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Production of volatile compounds in the fermentation of chardonnay musts inoculated with two strains of *Saccharomyces cerevisiae* with different nitrogen demands

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Abstract

This paper studies the behaviour of two strains of *Saccharomyces cerevisiae* with different nitrogen requirements in the production of esters and higher alcohols during alcoholic fermentation. To carry out the study a chardonnay must with a high content of nitrogen compounds was used. The results showed that the strain with the highest nitrogen demand produced a higher concentration of esters during fermentation and gave rise to a wine with a somewhat lesser content of higher alcohols. The formation of volatile compounds was probably related to the consumption of nitrogen by the strains as the nitrogen nutrients act as precursors in the synthesis of esters and alcohols and regulate their production.

Keywords: Assimilable nitrogen; Volatile compounds; Yeast strain

1. Introduction

Wine aroma is the result of the interaction of hundreds of compounds, whose individual concentrations move between 10^{-1} and 10^{-10} g/l (Rapp & Mandery, 1986). Among these, esters have a crucial importance as they provide pleasant aroma sensations. However, it is difficult to associate a particular property of aroma to any concrete ester. The main esters come from acetic acid and high alcohols and from saturated fatty acids and ethanol. Acetate esters have an aroma similar to that of fruit. Regarding to ethanol esters, small acyl chain esters are typically fruity or floral, while longer acyl chain esters are more soap-like. Higher alcohols are, in amount, the most extensive group of volatile compounds. They appear in variable concentrations in wine, and can be recognized by their strong and pungent smell. These compounds add a desirable complexity to the aroma in moderate concentrations (<0.3 g/l). In higher concentrations, they deteriorate the aroma in its global sensation (Rapp & Mandery, 1986; Wagener & Wagener, 1968).

The nitrogen compounds of must have an influence on the production of esters as amino acids and ammonium determine the pool of intracellular nitrogen which regulates the metabolic pathways of formation of esters (Henschke & Jiranek, 1993). Moreover, some amino acids are precursors in the formation of these volatile compounds (Boulton, Singleton, Bisson, & Kunkee, 1996). Acetate esters come from the reaction of acetyl-CoA with higher alcohols, which arise directly from neutral amino acids via Ehrlich reaction.Ethanol esters come from the reaction of acyl-CoA compounds with ethanol. These acyl-CoA compounds are normally generated through the metabolism of fatty acids, although they can also be synthesized through the carbon skeletons of certain amino acids (Boulton et al., 1996). Higher alcohols are also directly related to the nitrogen metabolism. After the deamination of amino acids in the cell, the resulting α-keto acids can be decarboxylated and reduced to form the corresponding alcohols through the Ehrlich pathway (Nykänen, 1986). Just as occurs with esters, the pool of intracellular nitrogen regulates the formation of higher alcohols (Large, 1986). On the other hand, the nitrogen stimulates the cellular multiplication

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since it increases the synthesis of cellular proteins. As well, it maintains the activity of the carriers of sugars in the yeast until the end of fermentation (Bisson, 1991). For that reason, in mediums with a deficiency of nitrogen, the addition of nitrogen nutrients usually turns into a higher production of esters, higher alcohols, and other volatile compounds (Henschke & Jiranek, 1993).

In the bibliography consulted, most of the authors study the relationship between the nitrogen compounds of must and the formation of volatile compounds during alcoholic fermentation (Ancín, Ayestarán, Corroza, Garrido, & González, 1996; Guitart, Hernández, Ferreira, Peña, & Cacho, 1999; Herráiz, Martín-Alvarez, Reglero, Herráiz, & Cabezudo, 1989; Nykänen, 1986; Ough & Bell, 1980; Rapp & Versini, 1991). However, we have not found any studies on the influence of the nitrogen demand of the inoculated yeast in the production of volatile compounds, in spite of the fact that the use of this enological technique is becoming ever more common. For that reason, in this present work it seemed an interesting question to study the production of esters and higher alcohols during the fermentation of a chardonnay must inoculated with two strains of *Saccharomyces cerevisiae* with different nitrogen demands. A must rich in nitrogen compounds was chosen in order to ensure that both yeasts would have sufficient nitrogen for their development. However, it is important to stress that this is a preliminary study on this subject and as such it will need further development where a wider number of yeasts and musts would be studied.

2. Materials and methods

2.1. Samples and vinification

The must used was *Vitis vinifera* var. *chardonnay* unsterilized. This must had 190 Nephelometric Units of Turbidity. The general parameters of the must are shown on Table 1. After adding SO_2 (80 mg/l), the must was divided into four aliquots of 5.0 l. The aliquots were inoculated with active dry yeasts *S. cerevisiae var. cerevisiae* (two with Na33 strain and two with D47 strain).

Table 1
Enological parameters of the chardonnay must (all parameters listed with standard deviation; $n = 6$)

pH	3.62 ± 0.01
Total acidity	4.5 ± 0.1 g/l[a]
Reducing sugars	231 ± 11 g/l
SO_2 free	22 ± 3 mg/l
SO_2 total	79 ± 5 mg/l
Ash	3.2 ± 0.7 g/l
Ash alkalinity	29 ± 2 mequiv/l

[a] Expressed as tartaric acid.

Na33 strain showed higher nitrogen demand than D47 strain in a synthetic medium (Manginot, Roustan, & Sablayrolles, 1998). Na33 strain was selected by the Estación de Viticultura y Enología de Navarra (a Government research center) from must originating from the Navarra region (North of Spain). D47 strain was selected by the Department of Microbiology from Montpellier Institut Coopératif du Vin (ICV), and comes from the French region of Côtes du Rhône. Both strains were commercialized by Lallemand (Madrid, Spain). The strains were inoculated in the must in a proportion of 0.2 g/l. To do this, 1.25 g of dry yeast was rehydrated in a sterile flask in 12.5 ml of distilled water with 0.125 g of sucrose (number of viable cells per gram $\geqslant 2 \times 10^9$); it was kept in this medium for 30 min at 35 °C. The must was inoculated with mixing, in order to get a homogeneous distribution. The fermentations were carried out in modular bioreactors of 5.0 l (Gallenkamp, Leicestershire, UK), at a controlled temperature (20 ± 2 °C). Samples were taken in all cases from the same point of fermentation so that they could be compared: initial must, must at 25% fermented sugars, must at 50% fermented sugars, must at 75% fermented sugars, final wine.

2.2. Polymerase chain reaction

This technique was used to identify Na33 and D47 strains and check their predominance in the fermentation of the musts. To do this, 5 ml samples of must were taken in the last phases of the fermentation (density 1.02 g/ml) and in the wine obtained. These samples were centrifuged at 5000 rpm for 3 min, the supernatant was eliminated, and the sediment was resuspended in 5 ml of sterilized water. It was centrifuged again, and the sediment was mixed with 1 ml of glycerol at 30% v/v for keeping at −40 °C. The polymerase chain reaction (PCR) analyses were done at the Sigmo laboratory of Nantes (France). The method used was that of Lavallée et al. (1994). From the results obtained in these analysis of PCR, Na33 and D47 strains predominated over the indigenous yeasts in all the samples.

2.3. Determination of assimilable nitrogen

This nitrogen was calculated by adding the nitrogen coming from ammonium and the amino acids and subtracting the nitrogen coming from proline, an amino acid which, although it is one of the most abundant, is hardly consumed under normal vinification conditions. Ammonium nitrogen was quantified using enzymatic test Kits (Boehringer Mannheim) according to the manufacturer's instructions. Amino nitrogen was calculated by taking into account the number of nitrogens of free amino acids cuantified by HPLC, following the

method described in Ancín, Ayestarán, and Garrido (1996).

2.4. Analysis of esters and higher alcohols

The volatile compounds of high volatility and high concentration (ethyl acetate, *n*-propanol, isobutanol, and isoamyl alcohols) were analyzed by direct inyection of the sample in a Gas Chromatograph with a Flame Ionization Detector. The compounds of the middle-range volatility and, in general, present in lesser concentrations than the former ones (2-phenylethyl acetate, ethyl lactate, ethyl octanoate, ethyl decanoate, diethyl matale, 2-phenylethanol, tyrosol, and triptofol), were previously extracted, concentrated, and then analyzed by GC–MS. The methods are described in Fraile, Garrido, and Ancín (2000).

2.5. General enological parameters

Analysis methods of general parameters are described by the Office International de la Vigne et du Vin (1990).

The results, outlined in Figures, are shown with their standard deviations and they are the arithmetic mean of six replicates, as the experiments were carried out in duplicate and three analysis were made for each sample.

3. Results and discussion

3.1. Evolution of assimilable nitrogen during the fermentation of chardonnay must

In this must the value of assimilable nitrogen was 350 mg/l. Several authors (Agenbach, 1977; Bely, Sablayrolles, & Barre, 1990) have established 140 mg/l as the minimum quantity of necessary assimilable nitrogen in order to complete the fermentation of musts with normal concentrations of sugar. As such, the chardonnay must used in this study provided sufficient nitrogen to the yeasts for their multiplication and metabolism.

In the course of fermentation, in the sample inoculated with Na33 strain the nitrogen was consumed in a greater quantity (309 mg/l) and faster than in the sample inoculated with D47 strain (277 mg/l) (Fig. 1). The consumption in both samples took place mainly during the fermentation of the first 25% of sugars, given that this stage coincides with the growth phase of yeast so that the microorganisms need nitrogen for their multiplication (O'Connor-Cox & Ingledew, 1989). During this stage, 278 mg/l of assimilable nitrogen was used in the Na33 sample and 159 mg/l in the D47 sample. During the fermentation from 25% to 50% of sugars, in the D47 sample 87 mg/l was consumed as against 15 mg/l in the Na33 sample. Consequently, during the first half of fermentation, in the sample inoculated with D47

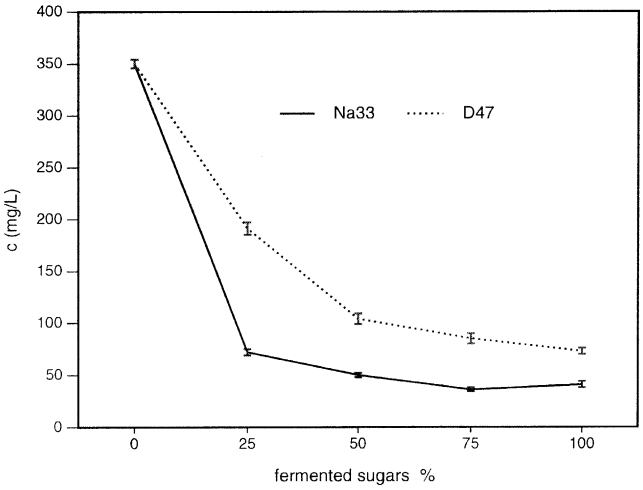

Fig. 1. Evolution of assimilable nitrogen during fermentation of chardonnay must inoculated with Na33 and D47 strains.

strain, less nitrogen was consumed (246 mg/l) and it was consumed more slowly than in the sample inoculated with Na33 strain (293 mg/l). During the second half of fermentation the use of nitrogen was scant in both samples, probably due to the presence of ethanol in the medium. Ethanol inhibits the transport systems of amino acids (D'Amore & Stewart, 1987; Ferreras, Iglesias, & Girbés, 1989), reduces the number of GAPs present in the plasma membranes of yeast (Ferreras et al., 1989), and it provokes the exit of the amino acids to the exterior cellular through a passive process of excretion (Bidan, Feuillat, & Moulin, 1986). The quantity of residual nitrogen in the wines was, therefore, determined by the utilization of nitrogen compounds during the first half of fermentation.

3.2. Formation of volatile compounds and their relationship with the assimilation of nitrogen

Esters. 2-Phenylethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl lactate, diethyl malate, and ethyl acetate were all quantified and among these, ethyl hexanoate was found below the limit of detection of the method in all the samples analyzed. The evolution of the total esters during fermentation is shown in Fig. 2a. Ethyl acetate has been excluded from the sum total of esters due to its distinct contribution to the aroma of wine (Cabrera, Moreno, Ortega, & Medina, 1988; Lema, García-Jares, Orriols, & Angulo, 1996). The total concentration of esters in the wines was 4.5 mg/l for the wine fermented with Na33 strain, and 3.9 mg/l for the wine fermented with D47 strain. Esters were mainly synthesized in both samples after the consumption of the first 25% of sugars (Fig. 2a) due to the fact that their formation is inhibited by the presence of oxygen (Jackson, 1994). The greatest synthesis took place during the

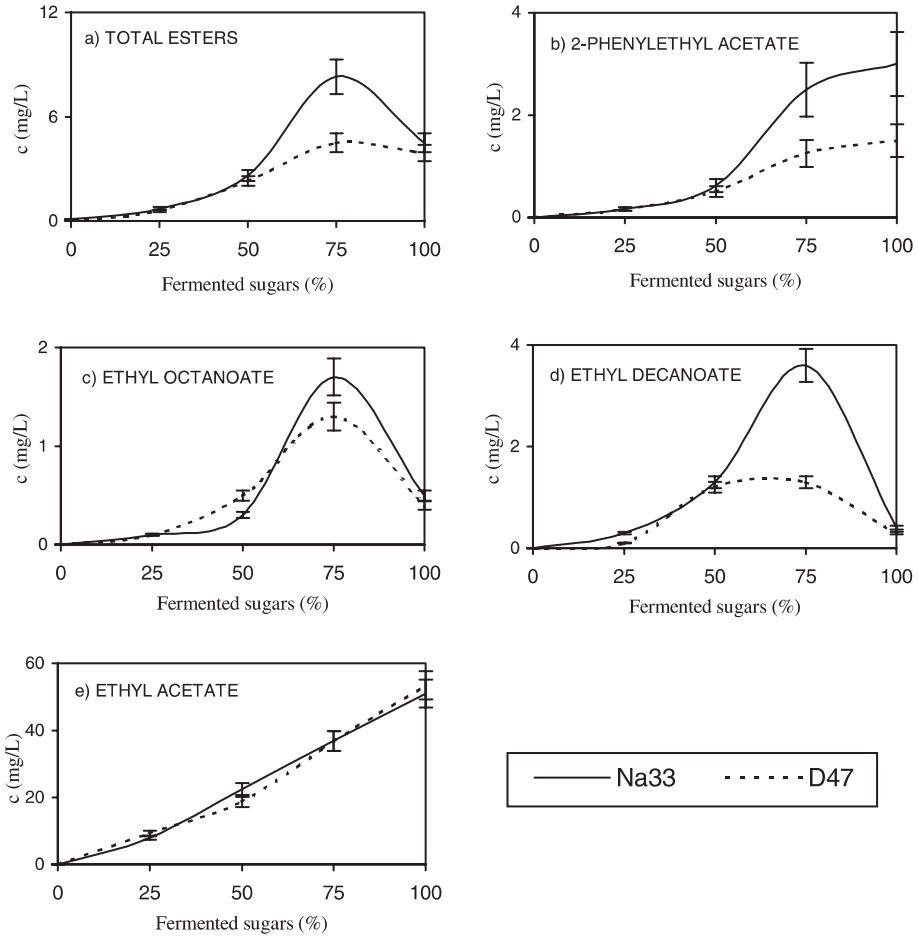

Fig. 2. Evolution of esters during fermentation of chardonnay must inoculated with Na33 and D47 strains.

fermentation from 50% to 75% of sugars, above all in the sample inoculated with Na33 yeast, which showed a greater capacity for generating these volatile compounds than D47 strain. However, at the end of fermentation, the concentration of esters descended sharply in the sample inoculated with Na33 strain, while they remained constant in the sample inoculated with D47 strain. Mauricio, Moreno, Zea, Ortega, and Medina (1997) observed that the hydrolytic action of the cellular esterases varied depending on the strain of *S. cerevisiae* used. In all probability, the activity of the esterases was greater in the Na33 strain than in the D47 strain.

2-Phenylethyl acetate was synthesized to a higher quantity in the sample inoculated with Na33 strain than in the sample inoculated with D47 strain; this difference could be specially appreciated during the fermentation from 50% to 75% of sugars (Fig. 2b). The concentration of 2-phenylethyl acetate in the wine fermented with Na33 strain was higher than the maximum value (1.1 mg/l) found by Snyman (1977) in wines. The production of ethyl octanoate and ethyl decanoate was also greater in the must inoculated with Na33 strain than in the one inoculated with D47 strain. Nevertheless, due to the hydrolysis observed at the end of fermentation, the concentrations in both wines were similar (Fig. 2c and 2d). Ethyl lactate and diethyl malate were synthesized at the end of fermentation and appeared in the wine in a small quantity. Their concentrations were 0.77 mg/l of ethyl lactate and 0.11 mg/l of diethyl malate for the wine fermented with Na33 strain, and 1.1 mg/l of ethyl lactate and 0.19 mg/l of diethyl malate for the wine fermented with D47 strain. Ethyl acetate showed a similar concentration in the two wines, independently of the strain used (Fig. 2e). In neither case was 150 mg/l reached, which is cited by Amerine and Roessler (1983) as a threshold for a vinegar smell in the wine.

In the bibliography consulted a positive correlation can be seen between the content of nitrogen in must and the concentration of esters in wine (Bell, Ough, & Kliewer, 1979; Guitart et al., 1999; Ough & Lee, 1981). In this present study, using a chardonnay must with a high content of nitrogen compounds, the strain with the higher nitrogen demand, Na33, produced a greater quantity of esters than D47 strain, likely due to the fact that it showed a more active general metabolism. The non-Saccharomyces indigenous yeast, which also grows

at the beginning of the inoculated fermentation, would also have contributed to the concentration of esters (Fleet & Heard, 1993).

Higher alcohols. n-Propanol, isobutanol, isoamyl alcohols, 2-phenylethanol, tyrosol, and tryptophol were analyzed. The evolution of the total higher alcohols during fermentation is shown on Fig. 3a, both for the musts fermented with Na33 strain and with D47 strain. In the wines, the concentration was slightly higher in the one fermented with D47 (300 mg/l) than in the one fermented with Na33 (269 mg/l). In neither case did they surpass the limit (300 mg/l) considered by some authors (Rapp & Mandery, 1986; Wagener & Wagener, 1968) as the point where the alcohols deteriorate the aroma of wine.

The production of *n*-propanol was considerably higher in the sample inoculated with D47 strain than with Na33 strain (Fig. 3b). The content of this alcohol in the wine is especially influenced by the yeast strain responsible for the fermentation (Giudici, Zambonelli, & Kunkee, 1993). Isobutanol was also found in higher concentration during the whole fermenting process in the sample inoculated with D47 strain (Fig. 3c), although the differences were less than in the case of *n*-propanol. The formation of isoamyl alcohols, majority components of the group, was somewhat greater in the

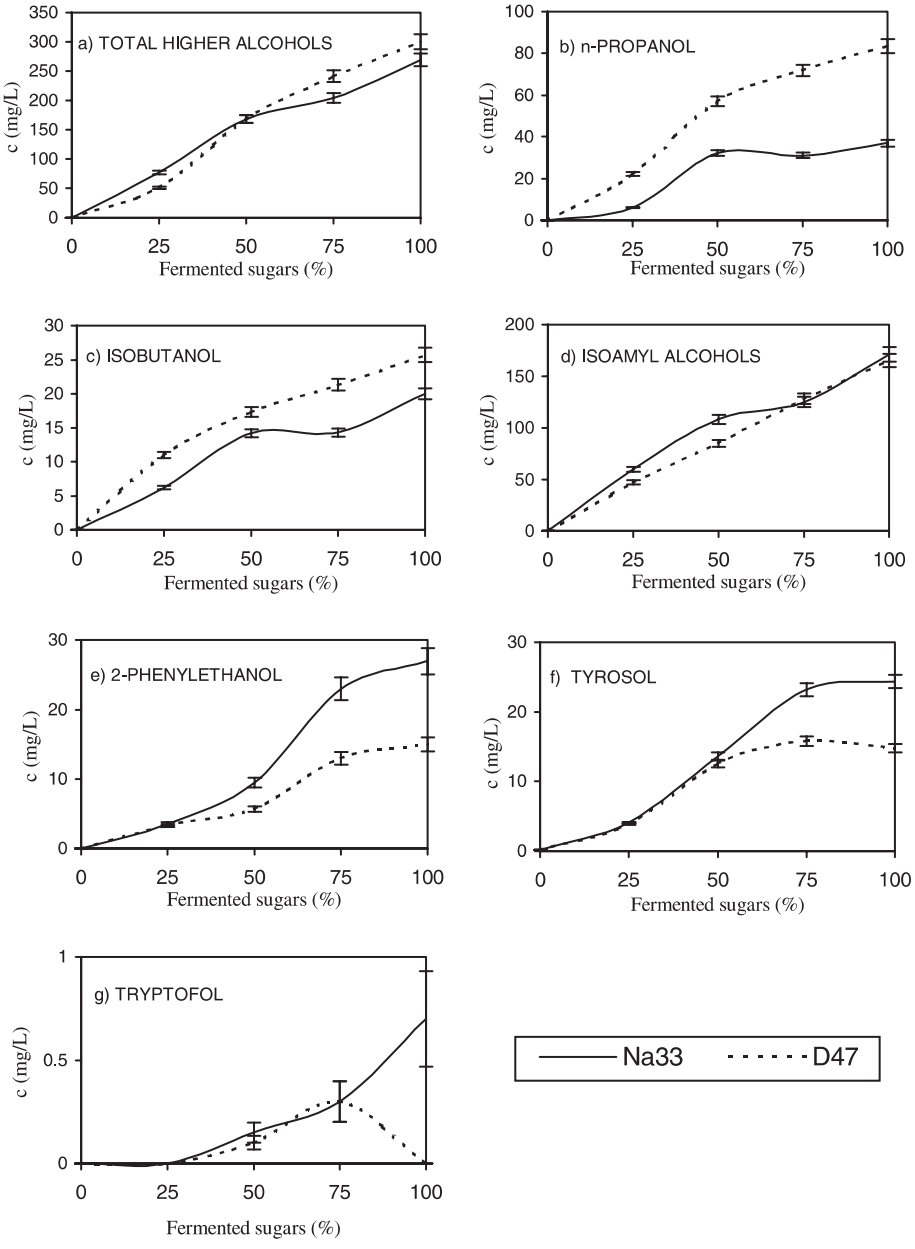

Fig. 3. Evolution of higher alcohols during fermentation of chardonnay must inoculated with Na33 and D47 strains.

must inoculated with Na33 strain during the first half of fermentation (Fig. 3d). However, during the fermentation from 50% to 75% of sugars, the concentration of isoamyl alcohols remained constant in this sample, while in the one inoculated with D47 strain the synthesis continued up to the end, so that in the wines the concentrations were similar.

2-Phenylethanol followed a similar evolution in the two samples during the fermentation of the first 25% of sugars, but from then on, its formation was always higher in the sample inoculated with Na33 strain (Fig. 3e). Houtman and du Plessis (1985) also found significant differences in the concentration of 2-phenylethanol using two yeast strains (We14 and We452) in five different musts. Tyrosol was synthesized to a greater extent in the sample inoculated with Na33 strain than with D47 strain during the fermentation from 25% to 75% of sugars (Fig. 3f). However, in the former case, the concentration of tyrosol remained constant in the final phase of the fermenting process, while in the sample inoculated with D47 strain it continued to form up to the end; the concentration of this alcohol in the final wine was similar. Tryptophol was synthesized in very low quantities during fermentation (inferior to 1 mg/l) and it was only found in the wine fermented with Na33 strain (Fig. 3g). The concentrations of the aromatic higher alcohols in the wines were within the intervals described by Nykänen, Puputti, and Suomalainen (1966), Ribéreau-Gayon and Sapis (1965) and Sapis and Ribéreau-Gayon (1969), which are 11–28 mg/l for tyrosol, 10–75 mg/l for 2-phenylethanol, and 0–0.8 mg/l for tryptophol, respectively.

Na33 strain consumed more nitrogen than D47 strain and synthesized a quantity slightly inferior of higher alcohols. Ough and Bell (1980) found a negative correlation between the content of nitrogen in must and the concentration of higher alcohols in wine. More recently, Guitart et al. (1999) observed that the levels of higher alcohols in wines made from the chardonnay variety were independent or slightly anti-correlated to the content of amino acids in the must. In our samples, the inverse relation between the nitrogen assimilated by the yeasts and the total concentration of higher alcohols in the wines may result from nitrogen repression of transamination, and hence, the Ehrlich pathway would have shown a reduced activity (Large, 1986). Here too, as occurs with the esters, the indigenous non-Saccharomyces yeast would have contributed to the synthesis of the higher alcohols, especially at the beginning of fermentation.

4. Conclusions

In this study we found that the yeast which consumed the assimilable nitrogen of must more quickly and to a greater degree (Na33) produced a greater concentration of esters during fermentation, being noteworthy the high formation of 2-phenylethyl acetate. The yeast with a lesser nitrogen demand (D47) produced concentrations of total higher alcohols somewhat higher than Na33 strain, although in neither of the two cases were the concentrations sufficiently high to have any negative effect on the aroma of wine. These differences could be due to the fact that the nitrogen nutrients act as precursors in the synthesis of esters and alcohols and regulate their formation. The results obtained reinforce the importance of taking into account the nitrogen demand of yeast in the selection criteria of commercial strains. The strains with high nitrogen requirements would be recommendable for the fermentation of musts rich in assimilable nitrogen, since, besides reducing the residual nitrogen—a source of microbiological instability—they could also strengthen the fruity aroma of wine through a greater formation of esters.

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