Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Production of aroma compounds by cryotolerant *Saccharomyces* species and hybrids at low and moderate fermentation temperatures

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Keywords
aroma compounds, cryotolerant, fermentation metabolites, fermentation temperature, *Saccharomyces* hybrids, *Saccharomyces*.

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2013/1854: received 16 October 2012, revised 21 December 2012 and accepted 2 January 2013

doi:10.1111/jam.12126

Abstract

Aim: Among the most important factors influencing wine quality are yeast strains and fermentation temperature. Fermentation at low temperature is presently used in winemaking to improve both aroma formation and retention. In this study, we have analysed the oenological characteristics of Tempranillo wines produced at 12 and 28°C by different *Saccharomyces* species and hybrids.
Methods and Results: Low temperature had a strong influence on yeasts fermentation kinetics, increasing fermentation times to more than 2 weeks. In some strains, glycerol production seemed to be positively influenced by low fermentation temperature. Analysis of the aroma composition of wines produced by different *Saccharomyces* species and hybrids revealed large differences depending on fermentation temperature.
Conclusions: Higher alcohols production seemed to be dependent on the strain. Production of acetate esters seemed to be favoured at 28°C, whereas production of ethyl esters was apparently preferred at low fermentation temperatures. The best aroma producers at 28°C were *Saccharomyces cerevisiae* strains, whereas *Saccharomyces uvarum* and some hybrids excelled at 12°C.
Significance and Impact of the Study: Our results suggest that fermentation temperature and yeast species are of crucial importance for production of metabolites influencing wine aroma.

Introduction

Wine fermentation is a complex ecological and biochemical process involving the sequential development of different yeasts and bacteria strains. Although different yeast species and genera are present in musts, only species of the *Saccharomyces* genus are responsible for the alcoholic fermentation (Lambrechts and Pretorius 2000). The physiological characterization of industrial *Saccharomyces* strains has showed that, in addition to their high fermentative capabilities, those yeasts also produce valuable secondary metabolites, which have an essential influence on wine quality. Among those metabolites, glycerol, ethanol, higher alcohols, acetates and ethyl esters are the most usually determined (Fleet and Heard 1993; Lambrechts and Pretorius 2000).

Saccharomyces cerevisiae is the main yeast species responsible for alcoholic fermentation, but closely related species such as *Saccharomyces uvarum* (or *Saccharomyces bayanus* var. *uvarum*) and natural hybrids between species of the *Saccharomyces* genus have been also found conducting wine fermentations at low temperatures (Sipiczki 2002, 2008; González *et al.* 2006). Moreover, the recently described *Saccharomyces* hybrids seem to be better adapted than *S. cerevisiae* to carry out fermentations at low temperatures (González *et al.* 2007; Gangl *et al.* 2009). However, wine fermentation at low temperature increases the probability of sluggish or stuck fermentations, which is a problem reported in case of fermentations conducted by *S. cerevisiae* (Novo *et al.* 2003). These risks can be reduced by the selection of wine yeasts able to ferment at low temperature while preserving the good quality of wines.

Cryotolerant *Saccharomyces* yeasts, such as *S. uvarum*, are good producers of glycerol and secondary aroma

compounds (Giudici *et al.* 1995; Antonelli *et al.* 1999; Sipiczki 2002; Tosi *et al.* 2009). Similarly, natural hybrids between *S. cerevisiae* and *Saccharomyces kudriavzevii* adapted to ferment at low temperature produce high amounts of glycerol and higher alcohols when compared to reference strains of their parental species (González *et al.* 2007; Gangl *et al.* 2009). Previous studies demonstrated that artificial hybrids between *S. cerevisiae* × *S. uvarum* produced intermediate amounts of glycerol compared with their *S. cerevisiae* and *S. uvarum* parentals (Zambonelli *et al.* 1997), while retaining the cryotolerance from *S. uvarum* (Kishimoto 1994). Regardless of the limited studies on the fermentative potential and aromatic profile generated by *Saccharomyces* hybrids, several strains (Lalvin W27, Lalvin W46 and Lalvin S6U) are being commercialized to perform fermentations at low temperature.

Wine fermentative aroma is the result of a complex mix of chemical compounds produced by yeast secondary metabolism (Lambrechts and Pretorius 2000; Swiegers and Pretorius 2005). The principal aromas in young wines are higher alcohols (fusel, marzipan and floral aromas) as well as acetate and ethyl esters (fruity and floral aromas). Higher alcohols can be synthesized either from intermediates of sugar metabolism, through anabolic reactions, or from branched-chain amino acids, through a multistep catabolic reaction, the Ehrlich pathway (Boulton *et al.* 1996; Dickinson *et al.* 1997, 2003; Eden *et al.* 2001). Ethyl ester compounds are produced by condensation of an alcohol and a coenzyme-A-activated acid (acyl-CoA; Swiegers and Pretorius 2005), while acetate esters result from the combination of acetyl-CoA with an alcohol, by the action of the alcohol acetyl transferases (Lambrechts and Pretorius 2000). The nature and amount of these aroma compounds depend on multiple factors, such as the nitrogen content of the must, temperature of fermentation and yeast strain (Lambrechts and Pretorius 2000; Swiegers *et al.* 2006).

This work explores the fermentative performance and aroma production by different cryophilic *Saccharomyces* species and hybrids at 12 and 28°C temperatures. Neutral 'Tempranillo' grape must was selected to test the fermentative aromas produced by the secondary metabolism of yeast. Concentration of metabolites ethanol, glycerol, acetic acid and acetaldehyde as well as higher alcohols, acetate esters and ethyl esters were measured.

Materials and methods

Yeast strains

The yeasts used in this study belong to different species of the genus *Saccharomyces* as well as diverse natural interspecific hybrids among these species. Table 1 shows references and origin of these yeasts.

Microvinifications

The yeast strains were cultivated in Erlenmeyer flasks containing 250 ml de GPY medium (0·5% peptone, 4% glucose, 0·5% yeast extract) at 25°C in an agitated incubator (Selecta, Barcelona, Spain). At the end of the exponential phase determined by the absorbance at 600 nm, 2×10^6 cells ml^{-1} were inoculated in each must flask. Fermentations were carried out in duplicate using 450 ml of Tempranillo grape must at pH of $3{\cdot}5 \pm 0{\cdot}1$. Before fermentation, must was clarified by sedimentation for 24 h at 4°C in the presence of 60 mg l^{-1} of sulfur dioxide. After separation, chemically pure glucose and fructose were added to raise the sugar content to 250 g l^{-1}. The must was then supplemented with 0·25 g l^{-1} of yeast nutrients (Lallemand, Montreal, QC, Canada). Yeast assimilable nitrogen was determined by the formol index method (Aerny 1997), and diammonium sulfate was added to reach a final concentration of 250 mg l^{-1}. Finally, must was sterilized adding dimethyl dicarbonate (Fluka, Buchs, St. Gallen, Switzerland) in a concentration of 1 ml l^{-1} must.

Tempranillo grape must was fermented at 12 and 28°C. Fermentations were carried out in duplicate (biological duplicate) and monitored by sugar consumption. Glucose and fructose concentrations were determined enzymatically in an Echo-Enosys analyzer (Tecnova S.A., Madrid, Spain). Fermentations were finished when concentration of reducing sugars was lower than 2 g l^{-1}. Samples taken at the last day of wine fermentation were used to determine concentrations of different metabolites using experimental duplicates.

Glycerol, ethanol, acetic acid and acetaldehyde determination

Glycerol concentration in wine was measured by liquid chromatography consisting of a GP40 gradient pump, an ED40 pulsed electrochemical detector and an AS3500 autosampler system (Dionex Corporation, Sunnyvale, CA, USA). The mobile phase consisted of water and sodium hydroxide 1 mol l^{-1} (52 : 48, V/V) at a flow rate of 0·4 ml min^{-1}. The anion-exchange CarboPac MA1 column (Dionex, 4 × 250 nm) with guard (4 × 50 nm) was used for chromatographic separation.

Ethanol concentration in wine was determined enzymatically (Boehringer Mannhein, Mannheim, Baden-Württenberg, Germany) using a spectrophotometer (Ultrospec 2100 pro; Amersham Biosciences, Freiburg, Baden-Württenberg).

Table 1 List of strains used in this study. Days of microvinification of Tempranillo grape must at 12 and 28°C

Species	Yeast strains	Origin	Fermentation days at 12°C	Fermentation days at 28°C
Saccharomyces cerevisiae	Lalvin T73, Lallemand, Montreal	Wine, Spain	21	6
	FCry,* Pascal Biotech, Paris	Wine, France	17	3
	FRCh,† Pascal Biotech, Paris	Sparkling wine, France	15	4
Saccharomyces uvarum	BMV58	Wine, Spain	21	6‡
	CECT 12600	Wine, Spain	17	4
	CECT 1969	Red currant, Holland	24‡	4‡
Saccharomyces kudriavzevii	IFO 1802	Decayed leaves, Japan	11	11
S. cerevisiae × *S. uvarum*	Lalvin S6U, Lallemand	Wine, Switzerland	14	6
S. cerevisiae × *S. kudriavzevii*	Lalvin W27, Lallemand	Wine, Switzerland	14	5
	AMH§	Wine, Germany	20	11
	HA 1841	Wine, Austria	21	7
	VIN7, Anchor Yeast, Cape Town	Wine, South Africa	23	6
S. cerevisiae × *S. uvarum* × *S. kudriavzevii*	CBS 2834	Wine, Switzerland	25	8

Strains W27, T73 and IFO 1802 have been used in González *et al.* (2007); VIN7 appears in King *et al.* (2008); HA 1841 was used in Gangl *et al.* (2009); AMH, Assmanhausen has been used in Egli *et al.* (1998).
*Fermol Cryophile.
†Fermol Reims Champagne.
‡Stuck fermentation.
§Assmanhausen.

Acetic acid and acetaldehyde concentrations in wine were determined enzymatically in a refrigerated Echo -Enosys analyzer (Tecnova S.A.).

Higher alcohols and esters determination

Extraction of higher alcohols and esters from wine samples was carried out by headspace solid-phase microextraction sampling using poly(dimethylsiloxane) (PDMS) fibres (Supelco, Sigma-Aldrich, St Louis, MO, USA) following the protocol of Rojas *et al.* (2001). Separation of alcohols and esters was carried out by gas chromatography using a Hewlett-Packard (HP) 5890 Series II gas chromatograph with a flame ionization detector and an HP-INNOWAX 30 m× 0·25 mm capillary column coated with 0·25 μm layer of cross-linked polyethylene glycol (Agilent Technologies Inc., Santa Clara, CA, USA). Carrier gas was helium (1 ml min^{-1}). Temperature programme was: 5 min at 35°C, 2°C min^{-1} to 150°C, 20°C min^{-1} to 250°C and 2 min at 250°C. Detector temperature was 300°C and injector temperature 220°C (splitless). Chromatographic signal was registered by a HP Vectra QS/16S detector and HP3365 Chemstation program.

Volatile compound concentrations were determined using calibration curves of the corresponding standard volatile compounds. Concentrations are given as the mean of two independent fermentations. 2-heptanone (0·05% w/v) was used as internal standard. The analysed compounds in elution order were: ethyl acetate, isobutyl acetate, isobutanol, isoamyl acetate, isoamyl alcohol, ethyl caproate (ethyl hexanoate), hexyl acetate, ethyl lactate, 1-hexanol, ethyl caprylate (ethyl octanoate), ethyl caprate (ethyl decanoate), diethyl succinate, phenylethyl acetate, benzyl alcohol and 2-phenylethanol.

Statistics

Statgraphics Centurion XV (StatPoint Inc., Waarenton, VA, USA) was used for variance analysis ANOVA and Fisher's least significant difference test (LSD test). These tests were applied to ethanol, glycerol and aroma production by the individual yeasts at 12 and 28°C and among all strains at 12 or 28°C. Heat map displaying differences in aroma production by individual strains at 12 and 28°C was generated using IBM SPSS Statistics v.19 (IBM, Madrid, Spain).

Results

Fermentation kinetics

The ability of cryophilic *Saccharomyces* strains and hybrids to ferment Tempranillo grape must at two different temperatures 12 and 28°C evaluated in fermentation days is summarized in Table 1. Comparison of fermentation days at both temperatures revealed that all strains fermented faster at 28°C than at 12°C, except *S. kudriavzevii* IFO 1802. This strain was the slowest at 28°C and

the fastest at 12°C, revealing its cryophilic character. Double hybrids S6U and W27 exhibited the shortest time difference between fermentations at 28 and 12°C, whereas double hybrid VIN7 and triple hybrid CBS 2834 showed the largest time difference. At 28°C fermentation temperature, *S. cerevisiae* FCry and double hybrid AMH were the fastest and slowest, respectively. The remaining strains reached the end of fermentation after 1 week at 28°C approximately. Strain *S. uvarum* CECT 1969 was not able to finish fermentation at any temperature. Moreover, at 12°C fermentation progressed very slowly and finally stopped at day 24 of fermentation, whereas at 28°C fermentation was stuck from day 4, although sugars were measured till day 24. Likewise, strain *S. uvarum* BMV58 was stuck at 28°C after day 6 of fermentation. The slowest fermenting yeasts were triple hybrid CBS 2834 at 12°C and double hybrid AMH at 28°C.

Main metabolites in wine

Glycerol, ethanol, acetic acid and acetaldehyde were determined to assess the effect of temperature and strain on the production of these compounds. Figure 1 shows ethanol and glycerol concentration at both temperatures 12 and 28°C.

Comparison of ethanol production between individual strains at the two temperatures of the study (12 and 28°C) showed statistically not significant differences (95% significance) except in case of strains FCry, CECT 1969 (stuck fermentation) and CECT 12600. Comparison of ethanol production between all strains at the same temperature, 12 or 28°C, revealed significant differences indicated in Fig. 1.

Comparison of glycerol production between individual strains at the two temperatures of the study (12 and 28°C) showed differences statistically significant (95% significance) in seven strains. *Saccharomyces cerevisiae* FCry and hybrids AMH, HA 1841 and CBS 2834 produced more glycerol at 28°C than at 12°C, whereas *S. uvarum* CECT 1969 (stuck fermentation) and CECT 12600, *S. kudriavzevii* IFO 1802 and double hybrid S6U produced more glycerol at 12°C than at 28°C. Differences in glycerol production between all strains at same temperature, 12 or 28°C, are indicated in Fig. 1.

The levels of acetic acid and acetaldehyde in all wines were below the sensorial thresholds, 0·7 g l^{-1} and 100 mg l^{-1}, respectively (Dubois 1994; Swiegers *et al.* 2005).

Aroma compounds

Tables 2 and 3 show the concentration of the different higher alcohols, acetate esters and ethyl esters produced

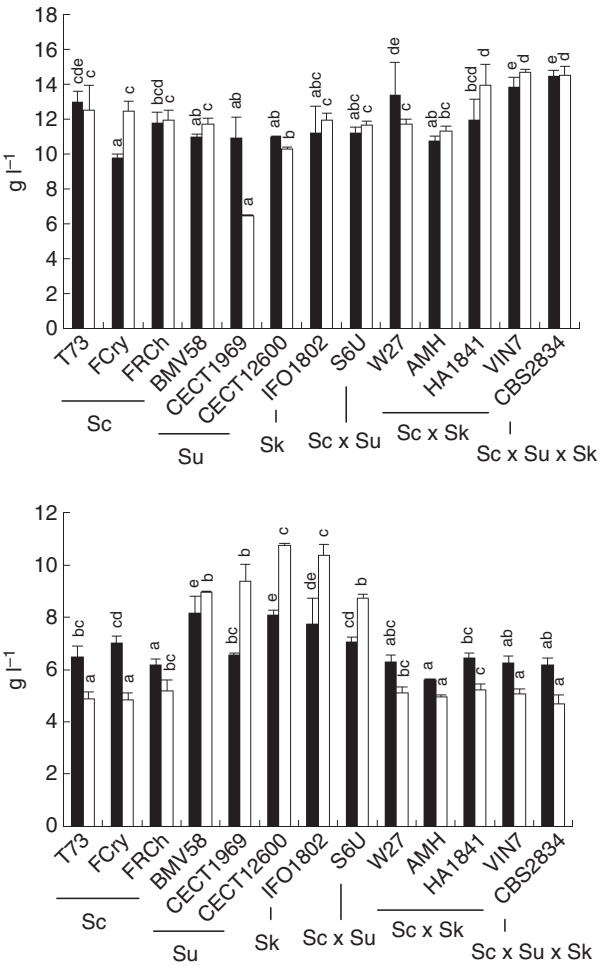

Figure 1 Comparison of ethanol and glycerol production at 12 and 28°C. Statistically significant differences regarding the concentration of ethanol and glycerol between strains at each temperature are indicated by labels at the top of the columns. (■) 28°C and (□) 12°C..

by *Saccharomyces* strains and hybrids at 12 and 28°C, respectively. Benzyl alcohol was solely detected in wines fermented at 12°C (Table 2). Most aroma compounds were produced below their odour thresholds at both temperatures. Hexyl acetate was produced below its odour threshold at 28°C (Table 3), but only strain FCry produced hexyl acetate above the detection threshold at 12°C (Table 2). Ethyl caprate was produced below its odour threshold at 12°C and only three strains produced it above its odour threshold at 28°C (Tables 2 and 3). *Saccharomyces uvarum* BMV58 produced the highest levels of aromas at 12°C reaching the highest scores in three compounds. *Saccharomyces cerevisiae* FRCh produced the highest levels of aromas at 28°C reaching the highest scores in five compounds.

Comparison of total higher alcohols and acetate and ethyl esters produced by the strains (data not shown)

Table 2 Production of aroma compounds by *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces kudriavzevii* and their hybrids at 12°C

Strains	Isobutanol 40*	Isoamyl alcohol 30*	1-Hexanol 8*	Benzyl alcohol 900†	2-Phenyl ethanol 14‡	Ethyl acetate 12·26†	Isobutyl acetate 1·6‡	Isoamyl acetate 0·03*	Hexyl acetate 0·115§	2-Phenylethyl acetate 0·250*	Ethyl caproate 0·014‡	Ethyl caprylate 0·005‡	Ethyl caprate 0·2‡	Diethyl succinate 200*
T73	33·199^{c}	212·528^{h}	3·597^{cde}	0·000^{a}	33·926^{ab}	56·477^{cd}	0·000^{a}	1·834^{f}	0·085^{h}	0·106^{a}	0·004^{e}	0·258^{b}	0·046^{b}	0·000^{a}
FCry	19·765^{b}	124·253^{b}	2·840^{ab}	0·000^{a}	27·182^{a}	37·629^{a}	0·107^{c}	3·085^{h}	**0·130**^{j}	0·210^{a}	0·007^{h}	**1·513**^{f}	0·066^{c}	9·455^{f}
FRCh	10·773^{a}	113·078^{a}	3·058^{ab}	0·000^{a}	32·207^{ab}	46·532^{ab}	0·000^{a}	1·011^{d}	0·028^{b}	0·120^{a}	0·007^{i}	0·552^{c}	0·068^{cd}	0·000^{a}
BMV58	63·624^{f}	276·098^{k}	3·861^{e}	**7·236**^{c}	**212·462**^{d}	133·757^{h}	0·130^{d}	1·296^{e}	0·064^{e}	**2·576**^{e}	0·004^{d}	0·266^{b}	0·085^{e}	0·404^{b}
CECT 1969	45·143^{d}	160·009^{d}	3·237^{bcd}	5·255^{b}	77·915^{c}	**135·110**^{h}	0·000^{a}	0·459^{bc}	0·031^{bc}	1·397^{c}	0·001^{a}	0·137^{a}	0·000^{a}	0·000^{a}
CECT 12600	48·818de	252·047^{j}	2·936^{ab}	0·000^{a}	208·137^{d}	72·928^{f}	0·106^{c}	1·282^{e}	0·057^{d}	2·216^{d}	0·005^{f}	1·016^{e}	0·073^{d}	2·048^{c}
IFO 1802	53·728^{e}	220·958^{i}	4·556^{f}	0·000^{a}	83·451^{c}	63·762de	**0·167**^{f}	2·141^{g}	0·079^{g}	0·634^{b}	0·006^{g}	0·503^{c}	0·097^{fg}	0·639^{b}
S6U	**123·343**^{g}	196·975^{g}	3·651de	0·000^{a}	72·773^{c}	69·558^{ef}	0·000^{a}	0·137^{a}	0·071^{f}	0·229^{a}	0·003^{c}	0·165^{a}	0·000^{a}	0·000^{a}
W27	59·356^{f}	**330·096**^{l}	**4·619**^{f}	0·000^{a}	51·851^{b}	51·433^{bc}	0·089^{b}	0·629^{c}	0·034^{c}	0·136^{a}	0·002^{b}	0·518^{c}	0·103^{g}	0·000^{a}
AMH	24·436^{b}	132·124^{c}	2·911^{ab}	0·000^{a}	28·985^{a}	58·437^{cd}	0·113^{c}	1·250^{e}	0·000^{a}	0·183^{a}	0·002^{a}	0·132^{a}	0·000^{a}	0·000^{a}
HA 1841	47·767^{d}	169·469^{e}	2·914^{ab}	0·000^{a}	43·039^{ab}	68·889^{ef}	0·000^{a}	0·357^{ab}	0·030^{bc}	0·125^{a}	0·004^{ef}	0·714^{d}	0·095^{f}	4·226^{d}
VIN7	30·740^{c}	186·771^{f}	2·666^{a}	0·000^{a}	42·767^{ab}	110·621^{g}	0·149^{e}	**4·979**^{i}	0·106^{i}	0·486^{b}	0·008^{i}	1·489^{f}	0·087^{e}	**10·133**^{g}
CBS 2834	48·087^{d}	194·092^{g}	3·117^{abc}	0·000^{a}	49·894^{b}	72·113^{ef}	0·000^{a}	0·190^{a}	0·069^{ef}	0·171^{a}	**0·010**^{j}	0·954^{e}	**0·113**^{h}	6·276^{e}

Numbers below the column heads indicate odour thresholds in mg l^{-1}. Amounts of aroma compounds are expressed in mg l^{-1}. Statistically significant differences regarding the concentration of different aroma compounds between strains at each temperature are indicated by super-indexes. Numbers in bold indicate the highest values of each aroma compound. Standard errors were always lower than 15% of the mean values. Statistically different groups were established with 95% confidence.

*Guth (1997a,b).

†Etiévant (1991).

‡Ferreira *et al.* (2002).

§Takeoka *et al.* (1996).

Table 3 Production of aroma compounds by *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces kudriavzevii* and their hybrids at 28°C

Strains	Isobutanol 40*	Isoamyl alcohol 30*	1-hexanol 8*	2-Phenyl ethanol 14†	Ethyl acetate 12·26‡	Isobutyl acetate 1·6†	Isoamyl acetate 0·03*	Hexyl acetate 0·115§	2-Phenylethyl acetate 0·250*	Ethyl caproate 0·014†	Ethyl caprylate 0·005†	Ethyl caprate 0·2†	Diethyl succinate 200‡
T73	29·092^{bc}	189·702^{e}	2·820^{cd}	91·127^{h}	72·393^{abc}	0·135^{abc}	4·589^{h}	0·071^{d}	1·269^{e}	**0·006**^{g}	1·588^{f}	0·150^{d}	0.641
FCry	42·511^{d}	240·006^{f}	2·273^{b}	96·004^{h}	115·409^{ef}	**0·247**^{g}	**6·026**^{i}	0·083^{e}	2·056^{g}	0·003^{f}	0·526^{e}	0·059^{b}	0·000^{a}
FRCh	37·811^{cd}	166·946^{d}	2·424^{b}	41·292^{cd}	86·012^{bcde}	0·163^{cd}	3·135^{f}	**0·089**^{e}	0·574^{bc}	**0·006**^{g}	**1·879**^{g}	**0·459**^{g}	**0·894**^{e}
BMV58	19·710^{ab}	71·188^{a}	3·039^{d}	82·359^{g}	81·642^{abcde}	0·156^{bcd}	1·357^{c}	0·085^{e}	1·789^{f}	0·003^{cde}	0·386^{d}	0·182^{e}	0·159^{c}
CECT 1969	13·973^{a}	56·472^{a}	2·945^{d}	36·294^{abc}	57·811^{ab}	0·113^{a}	0·808^{b}	0·071^{d}	0·433^{b}	0·001^{b}	0·330^{cd}	0·239^{f}	0·000^{a}
CECT 12600	17·054^{a}	126·783^{c}	2·285^{b}	**143·209**^{i}	46·188^{a}	0·113^{a}	1·686^{d}	0·067^{d}	**2·364**^{h}	0·002^{cd}	0·399^{d}	0·247^{f}	0·085^{b}
IFO 1802	31·803^{c}	114·584^{bc}	**3·849**^{e}	31·494^{ab}	125·340^{f}	0·178de	2·287^{e}	0·054^{c}	1·509^{e}	0·003^{def}	0·203^{bc}	0·042^{b}	0·000^{a}
S6U	47·339de	103·857^{b}	2·538^{bc}	39·119^{bc}	99·358^{cdef}	**0·247**^{g}	3·845^{g}	0·065^{d}	1·004^{d}	0·002^{c}	0·172^{b}	0·036^{b}	0·000^{a}
W27	55·896^{ef}	247·904^{f}	2·262^{b}	75·722^{g}	77·852^{abcd}	0·212^{f}	3·170^{f}	0·069^{d}	0·789^{cd}	0·003^{ef}	0·639^{e}	0·135^{d}	0·000^{a}
AMH	**113·793**^{l}	98·046^{b}	1·355^{a}	30·273^{a}	**403·215**^{g}	0·163^{cd}	0·032^{a}	0·028^{a}	0·115^{a}	0·000^{a}	0·000^{a}	0·000^{a}	0·000^{a}
HA 1841	94·448^{h}	**256·238**^{f}	2·237^{b}	62·541^{f}	103·178^{cdef}	0·213^{f}	1·775^{d}	0·055^{c}	0·355^{ab}	0·003^{cde}	0·607^{e}	0·124^{cd}	0·000^{a}
VIN7	67·173^{g}	240·070^{f}	2·360^{b}	55·680^{ef}	108·744^{def}	0·205^{ef}	3·162^{f}	0·032^{a}	0·361^{ab}	0·001^{b}	0·199^{b}	0·095^{c}	0·000^{a}
CBS 2834	61·002^{fg}	209·114^{e}	2·501^{bc}	48·490de	75·592^{abcd}	0·130^{ab}	0·625^{b}	0·042^{b}	0·334^{ab}	0·002^{cd}	0·384^{d}	0·098^{c}	0·000^{a}

Numbers below the column heads indicate odour thresholds in mg l^{-1}. Amounts of aroma compounds are expressed in mg l^{-1}. Statistically significant differences regarding the concentration of different aroma compounds between strains at each temperature are indicated by super-indexes. Numbers in bold indicate the highest values of each aroma compound. Statistically different groups were established with 95% confidence. Standard errors were always lower than 15% of the mean values.

*Guth (1997a,b).

†Ferreira *et al.* (2002).

‡Etiévant (1991).

§Takeoka *et al.* (1996).

demonstrated that strains *S. uvarum* BMV58 and CECT 12600 produced the highest amounts of total higher alcohols at 12°C, which were the highest in case of 2-phenyl ethanol (Table 2). Double hybrid VIN7 and *S. uvarum* CECT BMV58 and CECT 1969 were the highest producers of acetate esters at 12°C. VIN7 was the highest producer of isoamyl acetate, whereas BMV58 was the highest producer of 2-phenylethyl acetate (Table 2). Double hybrid VIN7 and *S. cerevisiae* FCry were the highest producers of ethyl esters at 12°C (Table 2). At 28°C double hybrid HA 1841 produced the highest amounts of higher alcohols. This strain was the highest producer of isoamyl alcohol and the second highest producer of isobutanol (Table 3). At 28°C *S. cerevisiae* FCry and FRCh were the highest producers of most acetate and ethyl esters, respectively (Table 3). The *S. cerevisiae* strains were among the poorest producers of 2-phenylethanol and 2-phenylethanol acetate at 12°C, whereas double hybrid AMH was the poorest producer of the same compounds at 28°C (Tables 2 and 3).

Aroma production by the individual yeasts at 12 and 28°C was compared statistically (confidence intervals of significant aroma production at any temperature were established at 95%) and results represented in a heat map (Fig. 2). In the case of higher alcohols, *S. uvarum*, *S. kudriavzevii* and double hybrid S6U produced significantly further higher alcohols at 12°C than at 28°C. Double hybrids W27 and AMH produced more isoamyl alcohol and 1-hexanol at 12°C; however, their production of 2-phenylethanol appeared not to be influenced by fermentation temperature, and their production of isobutanol showed different temperature dependence for each strain. The remaining strains produced similar (*S. cerevisiae* FRCh and triple hybrid CBS 2834) or higher (double hybrid HA 1841 and *S. cerevisiae* FCry) amount of higher alcohols at 28°C than at 12°C. Production of higher alcohols by *S. cerevisiae* T73 was apparently independent from the temperature except in the case of 2-phenylethanol production, which was favoured at 28°C. Production of acetate esters appeared to be higher at 28°C than at 12°C. In strains T73, FCry, Ha 1841 and VIN7, the higher phenylethanol production at 28°C corresponded with a higher production of the corresponding acetate. A similar correspondence at 12°C was observed

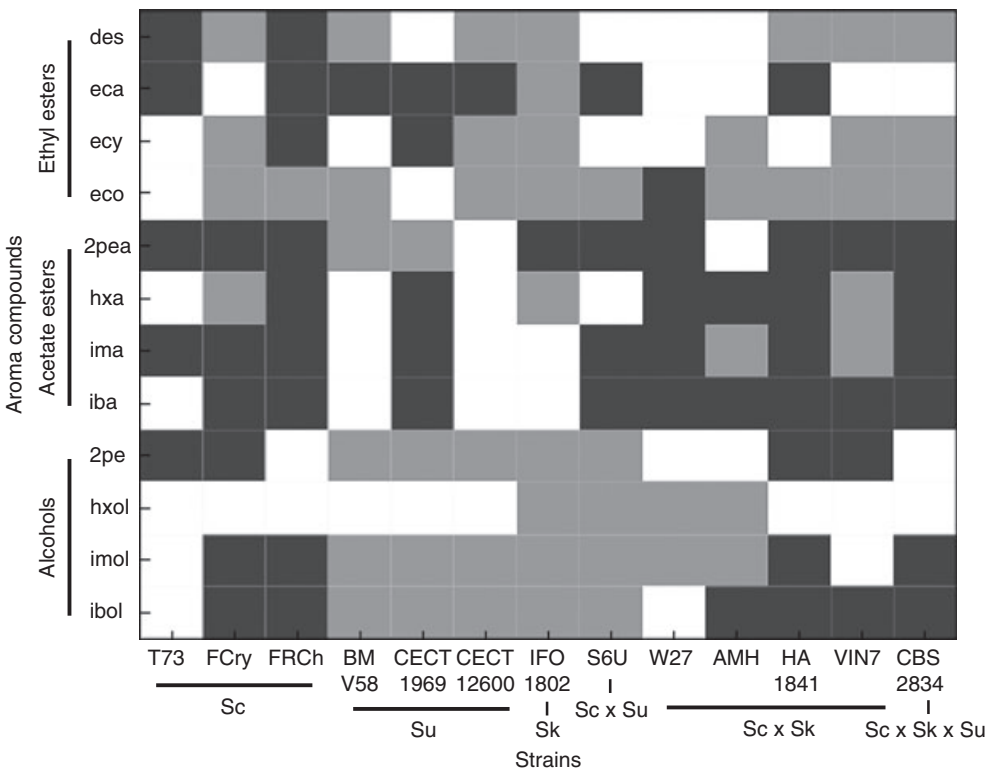

Figure 2 Heat map displaying the temperature at which every strain produced the highest amount of each aroma compound. Ethyl acetate was excluded from the total sum of esters due to its distinctive contribution to the aroma of wine (Cabrera *et al.*, 1998; Lema *et al.*, 1996). Acronyms of aroma compounds: ibol, isobutanol; imol, isoamyl alcohol; hxol, 1-hexanol; 2pe, 2-phenylethanol iba, isobutyl acetate; ima, isoamyl acetate; hxa, hexyl acetate; 2pea, 2-phenylethyl acetate; eco, ethyl caproate; ecy, ethyl caprylate; eca, ethyl caprate; des, diethyl succinate. Acronyms of species: Sc, *Saccharomyces cerevisiae*; Su, *Saccharomyces uvarum*; Sk, *Saccharomyces kudriavzevii*. (▪) 12°C; (■) 28°C and (□) no differences.

in strains BMV58 and CECT 1969. The remaining strains showed no alcohol–acetate correspondence. The *S. uvarum* strains showed different temperature preferences for acetate ester production. In the strains BMV58 and CECT 12600, generation of acetates seemed to be independent of the temperature, except in case of 2-phenylethyl acetate by BMV58. In contrast, CECT 1969 showed better production of acetates at 28°C except in the case of 2-phenylethyl acetate. *Saccharomyces kudriavzevii* and double hybrid S6U showed mixed temperature responses for acetate esters production although 2-phenylethyl acetate was favoured at 28°C. *Saccharomyces cerevisiae* strains and most hybrids showed a clear preference for acetate esters production at 28°C, and solely double hybrids VIN7 and AMH and *S. cerevisiae* FCry seemed to favour generation of hexyl and/or isoamyl acetates at 12°C. Ethyl esters production appeared to be favoured at 12°C for most strains except in case of ethyl caprate. *Saccharomyces cerevisiae* and *S. uvarum* strains showed mixed responses for ethyl esters production. Most hybrids showed preference for ethyl esters production at 12°C although generation of several compounds was indifferent of the temperature.

Discussion

Several authors have demonstrated that wines produced at low temperatures develop improved characteristics of taste and aroma due to greater retention of terpenes, a reduction in higher alcohols and an increase in the proportion of ethyl and acetate esters (Feuillat 1997; Llauradó *et al.* 2002; Torija *et al.* 2003). Accordingly, yeast producers and winemakers adapt to the new fermentation conditions by producing and using yeasts with good fermentation rates and enhanced aroma production at low temperatures.

The major constraints experienced by yeasts at low temperature fermentations are maintenance of the fermentative ability and the time required to reach the end of fermentation. *Saccharomyces cerevisiae* yeasts are good fermenters at moderate and high temperatures (Kishimoto 1994; Bertolini *et al.* 1996; González *et al.* 2007) although some strains of this species have been commercialized to ferment at low temperatures (http://www.vignevin.com). Other *Saccharomyces* species such as *S. uvarum* and *S. kudriavzevii* are considered cryotolerant yeasts and therefore better adapted to low fermentative temperatures (Giudici *et al.* 1998; Naumov *et al.* 2000; Pulvirenti *et al.* 2000; Belloch *et al.* 2008; Sampaio and Gonçalves 2008). Several studies have demonstrated the ability of hybrids between *S. cerevisiae* and *S. uvarum* or *S. kudriavzevii* to grow and ferment at low temperatures (Kishimoto 1994; Zambonelli *et al.* 1997; González *et al.* 2007; Belloch *et al.* 2008; Gangl *et al.* 2009; Arroyo-López *et al.* 2010).

In this study, we have compared fermentation dynamics as well as metabolite and aroma produced by several cryophilic *S. cerevisiae*, *S. uvarum*, *S. kudriavzevii* and natural hybrids between these species at two different fermentation temperatures, 12 and 28°C.

The results show that most *Saccharomyces* strains and hybrids were able to consume all reducing sugars at both fermentation temperatures. Strain *S. uvarum* BMV58 could not finish fermentation at 28°C, revealing the cryophilic character of this strain. Previous biometric studies based on physiological and technological properties of *S. uvarum* strains clearly indicated that this species had lower capacity to ferment at 24°C than *S. cerevisiae* (Masneuf-Pomarede *et al.* 2010). Moreover, earlier published data suggested that cryotolerant wine strains had low ethanol resistance at 25°C (Kishimoto 1994). Our results would be partially in agreement with this conclusion as some *S. uvarum* strains did not seem to be affected by high fermentation temperatures (e.g. CECT 12600). In addition, our results show that neither cryotolerant *Saccharomyces* species nor hybrids would be inhibited by ethanol at moderate or intermediate fermentation temperatures.

Production of glycerol by cryotolerant *S. uvarum* at low and moderate fermentation temperatures has been reported previously (Kishimoto 1994; Masneuf-Pomarede *et al.* 2010). Glycerol is one of the main metabolites produced in wine fermentation. This metabolite contributes positively to wine quality by providing slight sweetness, smoothness and fullness and reducing wine astringency (Ishikawa and Noble 1995; Remize *et al.* 2000). Glycerol is involved in osmoregulation (Ansell *et al.* 1997; Nevoigt and Stahl 1997) and adaptation to low-temperature growth in yeasts (Izawa *et al.* 2004). Our results showed that at low and moderate temperature of fermentation *S. uvarum*, *S. kudriavzevii* and double hybrid *S. cerevisiae* × *S. uvarum* Lalvin S6U were among the highest glycerol producers (Fig. 1). Similar studies comparing glycerol production by *S. cerevisiae*, *S. kudriavzevii, S. uvarum* and *Saccharomyces* hybrids support our findings (Kishimoto 1994; Bertolini *et al.* 1996; Zambonelli *et al.* 1997; González *et al.* 2007; Arroyo-López *et al.* 2010).

Low fermentation temperatures affect yeast metabolism and therefore final composition and quality of wine aroma (Llauradó *et al.* 2005; Beltrán *et al.* 2008). Several authors have attributed the improvement in the quality of wine aroma at low temperatures to a reduction in higher alcohols production and an increase in acetate and ethyl esters (Lambrechts and Pretorius 2000; Novo *et al.* 2003; Torija *et al.* 2003; Llauradó *et al.* 2004). Our results agree with those findings in case of *S. cerevisiae* and higher alcohols production although not in case of other *Saccharomyces* species and hybrids.

In contrast to previous studies, our results show that acetate esters production was apparently favoured at 28°C by all strains except in case of some *S. uvarum* strains and double hybrids (Fig. 2). Most strains showed an increase in acetate esters production at 28°C even when the corresponding alcohol production was favoured at 12°C, which might indicate an increased acetyltransferase (ATF) activity at higher temperatures. Nevertheless, some strains such as *S. cerevisiae* FCry, *S. uvarum* BMV58 and CECT 12600 and double hybrid VIN7 were very good acetate esters producers independent of the fermentation temperature (Tables 2 and 3). Finally, ethyl ester production was clearly favoured by fermentation at low temperatures except in case of ethyl caprate (Fig. 2). *Saccharomyces cerevisiae* strains were among the best producers of ethyl esters at 28°C, FRCh and T73, and 12°C, FCry (Tables 2 and 3).

Aroma production by double hybrids *S. cerevisiae* × *S. kudriavzevii* W27 and HA1841 was previously investigated (González *et al.* 2007; Gangl *et al.* 2009). Both studies reported that aroma production profile of these hybrids was similar to that of *S. kudriavzevii* at low fermentation temperature, whereas at moderate or high fermentation temperatures, they showed higher similarities with *S. cerevisiae*. Our results do not show such similarities between aroma profiles of hybrids and parental species except for higher alcohols production that were comparable to those of *S. cerevisiae* at 28°C and *S. kudriavzevii* at 12°C.

Fruity and floral aromas found in wines are essentially influenced by the amounts of 2-phenylethanol and 2-phenylethyl acetate. Increased production of 2-phenylethanol and 2-phenylethyl acetate by *S. uvarum* has been a demonstrated trait of this species (Bertolini *et al.* 1996; Masneuf *et al.* 1998; Antonelli *et al.* 1999; Gangl *et al.* 2009; Tosi *et al.* 2009). Similarly, the results of this study showed that the highest producers of these compounds at 12 or 28°C were some of the *S. uvarum* strains as well as *S. kudriavzevii* IFO 1802 and double hybrid S6U. In opposition, both compounds by most *S. cerevisiae* strains were higher at 28°C.

Comparison of aroma compounds produced by cryotolerant yeasts in natural Tempranillo must fermentation at low and moderated temperatures revealed that aromatic profile of wines is significantly affected by fermentation temperature and fermenting yeast. Production of higher alcohols and ethyl esters seemed to be favoured at 12°C, whereas production of acetate esters appeared to be favoured at 28°C. Strains *S. cerevisiae* FCry and double hybrid VIN7 were the best aroma producers at 12°C showing high production of acetate and ethyl esters while maintaining moderate levels of higher alcohols. On the contrary, at 28°C production of acetate and ethyl esters was lead by *S. cerevisiae* T73 and FRCh although both strains showed substantial production of higher alcohols.

Acknowledgements

AQ acknowledges the financial support from the Spanish Government project AGL2009-12673-CO2-01 and Generalitat Valenciana project PROMETEO/2009/019. CB acknowledges MICINN for a PTA2007 research contract. AG and JT acknowledge a CSIC I3P contract and a MICINN FPI grant, respectively.

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