eugenol	n.d.	n.d.	9.09 (3.7)[a]	10.16 (13.9)[b]	n.d.	n.d.	n.d.	n.d.
Nerolidol	n.d.	56.24 (0.4)[c]	11.08 (2.5)[a]	7.45 (17.3)[a]	9.49 (0.5)[a]	117.05 (1)[d]	18.06 (12.6)[b]	25.9 (7.8)[b]
Isocitronellol	n.d.	n.d.	4.19 (12.1)[a]	3.37 (18.8)[a]	n.d.	n.d.	n.d.	n.d.
β-Farnesese	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.73 (10.7)[a]	9.02 (8.8)[b]
Farnesyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.67 (15.6)	n.d.
β-Myrcene	n.d.	n.d.	n.d.	n.d.	n.d.	8.91 (10.1)	n.d.	n.d.
Cis-geranylacetone	13.12 (2)[a]	n.d.	n.d.	n.d.	n.d.	n.d.	120.37 (11.6)[c]	45.6 (8.4)[b]
Total	254.01	309.90	387.73	236.11	358.51	396.37	400.06	358.44
C_{13}-Norisoprenoids								
β-Damascenone	23.68 (16.9)[a]	44.77 (4.1)[b]	n.d.	21.06 (3.9)[a]	58.31 (4.5)[c,d]	51.64 (3.8)[b,c]	45.36 (5.2)[b]	62.72 (5.3)[d]
α-Ionone	13.68 (6.9)[a]	n.d.	378.93 (4.2)[c]	n.d.	188.31 (3.5)[b]	n.d.	n.d.	n.d.
α-Isomethyl ionone	n.d.	13.14 (10.2)[d]	3.35 (3.6)[b,c]	15.54 (0.6)[e]	3.67 (9.8)[c]	0.75 (28)[a]	0.76 (10.5)[a]	1.9 (25.1)[a,b]
β-Ionone	15.45 (7.6)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	55.22	57.90	382.27	36.60	250.29	52.39	46.12	64.63
Aldehydes								
Heptanal	2.45 (11.5)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hexylcinnamaldehyde	7.85 (2.3)[c]	n.d.	n.d.	5.42 (7.4)[b]	n.d.	n.d.	1.39 (23)[a]	n.d.
Total	10.30	0.00	0.00	5.42	n.d.	n.d.	1.39	0.00
Ketones								
2-Heptanone	16.50 (8.7)[c]	n.d.	n.d.	n.d.	4.86 (20.4)[b]	2.07 (23.2)[a]	n.d.	n.d.
2-Nonanone	16.80 (4.3)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Undecanone	3.88 (12.4)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	37.18	0.00	0.00	0.00	4.86	2.07	0.00	0.00

Compounds are listed according to their chemical classification

All concentrations are expressed in μg/l as the arithmetic mean ($n = 3$) and values in parentheses represent the corresponding percentage of relative deviation standard (RDS) for each condition

Different letters within the same row indicate significant differences ($p < 0.05$) between fermentations for the same volatile compound according to Tukey's honestly significant difference (HSD) test

MSD1: 1 % BSc562/99 % BDv566, MSD2: 10 % BSc562/90 % BDv566, MSC1: 1 % BSc562/99 % BCS403 and MSC2: 10 % BSc562/90 % BCs403

n.d. not detected

Table 4 Pearson correlations (PC) between aromatic compounds and xylanase, amylase and pectinase activities assayed in mixed cultures of *Saccharomyces cerevisiae* BSc562 and *Debaryomyces vanrijiae* BDv566: MSD1 and MSD2 (no significant correlation was found for β-glucosidase and protease activities)

Compounds	Xylanases	Amylases	Pectinases
Esters			
Ethyl butanoate	−0.64	−0.68	−0.66
Ethyl 2-butenoate	−0.99**	−0.99**	−0.98**
Ethyl 3-methylbutanoate	−1.00**	−0.99**	−0.98**
Ethyl pentanoate	−0.99**	−0.98**	−0.98**
Ethyl hexanoate	0.88*	0.88*	0.91*
Ethyl 2-hexenoate	−0.95**	−0.93**	−0.94**
Ethyl octanoate	0.98**	0.98**	0.97**
Ethyl nonanoate	−0.96**	−0.94**	−0.96**
Ethyl decanoate	−0.99**	−0.98**	−0.97**
Ethyl undecanoate	−0.47	−0.44	−0.49
Ethyl docecanoate	0.88*	0.91*	0.91*
Ethyl tetradecanoate	0.65	0.65	0.69
Ethyl pentadecanoate	0.49	0.44	0.46
Ethyl hexadecanoate	0.98**	0.96**	0.97**
Ethyl linoleate	0.03	0.07	0.09
2-Phenylethyl acetate	0.99**	0.98**	0.98**
Isobornyl acetate	−0.76	−0.75	−0.78
3-Methyl-1-butanol acetate	0.89*	0.90*	0.87*
Hexyl acetate	0.993**	0.988**	0.978**
3-Methyl-1-butanol acetate	0.89*	0.90*	0.87*
Methyl decanoate	−0.81*	−0.78	−0.80
Butyl octanoate	−0.35	−0.30	−0.29
3-Methylbutyl octanoate	0.75	0.79	0.77
Pentyl octanoate	−1.00**	−0.99**	−0.99**
Propyl decanoate	−0.45	−0.41	−0.39
Butyl decanoate	0.14	0.18	0.19
3-Methylbutyl pentadecanoate	0.99**	0.99**	0.98**
Isopropyl myristate	−0.26	−0.28	−0.22
Methyl octanoate	0.04	0.10	0.07
Butyl hexanoate	0.42	0.47	0.45
Isopentyl hexanoate	−0.99**	−0.99**	−0.98**
Propyl octanoate	0.02	0.07	0.04
Diethyl succinate	−0.68	−0.68	−0.64
Alcohols			
Cis-3-Hexen-1-ol	−0.93**	−0.93**	−0.95**
1-Hexanol	0.85*	0.81	0.82*
3-Methyl-1-pentanol	0.99**	0.99**	0.95**
4-Methyl-1-pentanol	−0.89*	−0.91*	−0.89*
(*S*)-3,4-Dimethylpentanol	−0.99**	−0.99**	−0.99**
2-Nonen-1-ol	0.99**	0.98**	0.98**
Ethylphenyl alcohol	0.99**	0.98**	0.98**

Table 4 continued

Compounds	Xylanases	Amylases	Pectinases
Acids			
3-Methylbutanoic acid	0.65	0.68	0.68
2-Methylbutanoic acid	−0.92**	−0.92**	−0.89*
n-Decanoic acid	0.99**	0.98**	0.98**
Eucalyptol	−0.93**	−0.90*	−0.90*
cis-Linalool oxide	−0.96**	−0.96**	−0.97**
β-Linalool	−0.89*	−0.88*	−0.90*
Hotrienol	−0.98**	−0.97**	−0.97**
Citronellol	−0.99**	−0.98**	−0.97**
Nerolidol	1.00**	0.99**	0.99**
β-Myrcene	0.99**	0.98**	0.98**
C_{13}-Norisop			
β-Damascenone	−0.88*	−0.85*	−0.88*
a-Ionone	−0.99**	−0.99**	−0.99**
a-Isomethyl ionone	−0.98**	−0.97**	−0.98**
Ketones			
2-Heptanone	−0.91*	−0.91*	−0.88*

* Significant correlation at $p < 0.05$

** Significant correlation at $p < 0.01$ (bilateral)

fermentations (MSD1 and MSD2), while the second one included values from the aromatic profile and enzymatic activity of *Saccharomyces–Candida* fermentations (MSC1 and MSC2).

Because correlation tests cannot discriminate between initial inoculum proportions of *S. cerevisiae* and non-*Saccharomyces*, a relationship was only established between the aromatic compounds and enzymatic activities.

A significant correlation was observed between determined aromatic compounds and xylanolytic, amylolytic and pectinolytic activities in *Saccharomyces–Debaryomyces* fermentations (MSD1 and MSD2), whereas no significant correlation was found for β-glucosidase and proteolytic activities (Table 4).

On the other hand, *Saccharomyces–Candida* fermentations (MSC1 and MSC2) showed a significant correlation between some aromatic compounds and xylanolytic, proteolytic and β-glucosidase activities, but no significant correlation was observed for amylolytic and pectinolytic activities (Table 5).

Below, results will be referred to and discussed with respect to significant positive or negative correlations between enzymatic activities and *S. cerevisiae/D. vanrijiae* or *S. cerevisiae/C. sake* mixed microvinifications.

Esters

Most of the acetate ester concentrations showed a statistically significant positive correlation with enzymatic activities in all mixed fermentations assayed (Tables 4, 5).

Ethyl esters are the second largest group of ester compounds produced by yeasts. Six of them showed statistically significant negative correlations with enzymatic activities assayed in all mixed fermentations (Tables 4, 5). However, in *S. cerevisiae/D. vanrijiae* fermentations, ethyl hexanoate and ethyl octanoate, associated with pleasant odors, showed a significant positive correlation with the enzymatic activities. Ethyl 3-methylbutanoate and ethyl pentanoate, recognized for their desirable effect on wine flavor, were also positively correlated to the enzymatic activities in MSC1 and MSC2 fermentations (Tables 4, 5).

Table 5 Pearson correlations (PC) between aromatic compounds and xylanase, β-glucosidase and protease activities assayed in mixed cultures of *Saccharomyces cerevisiae* BSc562 and *Candida sake* BCs403: MSC1 and MSC2 (no significant correlation was found for amylase and pectinase activities)

Compounds	Xylanases	Proteases	β-Glucosidases
Esters			
Ethyl butanoate	−0.83*	−0.84*	−0.85*
Ethyl 2-butenoate	−0.38	−0.45	−0.40
Ethyl 3-methylbutanoate	0.99**	0.88*	0.97**
Ethyl pentanoate	1.00**	0.88*	0.97**
Ethyl hexanoate	−0.21	−0.16	−0.16
Ethyl 2-hexenoate	−0.31	−0.45	−0.37
Ethyl octanoate	0.31	0.45	0.36
Ethyl nonanoate	−0.51	−0.45	−0.48
Ethyl decanoate	−0.99**	−0.90*	−0.97**
Ethyl docecanoate	−0.88*	−0.84*	−0.87*
Ethyl tetradecanoate	−0.71	-0.8	−0.79
Ethyl pentadecanoate	1.00**	0.88*	0.97**
Ethyl hexadecanoate	−0.99**	−0.89*	−0.97**
Ethyl linoleate	−0.99**	−0.90*	−0.98**
Hexyl acetate	0.98**	0.86*	0.95**
2-Phenylethyl acetate	0.45	0.46	0.44
Isobornyl acetate	0.65	0.45	0.59
3-Methyl-1-butanol acetate	0.97**	0.91*	0.97**
Methyl octanoate	−0.72	−0.76	−0.74
Butyl hexanoate	−0.09	−0.16	−0.09
Isopentyl hexanoate	−0.99**	−0.89*	−0.98**
Propyl octanoate	−0.65	−0.69	−0.67
Methyl decanoate	−0.35	−0.41	−0.36
Butyl octanoate	−0.75	−0.74	−0.75
3-Methylbutyl octanoate	−0.99**	−0.87*	−0.97**
Propyl decanoate	−0.83*	−0.80	−0.82*
Butyl decanoate	−0.67	−0.69	−0.68
3-Methylbutyl pentadecanoate	−0.93**	−0.90*	−0.94**
Isopropyl myristate	−0.97**	−0.81*	−0.93**
Diethyl succinate	−0.91*	−0.89*	−0.93**
Alcohols			
Trans-3-Hexen-1-ol	0.98**	0.90*	0.98**
Cis-3- Hexen 1-ol	−0.96**	−0.85*	−0.94**
1-Hexanol	−0.82*	−0.72	−0.79
4-Methyl 1-pentanol	−0.94**	−0.87*	−0.93**
(*S*)-3,4-Dimethylpentanol	−0.86*	−0.62	−0.77
Ethylphenyl alcohol	0.97**	0.82*	0.92**
Acids			
3-Methylbutanoic acid	−0.99**	−0.91*	−0.98**
n-Decanoic acid	0.99**	0.88*	0.97**
Terpenes			
Eucalyptol	0.97**	0.88*	0.97**

Table 5 continued

Compounds	Xylanases	Proteases	β-Glucosidases
α-Linalool	0.99**	0.89*	0.98**
β-Linalool	0.35	0.01	0.19
Citronellol	0.25	−0.13	0.11
Nerolidol	−0.77	−0.55	−0.67
β-Farnesese	−0.94**	−0.85*	−0.92**
Farnesyl acetate	0.98**	0.88*	0.97**
Cis-Geranylacetone	0.97**	0.86*	0.95**
C_{13}-Norsisop			
β-Damascenone	−0.97**	−0.77	−0.91*
α-Isomethyl ionone	−0.89*	−0.69	−0.83*
Aldehydes			
Hexylcinnamaldehyde	0.97**	0.87*	0.96**

* Significant correlation at $p < 0.05$

** Significant correlation at $p < 0.01$ (bilateral)

Higher alcohols

Most of the C_6 alcohols (*cis*-3-hexen-1-ol, *trans*-3-hexen-1-ol, 1-hexanol) detected in mixed cultures presented a significant negative correlation with the enzymatic activities assayed (Tables 4, 5). On the other hand, 2-phenylethanol was the most abundant volatile compound in the initial must and its concentration increased considerably after fermentation under all assay conditions, showing a significant positive correlation with enzyme activities in all mixed cultures assayed (Tables 4, 5).

Fatty acids

A significant positive correlation between *n*-decanoic acid and the enzymatic activities was found in all mixed cultures (Tables 4, 5).

Terpenes

Nerolidol and β-myrcene showed a significant positive correlation with enzymatic activities in MSD1 and MSD2 (Table 4). Similarly, α-linalool, *cis*-geranyl acetone and farnesyl acetate showed a significant positive correlation with enzymatic activities in MSC1 and MSC2 fermentations (Table 5).

C_{13}-Norisoprenoids

A negative correlation was observed between β-damascenone and α-isomethyl-ionone and enzymatic activities in all mixed cultures (Tables 4, 5). α-Ionone also showed a negative correlation with enzymatic activities in MSD1 and MSD2 (Table 4).

Multivariate statistics

A first exploratory method using Cluster Analysis (CA; Ward's method) showed that MSC2 can be completely separated from the other three mixed fermentations (MSC1, MSD1 and MSD2) (Fig. 1a).

Figure 1b shows that production of β-glucosidases and proteases by yeasts was positively related to the amount of esters and fatty acids but surprisingly negatively to the amount of C_{13}-norisoprenoids and ketones. Furthermore, CA evidenced an association between xylanases, amylases and pectinases and two groups of aromatic compounds: alcohols and terpenes.

Figure 1c reveals a clear separation of the four mixed fermentations along Factor 1 (x-axis). *Saccharomyces–Candida* fermentations (MSC1 and MSC2) were strongly correlated to the production of β-glucosidases and proteases by yeasts in addition to the amount of esters and fatty acids (represented only by *n*-decanoic acid). On the other hand, *Saccharomyces–Debaryomyces* fermentations (MSD1 and MSD2)

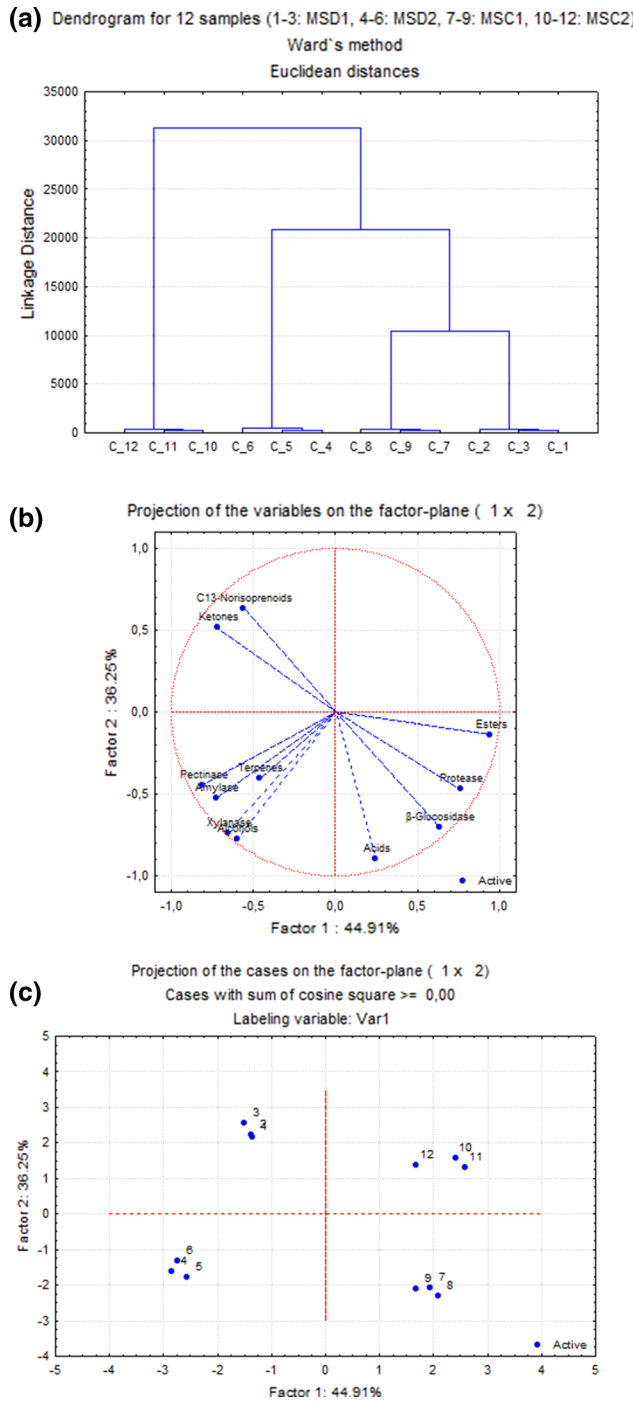

Fig. 1 **a** Cluster analysis (CA) of mixed fermentations (MSD1: 1 % BSc562/99 % BDv566; MSD2: 10 % BSc562/90 % BDv566; MSC1: 1 % BSc562/99 % BCs403 and MSC2: 10 % BSc562/90 % BCs403). **b** Projection of the variables on a factor plane (1 × 2) corresponding to factor analysis (FA) of mixed fermentations. **c** Projection of the cases (samples) on a factor plane (1 × 2) corresponding to factor analysis (FA) of mixed fermentations

were more associated with xylanolytic, amylolytic and pectinolytic activities, and the amount of alcohols, terpenes, ketones (represented by 2-heptanone) and C_{13}-norisoprenoids.

Factor 2 (y-axis) shows a further separation of the four mixed fermentations (Fig. 1c). MSD1 was associated with flower and tropical fruit aromas (norisoprenoids) and soap smells (2-heptanone), while MSD2 was correlated to varietal aroma compounds from the grapes (Pedro Giménez) and rose notes. MSC1 was associated with unpleasant odors such as cheese and rancid notes (fatty acids), whereas MSC2

was strongly correlated to fruity and floral aromas (esters).

All multivariate statistics showed associations between aromas and enzymatic activities in the mixed cultures assayed. All results of the multivariate analysis agree with those obtained with correlation analysis, despite the fact that correlation tests cannot discriminate between initial inoculum proportions of *S. cerevisiae* and non-*Saccharomyces* (Tables 4, 5).

Discussion

The wine industry is interested in mixed inoculations of non-*Saccharomyces* and *S. cerevisiae* yeasts because of technological and sensory reasons (Andorrà et al. 2010). Mixed cultures of *C. sake* BCs403, *D. vanrijiae* BDv566 and *S. cerevisiae* BSc562, strains characterized for their excellent enzyme production (Maturano et al. 2008, 2009a, b) were assayed in the present study. Our results indicate that enzyme activities fluctuated, making it impossible to establish trends over the process. Therefore, we decided to calculate the AUEC because it represents properly the enzymes behavior throughout the process (Douaiher et al. 2007). Based on the methodology used for the analyzing of the experimental data, we can assert that there is no direct relationship between enzymatic activities and biomass detected. Taking into account mixed cultures, non-*Saccharomyces* yeasts were detected until day 4–5, coinciding with the highest enzyme activity levels and lowest ethanol concentrations registered (data not shown). This is in agreement with previous studies carried out under the same conditions in our laboratory (Maturano et al. 2012) when all enzymatic activities were also detected at the end of the mixed fermentations. The results would indicate that the enzymes produced by non-*Saccharomyces* yeasts (especially xylanase and amylase activities) resisted increasing concentrations of ethanol during the fermentation process.

Aroma is one of the most important microbial enzyme contributions to wine. During the pure and mixed culture fermentation processes in the present study, yeasts released enzymes that produced secondary metabolites such as esters, higher alcohols, acids, terpenes, C_{13}-norisoprenoids, aldehydes and ketones.

The population size of non-*Saccharomyces* yeasts and their survival time in the fermentative medium can affect the wine quality (Bely et al. 2008; Andorrà et al. 2010). The present study emphasizes the relationship between enzymatic activities of *Saccharomyces* and non-*Saccharomyces* yeasts and aroma (an important quality parameter of wine). This is observed with two non-*Saccharomyces* strains, BDv566 and BCs403, in MSD2 and MSC1 mixed cultures, respectively. As shown in Figs. 1b, c and Tables 2 and 3, the highest levels of pectinases, amylases and xylanases in MSD2 were associated with the highest concentrations of terpenes and higher alcohols. On the other hand, in MSC1 mixed culture, β-glucosidase, protease and xylanase activities were associated with higher levels of esters and fatty acids.

In the present study, different aromatic compounds were negatively or positively correlated to enzymatic activities. However, the analysis applied does not allow discrimination between different inoculum ratios (MSD1 and MSD2; MSC1 and MSC2).

Acetate esters are the result of the reaction between acetyl CoA and higher alcohols that are formed during degradation of amino acids or carbohydrates (Perestrelo et al. 2006). This could explain the positive correlation between these compounds and carbohydrolases and proteases. Studies about the relationship between microbial enzymatic activities and acetate esters are scarce. Only a few studies examined the effect of polysaccharase-secreting recombinant *S. cerevisiae* strains on the wine aroma. Ganga et al. (1999) reported a significant increase in acetate esters in wines using a recombinant xylanolytic wine yeast. The authors attributed the release of a higher number of glycosidically bound precursors to the enzymatic degradation of the cell wall. Conversely, Louw et al. (2006) observed a decrease in acetate esters in fermentations carried out with recombinant glucanase- and xylanase-secreting strains. In the present study native non-*Saccharomyces* yeasts secreted the highest concentrations of carbohydrolases, and consequently, mixed conditions with these microorganisms could contribute to wines with high levels of acetate esters. Multivariate analysis highlighted MSC1 because of the secretion of β-glucosidases and proteases; highest levels of the corresponding aromatic compounds were observed under this condition.

Higher alcohols (*cis*-3-hexen-1-ol and 1-hexanol) that negatively correlated to enzymatic activities are associated with compounds that are formed during

pre-fermentation steps including harvesting, transport, crushing and pressing (Oliveira et al. 2006). This is not surprising since previous reports relate about biosynthesis of these compounds by enzymes not assayed in the present study (Sánchez Palomo et al. 2007). On the other hand, a significant positive correlation was observed between the main higher alcohol found in wine, 2-phenylethanol, that contributes to wine aroma with rose and honey notes, and enzyme activities in the mixed fermentations assayed (Tables 3a, b). Several authors have reported that this aromatic compound can originate from its glycosylated form present in grapes, rather than by alcoholic fermentation (Carballeira Lois et al. 2001; Oliveira et al. 2008). β-Glucosidase and carbohydrolase activities could be involved in the breakdown of non-aromatic precursors with the subsequent release of higher alcohols (Louw et al. 2006; Botelho et al. 2007; Kang et al. 2010). The MSD2 mixed culture stood out for its production of higher alcohols and this family of compounds was associated with xylanases, amylases and pectinases secreted by yeasts.

We know of no previous studies on the relationship of enzyme activities and fatty acid content in wines. Statistical analysis of our results indicated a positive correlation between enzyme activities and *n*-decanoic acid. Presence of this aroma compound may be the result of alterations in the must composition after the action of carbohydrolases affecting yeast metabolism (Louw et al. 2006). Fatty acids may usually be associated with unpleasant odors (cheese and rancid notes), but they also play a key role in balancing the flavor of the wine, because their presence in the fermentation medium partially prevents hydrolysis of the corresponding ethyl esters (Bertrand 1981; Gil et al. 1996). Because acid production could be affected by polysaccharide degradation in the must, would also influence the production of ethyl esters. This is reflected in the multivariate analysis. Ethyl esters and fatty acids were associated with MSC1, which showed the highest AUEC values of β-glucosidases and proteases.

Terpenes and C_{13}-norisoprenoids are important constituents of the wine aromatic profile and can be present in grape musts in a masked, nonvolatile form. They become visible as free aglycones through the action of β-glucosidase and carbohydrolase during fermentation (Ganga et al. 2001; Strauss et al. 2001; Mendes-Pinto 2009). Indeed, total terpene concentrations in grape must in the current study were significantly lower than in the wine fermentations assayed. Hence, MSD2 and MSC1 mixed cultures showed highest terpene concentrations, which were closely related, as mentioned previously, to the high AUEC values registered for the enzymatic activities assayed (Tables 2, 3). This fact, as mentioned before, may be related to the higher proportion of non-*Saccharomyces* populations present during the first fermentative stage under these mixed conditions (Table 1). Microbial enzymes, able to increase the amount of monoterpenyl-β-D-glucoside/-diglycoside precursors released in the must, were positively correlated to nerolidol and β-myrcene (in MSD1 and MSD2 mixed cultures) and α-linalool, *cis*-geranyl acetone and farnesyl acetate (in MSC1 and MSC2 fermentations), thus contributing to the wine aroma. Despite high values of C_{13}-norisoprenoides in MSD1 (BDv566 was most likely actively involved in the formation of norisoprenoid compounds) as reflected in Figs. 1b and c, statistical analysis revealed significant negative correlations with enzymatic activities (Table 4). A plausible explanation may be that C_{13}-norisoprenoides can also be formed by non-enzymatic reactions such as direct degradation of carotenoids (Mendes-Pinto 2009).

Conclusions

In the current study, mixed inoculations of locally-selected *Saccharomyces* and non-*Saccharomyces* yeasts affected the aroma profile of white wines elaborated with Pedro Giménez grapes.

Several aromatic compounds were negatively or positively associated with the enzymatic activities of the yeasts, and each mixed condition presented a particular aromatic profile. These results encourage the potential use of selected non-*Saccharomyces* wine yeasts in mixed starter cultures as a tool to enhance wine complexity. MSD2 and MSC1 mixed conditions showed the highest values of enzymatic activities that were associated with the main aromatic groups of interest in wine.

The findings of the present study contribute to a better understanding of the effect of enzymatic activities by yeasts on compound transformations that occur during wine fermentation, and thereby improve the comprehension of the microbial interactions in this

complex environment. Inoculation strategies and interactions between the different starter cultures that take place during the fermentation process need to be further examined, as well as the impact of enzymatic activities on the aromatic profile.

Sensory analyses are also necessary to establish a definitive relationship between the production of volatile compounds and the enzymatic activities performed by wine yeasts.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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