RESEARCH ARTICLE

Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: effects of yeast assimilable nitrogen on two model strains

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Received 18 November 2007; revised 5 June 2008; accepted 6 June 2008.
First published online 11 July 2008.

DOI:10.1111/j.1567-1364.2008.00412.x

Editor: Patrizia Romano

Keywords
Saccharomyces cerevisiae; assimilable nitrogen; fermentation aroma compounds.

Abstract

The contribution of yeast fermentation metabolites to the aromatic profile of wine is well documented; however, the biotechnological application of this knowledge, apart from strain selection, is still rather limited and often contradictory. Understanding and modeling the relationship between nutrient availability and the production of desirable aroma compounds by different strains must be one of the main objectives in the selection of industrial yeasts for the beverage and food industry. In order to overcome the variability in the composition of grape juices, we have used a chemically defined model medium for studying yeast physiological behavior and metabolite production in response to nitrogen supplementation so as to identify an appropriate yeast assimilable nitrogen level for strain differentiation. At low initial nitrogen concentrations, strain KU1 produced higher quantities of esters and fatty acids whereas M522 produced higher concentrations of isoacids, γ-butyrolactone, higher alcohols and 3-methylthio-1-propanol. We propose that although strains KU1 and M522 have a similar nitrogen consumption profile, they represent useful models for the chemical characterization of wine strains in relation to wine quality. The differential production of aroma compounds by the two strains is discussed in relation to their capacity for nitrogen usage and their impact on winemaking. The results obtained here will help to develop targeted metabolic footprinting methods for the discrimination of industrial yeasts.

Introduction

The role of yeasts in the fermentation of sugars into alcohol and carbon dioxide has been known for more than two centuries (Anderson, 1989). However, well over a half century elapsed before the role of yeast strain in the production of different wines was established by Pasteur in 1866 (Pasteur, 1866). While the principal yeast used in today's food and alcoholic beverage industries for the production of bread, beer, spirits, cider and wine is classified as *Saccharomyces cerevisiae*, it is well recognized that not all *S. cerevisiae* strains are suitable for the fermentation process and the ability to produce quality foods and beverages differs significantly among them. Because classical, physiological and genetic methods are of limited use in the characterization of wine-making yeast strains, researchers in wine biotechnology have been searching for new methods to reveal the genetic adaptations of industrial yeast strains of the same species, i.e. *S. cerevisiae* (Pretorius & Hoj, 2005). On the other hand, it is well established that *S. cerevisiae* produces different concentrations of aroma compounds as a function of fermentation conditions and must treatments, for example, temperature, grape variety, micronutrients, vitamins and nitrogen composition of the must. Moreover, many commercial yeasts produce undesirable off-flavors, such as H_2S, and a high concentration of higher alcohols, acetic acid and ethyl acetate, depending on the concentration of assimilable nitrogen present in the grape must (Bell & Henschke, 2005). Additionally, slow, sluggish and stuck fermentations have often been related to nitrogen deficiency

(Bisson, 1999). In the late 1970s, these fermentation problems were partially solved by the addition of ammonium salts to deficient musts, which not only increased fermentation rate but also the sensory desirability of the wines (Bell & Henschke, 2005). As a consequence, the routine addition of ammoniacal nitrogen to musts to correct nitrogen limitation is widely used by enologists in most wine regions of the world. Although today some useful methods are accessible for measuring the yeast assimilable nitrogen (YAN) content of grape musts in wineries, they are not commonly used, and so the cause and effect relationship that results in wines with an undesirable flavor is not usually correlated.

Initial studies have attempted to relate the yeast nitrogen demand concept with the profile of aroma compounds in wines. Paradoxically, the various studies reported to characterize the yeast aroma compounds of wines made with various wine yeasts have not considered the importance of nitrogen level of the grape must or the fermentation medium utilized (Rankine, 1967; Rankine & Pocock, 1969; Daudt & Ough, 1973; Di Stefano *et al.*, 1981; Cabrera *et al.*, 1988; Cavazza *et al.*, 1989; Giudici *et al.*, 1990; Herraiz *et al.*, 1990; Mateo *et al.*, 1991; Delteil & Jarry, 1992; Longo *et al.*, 1992; Lurton *et al.*, 1995; Lema *et al.*, 1996; Zoecklein *et al.*, 1997; Antonelli *et al.*, 1999; Vila *et al.*, 2000; Romano *et al.*, 2003). Moreover, many studies were conducted under conditions of excess nitrogen, above 300 mg N L^{-1} (Houtman & Du Plessis, 1986; Carrau, 2003). This experimental approach has, however, not proved to be suitable because *S. cerevisiae* yeast strains that show similar profiles of assimilable nitrogen consumption can nevertheless produce very different profiles of fermentation rate and aromatic compounds under industrial conditions of lower initial nitrogen levels (Jiranek *et al.*, 1991; Carrau, 2003; Taillandier *et al.*, 2007). There are few reports of investigations into the sensory characteristics of wines made with different strains under low nitrogen fermentation conditions (Carrau *et al.*, 1993; Medina *et al.*, 1997; Carrau, 2003). Only recently, have the first chemometric studies provided data on the significance of yeast aroma compounds to perceived flavor in wine (Smyth *et al.*, 2005).

From the chemical point of view, only a few studies have investigated the yeast aroma compounds of wines prepared under defined conditions of YAN contents similar to winemaking conditions (Bosso, 1996; Guitart *et al.*, 1999; Nicolini *et al.*, 2000a, b; Carrau, 2003; Bell & Henschke, 2005; Beltran *et al.*, 2005; Carrau *et al.*, 2005; Hernandez-Orte *et al.*, 2006; Vilanova *et al.*, 2007). Nevertheless, it is difficult to draw any firm conclusions concerning the relationship between YAN and the different aroma compounds produced by the yeast strains studied due to the different experimental conditions used.

The aim of this study was to characterize *S. cerevisiae* strains for the production of key yeast aroma compounds (esters, alcohols, acids and lactones) after fermentation of a chemically defined medium, which models the nutrient composition of grape must covering a wide range of initial nitrogen concentrations. The model yeast strains used in this study were selected from a wide group of strains because both showed similar nitrogen consumption when tested under different nitrogen conditions, but from a sensory and chemical point of view they produced different wines at a low nitrogen concentration (75 mg N L^{-1}). The differential behavior of yeast strains at different initial nitrogen concentrations was examined for a better understanding of this phenomenon in our model system. Application of these results for future discrimination of industrial yeast strains in relation to the type of aroma compounds produced will allow the development of data models for metabolic footprinting methods (Kell *et al.*, 2005; Carrau *et al.*, 2008).

Materials and methods

Yeast strains

Saccharomyces cerevisiae strains utilized were: Montrachet UCD 522 (University of California, Davis), referred to as M522 in this work, and KU1 [Uruguayan selected strain, (Carrau *et al.*, 1993; Medina *et al.*, 1997)]. Both strains are used in the commercial production of wine. Inocula were prepared in the same synthetic medium by incubation for 12 h in a rotary shaker at 150 r.p.m. and 25 °C. Inoculum size was 5×10^5 cells mL^{-1} of medium for both strains. The widely studied wine yeast strains, *S. cerevisiae* M522 and KU1, were characterized as strains having similar nitrogen demands (i.e. consuming a similar amount of nitrogen under 50–400 mg N L^{-1} of YAN).

Fermentation conditions

Chemically defined fermentation medium (nutrient components of grape juice) was prepared as described previously (Henschke & Jiranek, 1993), but modified as follows: the total nitrogen content was adjusted to a basic amount of 50 mgN L^{-1} with each amino acid and ammonium component added in the same proportions as indicated previously (Henschke & Jiranek, 1993). Media with YAN concentrations of 75, 125, 180, 250 and 400 mg N L^{-1} were made by increasing the basic concentration by supplementation with diammonium phosphate (DAP). None of these YAN amounts was a limiting concentration for complete fermentation of sugars by the yeast strains used. The final pH of each medium was adjusted to 3.5 with HCl. Equimolar concentrations of glucose and fructose were added to reach 120 g L^{-1} and the mixed vitamins and salts as described previously (Henschke & Jiranek, 1993). Tween 80 was excluded from the medium because it was not found to be necessary for complete fermentation and it had a negative

impact on the sensory characteristics of the resultant wines. Ergosterol was added as the only supplemented lipid at a final concentration of 10 mg L^{-1}. Fermentations were carried out in 125 mL of medium contained in 250-mL Erlenmeyer flasks, closed with Muller valves, filled with pure sulfuric acid. YAN was chosen as the variable for this investigation because it was found previously that this factor significantly affected production of fermentation aroma compounds by these yeasts under similar experimental conditions (Carrau, 2003). Static batch fermentation conditions were conducted at 20 °C in triplicate, simulating winemaking conditions. Fermentation activity was measured as CO_2 weight loss and expressed in grams per 100 mL, and total residual sugars were analyzed using the Fehling method (Zoecklein *et al.*, 1995). Once a day, samples were taken to measure cell growth in an improved Neubauer chamber. Samples for sensory and GC-MS analysis were taken 2 days after the end of fermentation, filtered through 0.45-μm pore membranes and SO_2 was added as 50 mg L^{-1} of sodium metabisulfite.

Sensory analysis

Fermentation products were subjected to sensory analysis through a paired preference test. Fermentations at 75 mg N L^{-1} were presented to a trained panel of 12–15 persons, in order to determine the significance of the sensory differences between the strains. Samples fermented with both strains with or without addition of YAN in the chemically defined medium containing the basic concentration of nitrogen (75 mg N L^{-1}) were presented to a group of five winemakers so as to evaluate aroma defects and positive characteristics. A free description of desirability and aroma characteristics was presented in the tasting sheet.

GC and GC-MS analysis

Higher alcohols

Higher alcohol and ethyl acetate analysis was performed by distillation of 50 mL of sample and direct injection of 0.5 μL of sample of the distillate and analyzed using the GC-FID with a glass column (2 m × 2 mm; Carbopack C, 60–80 mesh, 0.2% CW 1500, Supelco) in a Shimatzu C-17, equipped with EZ-CHROM software. The experimental conditions were as follows: program temperature 65 °C (5 min), 60–150 °C at 4 °C min^{-1}; injector temperature 200 °C; and detection temperature, 250 °C. The carrier gas was nitrogen (20 mL min^{-1}).

Aroma volatile compounds

Extraction of aroma compounds was performed using adsorption and separate elution from an Isolute (IST Ltd, Mid Glamorgan, UK) ENV+ cartridge packed with 1 g of a highly cross-linked styrene-divinyl benzene (SDVB) polymer. Treatment of samples and GC analysis were performed as described previously (Boido *et al.*, 2003).

Identification and quantification

The components of wine aromas were identified by comparison of their linear retention indices, with pure standards or data reported in the literature. Comparison of mass spectral fragmentation patterns with those stored on databases was also performed. GC-FID and GC-MS instrumental procedures using an internal standard (1-heptanol) were applied for quantitative purposes, as described previously (Boido *et al.*, 2003).

Statistical analysis

A stepwise discriminant analysis was carried out with the aroma compounds analyzed of the 12 wines produced using both strains in triplicate in a basic fermentation medium containing a initial YAN of 75 mg N L^{-1}, with and without an extra YAN addition of 63 mg N L^{-1} as DAP.

ANOVA for initial YAN concentration, yeast strain and aroma compounds were determined with STATISTICA 5.1. Differences of free volatile compounds were evaluated; the mean rating and least significant differences for initial YAN concentrations for each strain were calculated from an ANOVA.

Results

A preference sensory test was performed where significant differences were found between fermentations of KU1 and M522 ($P < 0.01$) at a low nitrogen concentration (75 mg N L^{-1}), while no significant differences were found between them at a higher nitrogen concentration (400 mg N L^{-1}). At a low nitrogen concentration, wines were described as fruity and pleasant for KU1 and unpleasant (soapy and sweaty) for M522, confirming our previous work with these two strains (Carrau, 2003). Table 1 shows the odor active values (OAV) of the main aroma compounds analyzed for these two nitrogen concentrations. On the left side, it can be seen that from the 7 compounds that resulted in higher OAVs for KU1 at a low YAN level, four were described as fruity (esters) and the others were medium-chain fatty acids, considered as precursors of fruity esters. Conversely, seven compounds that resulted in lower OAVs for KU1 at this YAN level were the higher alcohols and isoacids, corresponding to unpleasant aroma descriptions. At the higher nitrogen concentration, although wines could not be differentiated by the sensory analysis, M522 produced high concentrations (OAVs) of practically all the compounds, indicating that chemically, strains have a very

Table 1. Average OAV for the studied fermentations with the model strains at two YAN levels (75 and 400 mg N L^{-1})

				OAVs			
				At 75 mg N L^{-1}		At 400 mg N L^{-1}	
	Reference*	Threshold	Descriptor	Mont. 522	KU1	Mont. 522	KU1
Isoamyl acetate	1	30	Banana, pear	37.4	37.0	55.8	11.4
Ethyl acetate	2	7500	Fruity, solvent	1.01	1.5	3.4	2
ethyl hexanoate	1	14	Apple, fruit	23.0	33.3	38.3	20.0
Ethyl octanoate	1	5	Pineapple, pear, soapy	77.7	143.9	164.5	78.5
Ethyl decanoate	1	200	Grape	0.0	0.0	0.2	0.1
2-Methyl-1-propanol	1	40 000	Wine, solvent	0.6	0.3	0.23	0.07
2-Methyl-1-butanol	2	30 000	Whiskey, malt, burned	11.2	7.4	3.7	1.3
3-Methyl-1-butanol	1	30 000	Alcohol, nail polish	3.6	2.5	2.3	1.8
β-Phenylethyl alcohol	1	14 000	Honey, rose, spicy	6.2	4.8	1.09	0.36
1-Propanol	3	83 0000	Alcohol, pungent	0.0	0.0	0.0	0.0
Isobutyric acid	4	50	Rancid, butter, cheese	39.9	30.0	21.7	6.4
Isovaleric acid	1	33	Sweat, acid, rancid	85.8	71.7	14.4	7.9
Butanoic acid	1	173	Rancid,cheese, sweat	7.2	12.5	7.9	3.2
Hexanoic acid	1	420	Sweat, acid, rancid	5.3	9.5	16.7	5.7
Octanoic acid	1	500	Sweat, cheese	7.0	13.4	25.2	7.3
Decanoic acid	1	1000	Rancid, fatty	0.3	1.5	4.9	0.8
γ-Butyrolactone	3	100 000	Caramel, coconut	0.0	0.0	0.0	0.0
Ethyl-4-hydroxybutanoate	5	ND	Caramel	ND	ND	ND	ND
β-Phenylethyl acetate	3	250	Rose, honey, tobacco	0.8	0.9	2.1	0.4
3-Methylthio-1-propanol	1	1000	Sweet, potato	5.0	2.4	1.0	0.1

*For ref.: 1, the threshold determination was performed in a 11% water/ethanol solution containing 7 g L^{-1} glycerol, 5 g L^{-1} tartaric acid and pH adjusted to 3.4 with 1 M NaOH (Ferreira *et al.*, 2000); 2, Etievant (1991); 3, the threshold determination was performed in 14% ethanolic solution (Moreno *et al.*, 2005); 4, the threshold determination was performed in water (van Gemert, 2003); 5, Carrau (2003).
ND, not determined.

different behavior at this nitrogen concentration. On the other hand, addition of YAN to the low nitrogen concentration (75 mg N L^{-1}) of 63 mg N L^{-1} as DAP (resulting in a final YAN of 138 mg N L^{-1}) shows that the M522 strain requires a higher nitrogen concentration to produce a similar aroma profile when compared with KU1, as shown in Fig. 1. A discriminant analysis using 28 compounds determined using GC-MS shows a clear discrimination in three fermentation groups. The discriminant compounds in this analysis were: β-phenylethyl alcohol, β-phenylethyl acetate, the isobutanoic (isoC4) and isovaleric acids (isoC5), ethyl octanoate and 2-ethyl hexanol. The YAN addition to M522 resulted in fermentations with a response similar to that of unsupplemented KU1, as the discriminant analysis could not separate these two cases.

Conditions of growth and fermentation activity could affect the profile of volatile yeast metabolites that contribute to wine aroma. The growth and fermentation kinetics of the two strains were first characterized over the wide range of nitrogen concentrations utilized in this study (50–400 mg N L^{-1}). No significant differences in cell growth were found between strains (Fig. 2a), but a significantly smaller total population was found for both strains in fermentations at an initial YAN of 75 mg N L^{-1}. Fermentation activity (CO_2 evolution rate) of the two strains was found to depend on the initial YAN concentration up to 125 mg N L^{-1}, (Fig. 2b). All fermentations were completed (final residual sugars below 2 g L^{-1}) at low nitrogen concentrations and fermentation curves were not significantly different for strains at higher YAN concentrations (data not shown).

Production of higher alcohols and isoacids in response to initial nitrogen concentration

The sum of the higher alcohols, 2-methyl-1-propanol, 2-methyl-1-butanol and β-phenylethyl alcohol is depicted in Fig. 3a. Both strains produced a similar profile for higher alcohols in relation to initial nitrogen, except that at each nitrogen concentration, strain M522 produced significantly higher concentrations of these compounds. At low initial nitrogen concentrations (50–75 mg L^{-1}), a direct relationship was observed whereas at higher initial nitrogen concentrations an inverse relationship existed. Above *c.* 250 mg N L^{-1}, the production of higher alcohols reached a plateau. 3-Methyl-1-butanol, which was produced at a considerably higher concentration than the remaining alcohols, showed a slightly different production profile (Fig. 3c) in that its concentration slightly changed up to 200 mg N L^{-1} and above this concentration an inverse relationship existed

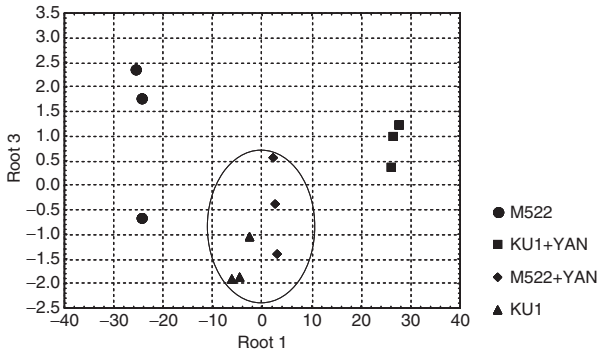

Fig. 1. In the following experiment, the two selected strains were fermented with and without the addition of 63 mg N L^{-1} of YAN. The graph shows the discriminant analysis, where 28 compounds determined using GC-MS were used. The compounds that were discriminant in this analysis were: β-phenylethyl alcohol, β-phenylethyl acetate, the isobutanoic and isovaleric acids, ethyl octanoate and 2-ethyl hexanol.

up to the highest nitrogen concentration tested (400 mg L^{-1}). The production of isoacids by the two yeast strains in response to initial nitrogen was also studied. Figure 3b, which shows the sum of isobutanoic (isoC4) and isovaleric (isoC5) acids, indicates a behavior similar to higher alcohols for both strains. Strain M522 produced significantly higher concentrations of these compounds than KU1. Interestingly, an inverse relationship between growth (Fig. 2a) and higher alcohols and isoacids profiles (Fig. 3a and b) was shown.

Production of 1-propanol in response to different initial YAN concentrations

A clear exception among the higher alcohols is 1-propanol as shown in Fig. 4. In this case, a positive relationship between 1-propanol production and initial YAN was observed across the whole range of nitrogen concentrations studied. In contrast to the trends shown by the other higher alcohols, the behavior of each strain is significantly different at moderate to high YAN concentrations exceeding *c.* 150 mg N L^{-1}. Strain KU1 produced higher concentrations of 1-propanol at moderate to higher concentrations of YAN.

Production of esters and fatty acids in response to different initial YAN concentrations

The production of esters (sum of isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate) and fatty acids (C4, C6, C8 and C10) by the two strains M522 and KU1 is shown in Fig. 5. These results indicate a similar behavior for the two groups of compounds; however, the two strains produced different patterns in relation to the initial YAN concentration. KU1 produced a higher concentration of these compounds at lower YAN concentrations relative to M522 up to 125 mg N L^{-1}, beyond which significantly lower concentrations of volatiles were produced by KU1. Although ethyl acetate showed a profile similar to those observed for the other esters studied, it was produced at a relatively higher concentration and so its response pattern is presented separately in Fig. 5c. Interestingly, as can be seen, the behavior of esters and fatty acids, mainly the ethyl acetate, is the reverse of that observed for 1-propanol for each strain (see Fig. 4). KU1 showed a reverse behavior compared with M522 in these compounds.

Other esters and alcohols

Phenylethyl acetate is presented separately because its formation pattern, in response to the initial nitrogen concentration in the medium, differed from that for the other esters, showing a more similar profile to those of the higher alcohols. While 3-methylthio-1-propanol showed a response profile similar to the other higher alcohols, as shown in Fig. 2a, the behavior of the two strains was significantly different for these compounds as, shown in Fig. 6. KU1 produced lower concentrations of these compounds at all initial YAN concentrations.

Production of γ-butyrolactone and ethyl-4-hydroxybutanoate in response to initial YAN concentrations

Figure 7a shows γ-butyrolactone production, one of the main lactones present in wines, which showed a behavior similar to the higher alcohols. M522 produced higher concentrations of this lactone at all YAN concentrations, with a maximum of 125 mg N L^{-1} compared with 75 mg N L^{-1} for the higher alcohols. Figure 7b shows the behavior of ethyl-4-hydroxybutanoate, a compound that could be metabolically related to γ-butyrolactone as discussed below. The profile of lactone production by M522 was consistent with this similar metabolic relationship between the two compounds. However, this is not the case for KU1, where a reverse behavior is observed at a YAN of 125 mg N L^{-1}.

Discussion

Researches on the chemical identification of aroma compounds in wine derived from the metabolic activity of yeasts have been reported widely in the literature during the last decades (Houtman & Du Plessis, 1986; Rapp & Versini, 1996; Lambrechts & Pretorius, 2000; Swiegers *et al.*, 2005). From these studies, it can be concluded that various fermentation products, including ethyl esters, acetates, higher alcohols, fatty acids and thiols, are especially important to the sensory perception of different wine types (Ferreira *et al.*, 1996; Guth & Sies, 2002; Smyth *et al.*, 2005; Cozzolino *et al.*, 2006). While this research provides important information on the sensory significance of yeast volatile

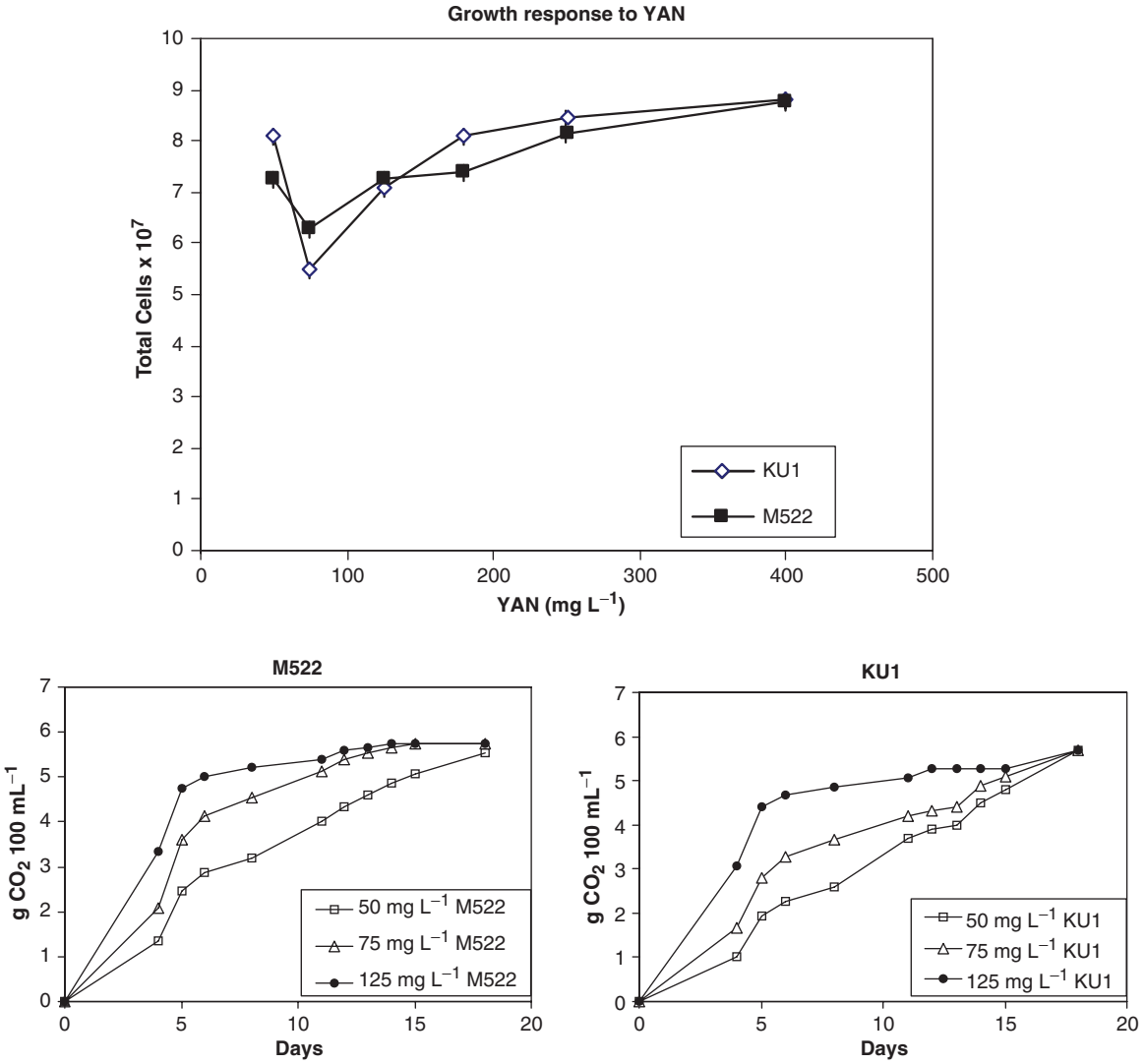

Fig. 2. (a) Growth response to different nitrogen concentrations for M522 and KU1 strains. Results are the average of duplicate fermentations. (b) Fermentation rate curves, determined by culture weight loss due to CO_2 evolution for M522 and KU1 at low YAN concentrations (50–125 mg N L^{-1}). Results are the average of duplicate fermentations and SEs of the mean $< 5\%$.

compounds, more focused research is required on the physiological regulation of these compounds for the benefit of the alcoholic beverage industries, such as wine and beer production.

A lack of recognition of the importance of defining the nitrogen content of media in relation to aroma compounds has produced considerable discrepancies and misunderstandings in the literature. The results presented in this work will facilitate a better understanding of the importance of the appropriate YAN concentration for yeast characterization in relation to aroma compounds.

Table 1 shows the OAV of the compounds that are shown in the different figures. Although most of the compounds studied showed significant chemical and OAV differences between strains, some of them may not contribute to the sensory characteristics of a wine at the studied concentrations. This is the case for ethyl decanoate, 1-propanol and γ-butyrolactone. However, from the chemical point of view some interesting changes in the behavior of both strains for all the compounds studied may contribute to chemical yeast discrimination if an appropriate nitrogen concentration is used in a chemically defined medium. In the case of higher alcohols's production, except for 1-propanol, the response to the initial nitrogen concentration varies between reports, but, in general, is described by an inverse relationship. However, a nitrogen-dependent biphasic response pattern of higher alcohol production involving a direct positive relationship at low initial nitrogen concentrations and an

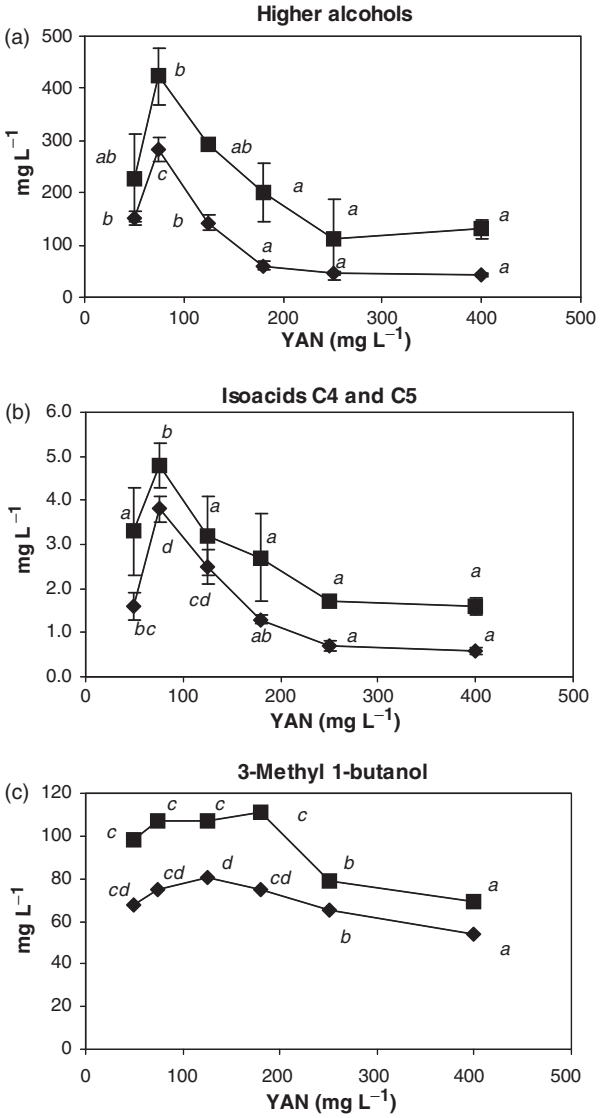

Fig. 3. Relationship between the accumulation of higher alcohols and isoacids in wines made with two *Saccharomyces cerevisiae* yeast strains in response to the initial nitrogen concentration. (a) Sum of 2-methyl-1-propanol, 2-methyl-1-butanol and β-phenylethyl alcohol. (b) Sum of the isobutanoic and isovaleric acids and (c) the major alcohol 3-methyl-1-butanol. These compounds were produced by yeast strains M522 (■) and KU1 (◇) in an artificial grape must medium and measured using GC and GC-MS as described in Material and methods. Letters at each point indicate the significant differences ($P < 0.05$) according to an LSD test of ANOVA calculated for each strain. Error bars indicate SD.

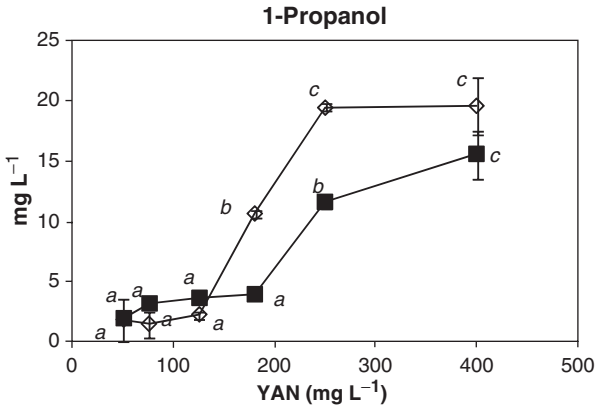

Fig. 4. Relationship between 1-propanol production and initial YAN concentration for M522 (■) and KU1 (◇) yeast strains. Letters at each data point indicate the level of significant difference ($P < 0.05$) according to an LSD test of ANOVA calculated for each strain. Error bars indicate SD of the mean value.

inverse relationship at higher initial concentrations was described for beer yeasts many years ago by Ayrapaa (1967, 1968, 1971). Interestingly, little attention has been directed toward this phenomenon since these studies were published. In fact, most of the recent studies were performed using higher concentrations of nitrogen (exceeding 100 mg N L^{-1}), with which only an inverse response is usually observed. Moreover, we believe that our report is the first to describe comprehensively the behavior of isoacid production in response to different initial nitrogen concentrations. The nitrogen-dependent common trend for production of isoacids and higher alcohols suggests that their metabolism and production may be coordinated as reported previously (Nordstrom, 1964; Reazin *et al.*, 1970). On the other hand, we suggest that the significantly higher production of higher alcohols and isoacids by M522 could reflect a less efficient usage of nitrogen, resulting in an increase of carbon flux related to branched-chain amino acid metabolism by this strain. These model strains would be useful for further studies for a better understanding of the different genetic (*BAT* and *ARO* genes) and/or physiological [NAD(P)H/NAD(P) balances] adaptations that could explain the differences found here. Our results suggest that KU1, which produces less higher alcohols and isoacids at all the nitrogen concentrations tested, when compared with M522, might regulate more effectively the carbon flux at any given nitrogen availability–resulting in the excretion of less quantities of 'carbon metabolic wastes' (Ribereau-Gayon *et al.*, 2000) from the cell at all initial nitrogen concentrations.

In addition, the direct positive relationship of these compounds at a low initial concentration could be explained by the very low yeast fermentation activity in this situation, as shown in Fig. 2, which is induced by the low biosynthetic capacity of the cell. These results are in agreement with the first gene expression analysis conducted in an industrial strain (Backhus *et al.*, 2001), where, at a low nitrogen level (53 mg N L^{-1}) compared with a high nitrogen level (400 mg N L^{-1}), cultures display greater expression of genes involved in translation and in oxidative carbon metabolism,

suggesting that respiration is more nitrogen conserving than fermentation (Backhus *et al.*, 2001).

In contrast to other higher alcohols, 1-propanol is known to be formed by the condensation of pyruvic acid and acetyl CoA (Nykanen, 1986). Our results are in agreement with previous studies (Ayrapaa, 1968; Margheri *et al.*, 1984); however, the formation of 1-propanol varied in relation to nitrogen level for the two strains (Fig. 4). Strain M522 produced relatively higher concentrations of 1-propanol at lower nitrogen concentrations but relatively less at higher nitrogen levels. This reversal in production in relation to nitrogen availability does not appear to have been reported before. Moreover, the fact that the relative concentration of 1-propanol produced in response to nitrogen is generally reversed for ethyl acetate with respect to each of the strains would be an interesting topic for further research.

In addition, our results indicated an indirect relationship between higher alcohol and isoacid production and growth (see Figs 2a and 3a). The effect of some aromatic alcohols, such as tryptophol, isoamyl alcohol and β-phenylethyl alcohol, acting as auto-signaling molecules capable of stimulating morphogenesis in *S. cerevisiae* (Hazelwood *et al.*, 2008), may explain this behavior. Furthermore, a quorum-signaling pathway linking environmental sensing and entry into the stationary phase in *S. cerevisiae* has been described recently (Chen & Fink, 2006). As can be seen in our data, these results might help to explore the occurrence of other putative quorum-sensing molecules in *S. cerevisiae*.

Production of esters and fatty acids

Yeasts synthesize fatty acids by the hydrolysis of the acyl-CoA derivatives and esters by esterification of activated fatty acids and alcohols. The results obtained here showed a clear metabolic correlation between these compounds (Fig. 5). Acetate esters are described as having fruity aromas and are considered as pleasant flavors from a sensory point of view (Smyth *et al.*, 2005). The results obtained in the present work may provide an explanation for the more pleasant sensory character of the wines obtained with KU1 at a low nitrogen concentration compared with M522, which are also in agreement with the OAVs shown in Table 1. The behavior of strain KU1, in which higher concentrations of these compounds are produced when nitrogen availability is low, contradicts the concept raised in many reports stating that the increase in ester production is directly related to the increase of nitrogen in the must. On the other hand, this contradictory behavior of KU1 could also explain why several studies did not observe a consistent correlation between YAN grape musts and esters and fatty acid concentration (Rapp & Versini, 1996; Lambrechts & Pretorius, 2000). Esters and fatty acids have also been considered 'metabolic wastes' with a potentially toxic effect on the cell

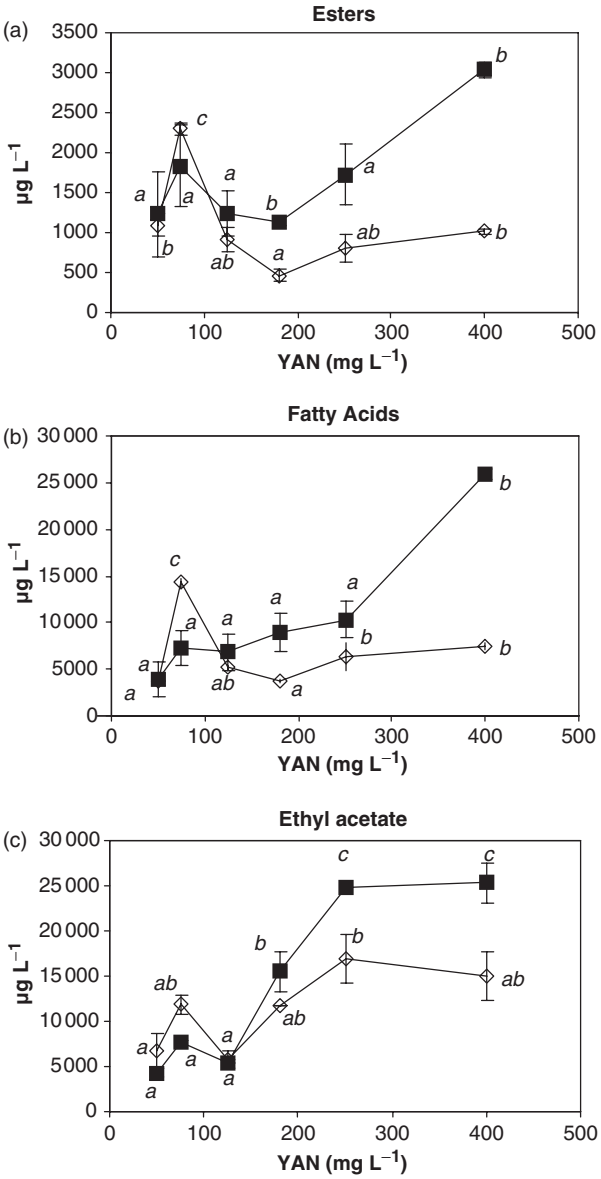

Fig. 5. Relationship between the production of esters and fatty acids and initial YAN concentration for M522 (■) and KU1 (◇) yeast strains. (a) Sum of esters (isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate); (b) sum of fatty acids (C4, C6, C8 and C10); and (c) ethyl acetate. Letters at each data point indicate the level of significant difference ($P < 0.05$) according to an LSD test of ANOVA calculated for each strain. Error bars indicate SD of the mean value.

(Peddie, 1990). A limited production of these compounds by KU1 at a high nitrogen concentration compared with M522 can be understood as an efficient behavior of this strain in relation to nitrogen usage. More interestingly, the profiles of fatty acids and esters obtained with KU1 are quite similar to the higher alcohol and isoacid profiles shown in Fig. 3a, b.

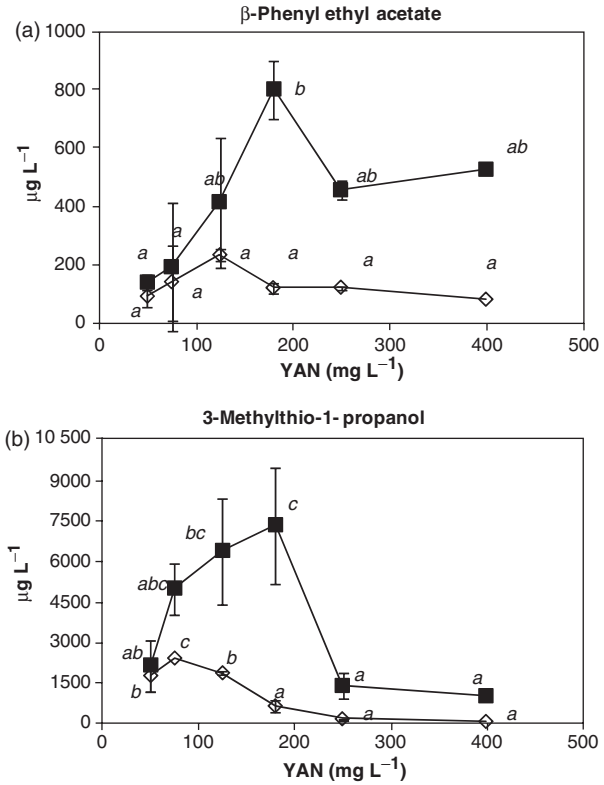

Fig. 6. Production of other volatile compounds that show variability in response to the initial YAN concentration by M522 (■) and KU1 (◇) yeast strains. (a) β-phenylethyl ester and (b) the 3-methylthio-1-propanol. Letters at each data point indicate the level of significant difference ($P < 0.05$) according to an LSD test of ANOVA calculated for each strain. Error bars indicate SD of the mean value.

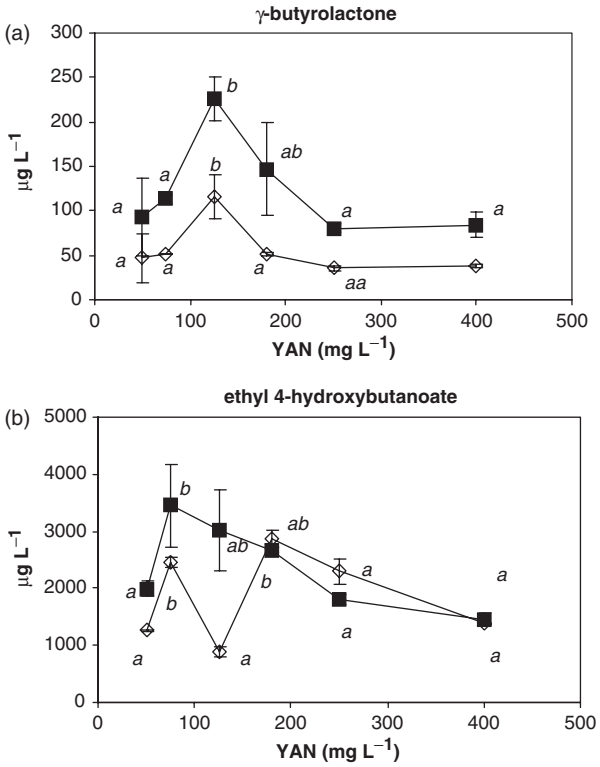

Fig. 7. Production of γ-butyrolactone (a) and ethyl 4-hydroxybutanoate (b) in response to the initial YAN concentration by M522 (■) and KU1 (◇) yeast strains. Letters at each data point indicate the level of significant difference ($P < 0.05$) according to an LSD test of ANOVA calculated for each strain. Error bars indicate SD of the mean value.

Production of γ-butyrolactone, 3-methylthio-1-propanol and ethyl 4-hydroxybutanoate

Limited information about the production of these compounds by yeast can be found in the literature. Although γ-butyrolactone was characterized as being negatively related to YAN level in grape must (Bosso, 1996), our results, which were obtained over a very wide range of nitrogen concentrations, showed a direct relationship with low nitrogen levels. Such low nitrogen levels have not been considered in other reports. The profile of γ-butyrolactone production also resembles that of higher alcohols and isoacids. The relation of γ-butyrolactone with ethyl-4-hydroxybutanoate production was proposed previously (Muller *et al.*, 1973); however, an important strain difference was observed for KU1 in ethyl-4-hydroxybutanoate production. Recently, 3-methylthio-1-propanol production was described to be negatively related to the nitrogen concentration of the medium (Moreira *et al.*, 2002). However, our results showed a different behavior for M522 at lower nitrogen levels, indicating a positive relation with this thioalcohol (Fig. 6b), which is more consistent with the general behavior of higher alcohols shown in Fig. 3.

In summary, depending on the yeast strain utilized, the formation of aroma compounds presented different responses when nitrogen was added to the medium. Nitrogen addition could also decrease ester and fatty acid concentration during fermentation of a strain, such as KU1, a phenomenon not described previously to the best of our knowledge.

From a chemical point of view, in our artificial medium, it was possible to obtain a clear discrimination between both model strains evaluated through the analysis of key compounds produced after fermentation like higher alcohols, fatty acids, ethyl esters and lactones. Major differences between strains in relation to aroma compound concentrations were obtained at a higher nitrogen level ($400\ mg\ N\ L^{-1}$). Based on these two strains and the appropriate conditions determined in this work, our results will allow the development of data models for metabolic footprinting methods for industrial yeast strain discrimination.

From a biotechnological point of view, the results obtained had shown a better adaptation of strains such as KU1

for the fermentation of grape varieties with considerably variable character such as most of the red *Vitis vinifera* and some white grapes such as Sauvignon Blanc, Chardonnay, Riesling and Muscats. On the contrary, strains like M522 would be more suitable for fermentation of neutral varieties, in which fermentation aromas such as esters and fatty acids would contribute toward improving the fruity intensity of the wine when the nitrogen level of these grape musts is increased.

Acknowledgements

We would like to thank Prof. Patrick Moyna and Dr Carina Gaggero. for the critical comments made for this manuscript We would also thank PDT, INIA and CSIC of UdelaR for financially supporting this project.

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