Use of Nitrogen Compounds in Spontaneous and Inoculated Wine Fermentations

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In this paper the use of nitrogen compounds in garnacha must inoculated with active dry wine yeast *Saccharomyces cerevisiae* subsp. *cerevisiae* strain Na33 has been studied. The results are compared to garnacha must fermented with indigenous yeasts (control must). In the samples where the inoculated yeast predominated, no qualitative differences were appreciated in the use of amino acids with respect to the control samples, although there were quantitative differences. In the musts where the Na33 strain dominated, a lesser quantity of amino acids were consumed at the beginning of fermentation than in the control samples. For that reason, probably, this yeast showed problems in competing for the nitrogen nutrients of the must; this could have made its implantation in one of the inoculated samples more difficult. At the end of fermentation the Na33 strain continued to consume amino acids at high concentrations of ethanol. Its high tolerance to this toxic could be favored by the production and rehydration of dry wine yeast in the presence of air.

Keywords: *Free amino acids; dry wine yeast; Saccharomyces cerevisiae strain Na33; garnacha must*

INTRODUCTION

Nitrogen compounds are essential to the growth and metabolism of yeast. The nitrogen fraction of must is complex and variable and is involved in the kinetics of fermentation and aroma production (Dukes et al., 1991; Rapp and Versini, 1991). The principal nitrogen sources in must are ammonium ions and free amino acids; the proteins themselves do not serve as sources of nitrogen for yeast growth. Many factors affect the assimilation of nitrogen compounds from a mixture, including must composition and yeast strain (Jiranek et al., 1990; Ough et al., 1991). Must clarification clearly affects composition since it reduces nutrients, eliminating fatty acids and especially many unsaturated fats (Ancín et al., 1998; Ayestarán et al., 1998). As a result, the yeast transport system is affected. For this reason, in excessively clarified musts, problems have been observed in the use of amino acids by the yeast, although this treatment does not eliminate these compounds from the medium (Ayestarán et al., 1995). Significant differences were found between strains of *Saccharomyces cerevisiae* in the pattern of amino acids utilized and total nitrogen demand in a synthetic must with limiting nitrogen concentrations (Jiranek et al., 1991). Likewise, for a few amino acids, differences among strains of *S. cerevisiae* in the kinetics of individual amino acid accumulation were also evident (Jiranek et al., 1995).

It is important to know the nitrogen consumption by the yeast strains, since it allows us to pair strains with musts with regard to nitrogen requirements and the nitrogen content. If these requirements are not known, we run the risk of adding compounds in an empirical way, as occurs in the majority of cellars. If large amounts of ammonium salts are added to the must, the wine will have high amounts of residual nitrogen, which is responsible for the microbiological instability of the product and the elevated production of ethyl carbamate (Ough et al., 1988a,b; Monteiro et al., 1989). On the other hand, since the nitrogen compounds are not usually in high quantities in the must, they can be limiting factors for the growth of the yeasts. Consequently, there could be a relationship between the implantation of an inoculated yeast strain and its capacity to compete with the indigenous ones in the uptake of nitrogen compounds, particularly at the beginning of fermentation.

Years of experience have shown that wine quality is significantly affected by the particular strain of *S. cerevisiae* conducting the fermentation (Fleet, 1994). The inoculated fermentations produce reproducible quality wines, and greater flexibility exists in control over wine sensory quality through selection of the yeast strain (Kunkee and Goswell, 1977). It is generally recognized that the use of dry wine yeast has taken on increasing importance in the wine industry over the last few years. It is particularly interesting to study selected strains native to different regions and their behavior in the fermentation of musts from grape varietals from these same regions. Several groups have studied the nitrogen needs of different yeasts in different synthetic media (Ingledew, 1975; Pekur et al., 1981; Ingledew and Kunkee, 1985), but it is also important to know these needs in musts used for vinification. For these reasons, in this paper the use of nitrogen compounds in garnacha must inoculated with active dry wine yeast *S. cerevisiae* subsp. *cerevisiae* strain Na33 has been studied. This strain is native to Navarra, and was selected among 1440 yeasts isolated between the years 1989 and 1992. Likewise, the must used is also from this same geographical zone in the North of Spain. The results are

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Table 1. Characteristics of Musts and Wines

sample	reducing sugar (g/L ± SE)	pH ± SE	volatile acidity (g/L[a] ± SE)	free SO_2 (mg/L ± SE)	alcohol (v/v % ± SE)
control 1	234 ± 11	3.14 ± 0.01		14 ± 2	0.50 ± 0.01
NaA1	222 ± 11	3.11 ± 0.01		12 ± 2	0.30 ± 0.01
NaB1	240 ± 11	3.14 ± 0.01		13 ± 2	0.30 ± 0.01
control 2	158 ± 7	2.99 ± 0.01		9 ± 1	4.5 ± 0.1
NaA2	168 ± 8	3.11 ± 0.01		9 ± 1	3.1 ± 0.1
NaB2	158 ± 7	2.99 ± 0.01		11 ± 2	5.6 ± 0.2
control 3	122 ± 6	3.10 ± 0.01		11 ± 2	8.9 ± 0.2
NaA3	111 ± 5	3.18 ± 0.01		14 ± 2	9.7 ± 0.3
NaB3	128 ± 6	3.02 ± 0.01		12 ± 2	8.9 ± 0.2
control 4	59 ± 3	3.03 ± 0.01	0.30 ± 0.01	11 ± 2	12.2 ± 0.3
NaA4	56 ± 3	3.23 ± 0.01	0.30 ± 0.01	9 ± 1	11.7 ± 0.3
NaB4	66 ± 3	3.01 ± 0.01	0.40 ± 0.01	19 ± 3	12.2 ± 0.3
control 5	1.4 ± 0.1	2.95 ± 0.01	0.30 ± 0.01	11 ± 2	12.4 ± 0.3
NaA5	2 ± 0	3.09 ± 0.01	0.40 ± 0.01	12 ± 2	12.3 ± 0.3
NaB5	2 ± 0	3.08 ± 0.01	0.30 ± 0.05	11 ± 2	12.7 ± 0.4

[a] As g/L acetic acid.

compared to garnacha must fermented with indigenous yeasts (control must). Five samples were studied, two fermented with indigenous must yeasts and three inoculated with the previously mentioned strain.

MATERIALS AND METHODS

Samples and Vinification. The must used was *Vitis vinífera* subsp. *garnacha* kept in contact with skin for 17−20 h, and later treated with SO_2 (80 mg/L). The must had 1390 nephelometric units of turbidity (NTU). The sample was divided into five aliquots of 5.0 L; three were inoculated with active dry *S. cerevisiae* subsp. *cerevisiae* (Na33 strain) selected by the Estación de Viticultura y Enología de Navarra and commercialized by Lallemand España; the other two were exclusively fermented by indigenous yeasts (control must). According to several studies performed by the Estación de Viticultura y Enología de Navarra, we know that the selected yeast has a neutral phenotype, is resistant to wild killer yeasts, and has a powerful alcohologenic effect, low H_2S production, and volatile acidity. In studies carried out in a synthetic medium, this yeast showed a high demand for nitrogen compounds during fermentation, although the kinetics of consumption of these compounds was slow.

The Na33 strain was inoculated in the must in a proportion of 0.2 g/L. For 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/mL $\geq 2 \times 10^9$); it was kept in this medium for 30 min at 35 °C. The must was inoculated while being mixed to obtain a homogeneous distribution. The fermentations were done in modular bioreactors of 5.0 L (Gallenkamp, Leicesterhire, U.K.) at a controlled temperature of 18 ± 2 °C. In the rosé wines, the concentration of reducing sugars fell below 2.5 g/L.

Preparation and HPLC Analysis of Free Amino Acids. Analysis was performed with a Waters high-pressure liquid chromatograph (Waters Chromatography Division, Milford, MA) equipped with two 510 pumps, a 717 Plus Autosampler, and a 486 UV−vis detector used at 254 nm. The Pico·Tag method used is described by Ancín et al. (1996).

Polymerase Chain Reaction (PCR). This technique was used to identify the Na33 strain and check its predominance in the fermentation of the inoculated musts. For 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 PCR analyses were done at the Sigmo Laboratory of Nantes (France). The method used was that of Lavallée et al. (1994).

Total Nitrogen, Nitrogenous Fractions, and Enological Parameters. Total nitrogen was determined with the Kjeldahl method (A40), reported by the Office International de la Vigne et du Vin (1990). The samples were mineralized with H_2SO_4, with $CuSO_4$ as catalyst and K_2SO_4 to raise the boiling point. Distillation of the total nitrogen was performed with Tecator automatic steam equipment (Tecator AB, S-26321 Höganäs, Sweden). Ammonium nitrogen was quantified using enzymatic test kits (Boehringer Mannheim) according to the manufacturer's instructions. Amino nitrogen was calculated by determining free amino acids by HPLC following the Pico·Tag method. Enological parameters are described as reported by the Office International de la Vigne et du Vin (1990).

All determinations were performed in triplicate on representative samples of musts and wines. The results are the arithmetic mean of the studied samples, and those presented in the tables are with standard errors (SEs). To improve clarity, the results presented in histograms do not include SEs; however, the coefficients of variation for amino acid data obtained by the method described were between 1% and 13%.

RESULTS AND DISCUSSION

PCR as a Quality Control Tool To Identify Inoculated Commercial Wine Yeast Strains. The results of the PCR indicated that, in two of the three inoculated samples, the Na33 strain was predominant during the fermentation. This did not occur in the other sample. The predominance of an inoculated strain depends on the composition of the medium and on the specific conditions of vinification, for example, the timing and concentration of added sulfur dioxide. In our case, the dose of SO_2 added to the must was 80 mg/L and the method of vinification was the same for all the samples; nonetheless, the behavior of the inoculated strain was different in one of the samples. These results coincide with the conclusions of Loiseau et al. (1987), Delteil and Aizac (1988), and Fleet (1990). These workers, in genetically marked strains of *S. cerevisiae*, also observed that the predominance of the inoculated strain is not always assured.

Arising from these results, the samples were named in the following way: (a) NaA, sample where the inoculated strain did not predominate; (b) NaB, arithmetic mean of the two samples where the inoculated strain predominated; (c) control, arithmetic mean of the two samples uninoculated. The different stages of sampling were designated by numbers: 1, initial must; 2, must at 25% of fermented sugars; 3, must at 50% of fermented sugars; 4, must at 75% of fermented sugars; 5, recently obtained wine.

General Parameters and Fermentation Kinetics. Table 1 shows that the initial pH of the must was

Table 2. Features of the Fermentation Kinetics in the Samples

	dt_{5-50} (days)	dt_{0-99} (days)	Vf_{5-50}[a] (%/days)	Vf_{0-99}[b] (%/days)
control	4	14	11.2	7.1
NaA	4	16	11.2	6.2
NaB	4	13	11.2	7.6

[a] Average percentage of sugar used daily during the fermentation time required from 5% to 50% of the total. [b] Average percentage of sugar used daily during the fermentation time required from 0% to 99% of the total.

Table 3. Total Nitrogen and Nitrogenous Fractions of Musts and Wines

	total nitrogen (mg/L ± SE)	ammonium nitrogen (mg/L ± SE)	amino nitrogen (mg/L ± SE)
control 1	450 ± 12	86 ± 3	246 ± 3
NaA1	475 ± 11	86 ± 3	247 ± 3
NaB1	450 ± 15	90 ± 4	247 ± 2
control 2	323 ± 9		103 ± 4
NaA2	375 ± 11		100 ± 3
NaB2	481 ± 14		111 ± 2
control 3	430 ± 14		72 ± 11
NaA3	454 ± 11		81 ± 1
NaB3	559 ± 24		94 ± 3
control 4	380 ± 23		101 ± 4
NaA4	426 ± 14		97 ± 3
NaB4	304 ± 33		99 ± 2
control 5	130 ± 20		92 ± 3
NaA5	160 ± 12		95 ± 2
NaB5	105 ± 16		81 ± 1

between 3.1 and 3.6, similar to what was reported by Amerine and Ough (1976) for this product. The free SO_2 concentration in the must was similar in all the samples. In the wine from the control must, the pH was slightly lower than those of the other samples; in any case, the pH of all the wines was low and below 3.6, a value considered limiting for correct product conservation. The free SO_2 concentration in the wines was very similar to that in the initial musts. On the other hand, there were no differences in the values of volatile acidity nor in the alcohol concentration in the resulting wines.

To characterize the kinetics, the fermentation rates were calculated from fermentation curves as an average percentage of the daily consumed sugar in the ranges of 5−50% (vf_{5-50}) and 0−99% (vf_{0-99}) of total sugars (Houtman and du Plessis, 1985). These results are shown in Table 2. The fermentation rate was similar in all the samples.

Total Nitrogen and Nitrogenous Fractions. The results are presented in Table 3. The assimilable nitrogen of these musts had an average value of 334 mg/L, 87 mg/L of which corresponded to ammonium nitrogen and 247 mg/L to amino nitrogen. The ammonium nitrogen of the must represented 19% of the total nitrogen, and the amino nitrogen 54% of the total; the latter value is slightly higher than those found by Gorinstein et al. (1984) in Semillon and Carignane must. The existence of assimilable nitrogen in sufficient quantities for a good development of the yeast would have favored fermentation at the same rate in all samples. Jiranek et al. (1991) observed that the differences in fermentation rates (using different yeast types) were lower at higher concentrations of assimilable nitrogen.

The ammonium nitrogen was totally consumed in all the samples at the beginning of the fermentation process (while 0−25% of the sugars were consumed) (Table 3). This nitrogen is the main source of inorganic nitrogen assimilable by the yeast in the growing phase (O'Connor-Cox and Ingledew, 1989). The consumption of amino nitrogen during the fermentation was higher in the NaB sample (166 mg/L) than in the other two (NaA, 152 mg/L; control, 154 mg/L) (Table 3). This was slightly higher than that observed by Dizy and Polo (1996) in white grape must (malvar variety), and that found in the synthetic medium with an excess of amino nitrogen (Jiranek et al. 1990). The utilization of this amino nitrogen was also different in NaB than in the control; the NaA followed an intermediate behavior between those of the other two. In this sense, during the first half of fermentation (until 50% of the sugars consumed), more amino nitrogen was consumed in the control sample (174 mg/L) than in the NaB sample (153 mg/L); the NaA sample consumed 166 mg/L (Table 3). In the second half of the fermentation, the amino nitrogen consumption of NaB continued (13 mg/L), while it was excreted in the other two (control, 20 mg/L; NaA, 14 mg/L) (Table 3). This means that, in the sample where Na33 predominated, the amino nitrogen was consumed more slowly but continued until the end of fermentation. Thus, the Na33 strain was better at resisting the toxic effect of ethanol that inhibits the amino acid transport system as well as that of glucose, maltose, and ammonium (Leao and van Uden, 1984; van Uden, 1985), as it alters some of the components of the transport proteins (van Uden, 1989).

Evolution of the Amino Acids Found in Proteins (AaP), during the First Half of Fermentation. During the use of the *first 25% of the sugars* (Figure 1a

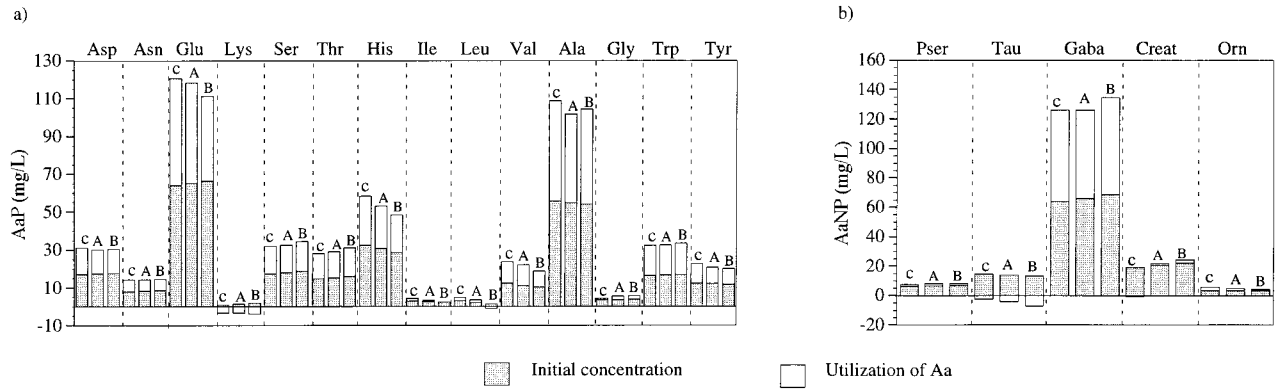

Figure 1. Initial concentration of amino acids in the musts. Assimilation of amino acids (positive values in the graph) or excretion of amino acids (negative values in the graph) at 25% sugar fermented. (a) Amino acids found in proteins (AaP). (b) Amino acids not found in proteins (AaNP). C, control sample; A, sample where the strain Na33 did not predominate; B, sample where the strain Na33 predominated.

Table 4. Concentration (mg/L) of Arginine and Proline in Garnacha Must and during Fermentation

	Arg (mg/L ± SE)					Pro (mg/L ± SE)				
	stage 1	stage 2	stage 3	stage 4	stage 5	stage 1	stage 2	stage 3	stage 4	stage 5
control	384 ± 6	7 ± 2	2.1 ± 0.8	2.9 ± 0.6	3.6 ± 0.3	484 ± 14	664 ± 30	473 ± 54	629 ± 27	622 ± 21
NaA	388 ± 5	10 ± 2	2.0 ± 0.2	1.8 ± 0.3	9.9 ± 0.2	485 ± 9	625 ± 14	545 ± 9	591 ± 17	589 ± 12
NaB	393 ± 5	20.7 ± 0.2	2.5 ± 0.4	1.1 ± 0.3	1.0 ± 0.1	486 ± 11	636 ± 18	600 ± 11	622 ± 13	576 ± 7

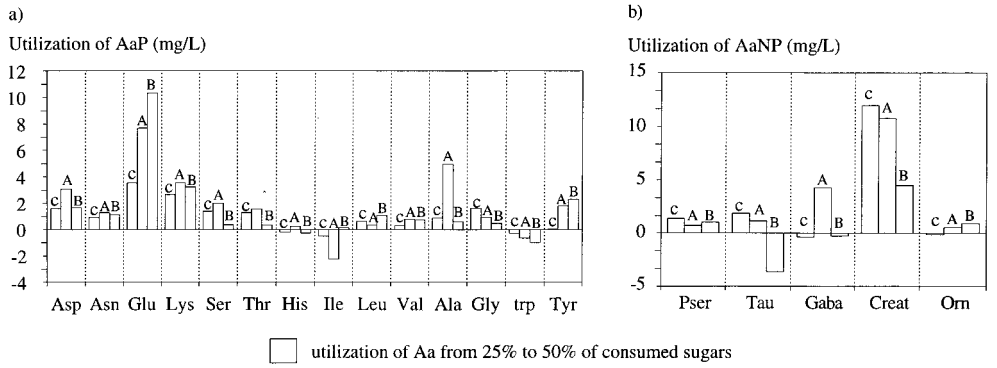

Figure 2. Assimilation of amino acids (positive values in the graph) or excretion of amino acids (negative values in the graph) from 25% to 50% of fermented sugars. (a) Amino acids found in proteins (AaP). (b) Amino acids not found in proteins (AaNP). C, control sample; A, sample where the strain Na33 did not predominate; B, sample where the strain Na33 predominated.

and Table 4), qualitatively the utilization of amino acids was the same in the three samples; quantitatively, the consumption was slightly higher in the control sample (94%) and in the NaA sample (91%) than in the NaB sample (88%). The amino acids most consumed were the same in the three samples studied (aspartate, glutamate, serine, threonine, histidine, alanine, tryptophan, arginine). These results, except that of lysine, agree with those found by Jiranek et al. (1995) who observed that these amino acids were the most consumed by nine yeast strains in a chemically defined grape juice. In the previously cited work, lysine was initially found in relatively high concentrations and was consumed from the beginning of fermentation. In our musts, this amino acid was found in low concentrations and was excreted in the beginning of fermentation. This excretion may be due to several causes such as the capacity of the yeast to transport and hydrolyze (inside the cell) small di- and tripeptides (Cooper, 1982), as well as the interchange of these amino acids between the intracellular pool and the external medium.

In the three samples, arginine was consumed in great amounts (control sample, 98%; NaA, 97%; NaB, 95%) (Table 4). These results are in agreement with those reported by Jiranek et al. (1990) who observed that arginine is the best source of nitrogen for wine yeast, irrespective of strain and conditions. This amino acid, in addition to being in the majority, is completely degraded and provides three nitrogens for cellular biosynthesis, since the fourth nitrogen atom ends up as the imino nitrogen of proline (Monteiro and Bisson, 1991). Glutamate was consumed substantially in the control sample and in NaA (89% and 82%, respectively) and a bit less in NaB (68%) (Figure 1a). The high use of this amino acid coincides with the results of different authors (LaRue and Spencer, 1967; Cooper, 1982); glutamate is considered a good source of nitrogen for yeast since it enters directly into the intracellular pool during its growth, even in the presence of ammonia (Large, 1986). Alanine was another amino acid consumed substantially in the three samples (Figure 1a). This demonstrates the importance of medium composition for yeast since alanine was one of the least consumed along with glycine, tyrosine, and tryptophan in a synthetic medium rich in amino acids (Jiranek et al., 1990).

The excretion of proline was also important in this first phase of fermentation in the three samples (Table 4). This amino acid, along with ornithine and urea, is an intermediate product in the degradation of arginine (Ough et al., 1988a,b). The liberation of proline may be due to the metabolism of arginine that was consumed in high concentration in the first phase of fermentation.

From 25% to 50% of the fermented sugars (Figure 2a and Table 4), a lesser quantity of amino acids were consumed in the NaB sample (76.6 mg/L) than in the control sample (211 mg/L). In the NaA sample 116 mg/L was consumed. The most used amino acids in NaB were arginine (18.2 mg/L), proline (36.4 mg/L), and glutamate (10.3 mg/L). The consumption of proline stood out in the control sample and NaA (control, 191 mg/L; NaA, 80.4 mg/L); arginine and glutamate were also used in these samples, although less than in NaB. During this phase, when the conditions may still be aerobic, there were no problems in the use of proline since proline oxidase would have had O_2 for activity (Cooper, 1982; Ingledew et al., 1987). The lower consumption of proline in NaB was probably due to the fact that, in this medium, a greater amount of unconsumed amino acid was present, which would have inhibited proline permease (Jiranek et al., 1990).

In this first half of fermentation the lower consumption of AaP in the NaB sample could have been due to the fact that this strain consumed the amino acids more slowly than the indigenous strains of *S. cerevisiae* of the control sample. Consequently, in a complex matrix such as must, this strain followed the same tendency as in a synthetic medium (results commented on in the Materials and Methods). This fact probably decreased the capacity of this yeast to compete with the indigenous yeasts for the nitrogenous nutrients of the must. Since these nutrients can be growth limiting for these microorganisms, the Na33 strain could present difficulties in its implantation, as has been proved experimentally. In the NaA sample, the utilization of amino acids followed an intermediate evolution between those of the control

Table 5. Utilization of Amino Acids during the Course in Which 50−75% Sugars Were Fermented (+, Consumption; −, Excretion)

AaP	AaNP	control sample (mg/L ± SE)	NaA sample (mg/L ± SE)	NaB sample (mg/L ± SE)
Asp		*a*	−0.9 ± 0.1	0.6 ± 0.1
Asn		−1.5 ± 0.6	−1.8 ± 0.1	−0.6 ± 0.1
Glu		−9 ± 1	−8.7 ± 0.6	−11.4 ± 0.8
Lys		*a*	−1 ± 0.1	1.5 ± 0.07
Ser		−0.7 ± 0.6	−0.9 ± 0.2	0.5 ± 0.1
Thr		−0.7 ± 0.2	−1.7 ± 0.1	−0.5 ± 0.3
His		3 ± 2	4.8 ± 0.6	6.1 ± 0.4
Ile		−0.7 ± 0.6	*a*	−0.5 ± 0.4
Leu		*a*	0.5 ± 0.2	0.4 ± 0.1
Val		*a*	0.2 ± 0.1	0.3 ± 0.1
Ala		−2 ± 1	−2.0 ± 0.5	0.7 ± 0.6
Gly		*a*	−0.17 ± 0.06	0.75 ± 0.05
Trp		*a*	0.2 ± 0.1	0.3 ± 0.2
Tyr		0.7 ± 0.3	0.6 ± 0.1	0.4 ± 0.3
	Pser	*a*	0.3 ± 0.2	*a*
	Tau	*a*	2.6 ± 0.8	10 ± 2
	Gaba	0.9 ± 0.6	1 ± 0.1	1.6 ± 0.5
	Creat	−9 ± 2	−11.5 ± 0.7	−1.4 ± 0.6
	Orn	*a*	0.3 ± 0.1	0.6 ± 0.1

[a] No significant difference.

Table 6. Utilization of Amino Acids during the Course in Which 75−100% Sugars Were Fermented (+, Consumption; −, Excretion)

AaP	AaNP	control sample (mg/L ± SE)	NaA sample (mg/L ± SE)	NaB sample (mg/L ± SE)
Asp		0.5 ± 0.3	*a*	1.5 ± 0.1
Asn		−2.4 ± 0.6	−4 ± 0.2	−0.2 ± 0.1
Glu		5.4 ± 0.9	1.7 ± 0.8	19 ± 1
Lys		0.3 ± 0.2	−0.9 ± 0.3	0.07 ± 0.03
Ser		−1 ± 0.4	−0.6 ± 0.2	0.8 ± 0.08
Thr		−0.6 ± 0.3	*a*	1.2 ± 0.3
His		2 ± 0.6	*a*	2.2 ± 0.2
Ile		1.9 ± 0.3	2.2 ± 0.8	1.7 ± 0.3
Leu		−0.9 ± 0.1	−2.1 ± 0.2	0.13 ± 0.06
Val		−0.2 ± 0.1	−0.7 ± 0.1	0.3 ± 0.1
Ala		−5 ± 1	−9.3 ± 0.4	1.5 ± 0.2
Gly		−1.2 ± 0.3	−1 ± 0.1	0.12 ± 0.04
Trp		0.2 ± 0.1	0.4 ± 0.2	*a*
Tyr		*a*	−1.4 ± 0.2	*a*
	Pser	−4.6 ± 0.6	−4.3 ± 0.1	−4.2 ± 0.4
	Tau	6 ± 1	5.9 ± 0.4	9.5 ± 0.8
	Gaba	−4.2 ± 0.6	−4.8 ± 0.3	0.2 ± 0.1
	Creat	*a*	*a*	0.6 ± 0.5
	Orn	*a*	0.2 ± 0.1	0.3 ± 0.1

[a] No significant difference.

and NaB samples; consequently, although the yeast did not predominate, it seems that it contributed to the fermentation process.

Evolution of the Amino Acids Found in Proteins (AaP), during the Second Half of Fermentation. *From 50% to 75% of the fermented sugars* (Tables 4 and 5), differences were observed in the use of amino acids between NaB and the other two. Excretion of amino acids predominated over consumption in the control sample and NaA; the quantity of excreted amino acids, except for proline, was 15.4 mg/L for the control and 17.2 mg/L for NaA, while the consumption was 3.7 mg/L for the control and 6.5 mg/L for NaA. In NaB more amino acids were consumed than in the control and NaA (13 mg/L). In all the samples, histidine was the most consumed, although in more concentration in NaB than in the other two (control, 3 mg/L; NaA, 4.8 mg/L; NaB, 6.1 mg/L); this consumption was produced even though this amino acid is not a good source of nitrogen for yeast (Watson, 1976; Perkur et al., 1981; Ingledew et al., 1987) and it is apparently not even degraded by many yeasts (Cooper, 1982; Large, 1986). Proline was excreted in the control and NaA, while its concentration was not modified in NaB.

The last phase of fermentation (*from 75% to 100% of the fermented sugars*) (Tables 4 and 6) followed the same tendency as the previous phase, and we continued to find differences in the use of amino acids between NaB and the other two. Thus, in NaB all the amino acids were consumed and there was only a small excretion of asparginine. In this sample, glutamate was substantially consumed (19 mg/L), and the rest of the amino acids were consumed in lower quantities. In the other two samples the majority of the amino acids were excreted.

In this second half of fermentation, the greater consumption of AaP in the sample where the inoculated yeast predominated, as compared to the control sample, could have been due to the fact that the Na33 strain showed a high tolerance for the toxic effects of ethanol. Several researchers have noticed that tolerance to ethanol is a strain-dependent characteristic (Beavan et al., 1982; Jones, 1989). Moreover, this tolerance would have been favored by the presence of oxygen in the production of the yeasts and for obtaining the inoculum. Since active dry wine yeasts are grown under fed-batch conditions in the presence of air, they contain a reservoir of unsaturated fatty acids and esterols. These compounds play an important role in the structure of the plasma membrane of yeast, increasing its resistance to the toxic effects of ethanol (van Uden, 1989).

Evolution of Amino Acids Not Found in Proteins (AaNP), during the First Half of Fermentation. *From 0% to 25% of the fermented sugars* (Figure 1b), there was a similar evolution in the three samples; γ-aminobutiric acid was the most consumed at about 60 mg/L. Similarly, ornithine was consumed a bit more in the control (2.4 mg/L) and NaA (1.5 mg/L) than in NaB (0.9 mg/L); phosphoserine was consumed in a similar manner in the three samples, and taurine was also excreted in the three, although more in NaB (7.1 mg/L) than in NaA (4 mg/L) or the control (2.4 mg/L).

From 25% to 50% of the fermented sugars (Figure 2b), there was less consumption of amino acids in the NaB sample (6.4 mg/L) than in the control sample (15.3 mg/L) or in the NaA sample (17.5 mg/L). The main consumption was of creatinine in the three samples, although more in the control (12 mg/L) and NaA (10.8 mg/L) than in NaB (4.5 mg/L). Taurine was excreted in NaB, while it was consumed in a similar manner in NaA and control.

Evolution of Amino Acids Not Found in Proteins (AaNP), during the Second Half of Fermentation. *From 50% to 75% of the fermented sugars* (Table 5), the same tendency existed in these amino acids as in AaP. In NaB more amino acids were consumed (12.2 mg/L) than excreted (1.4 mg/L); in the other two samples, the quantity of excreted amino acids was greater than that consumed. In the three samples the amino acid most excreted was creatinine, although more in NaA and the control than in NaB. The most consumed amino acid was taurine, especially in NaB (10 mg/L).

In the last phase of the fermentation (*from 75% to 100% of the fermented sugars)* (Table 6), as in the previous stage, more amino acids were consumed in NaB than in the other two. Taurine was the most consumed amino acid in the three samples.

AaNP are distinguished from AaP in that they do not incorporate directly into proteins, and the yeasts use them only as a source of nitrogen and carbon. The consumption of AaNP was less than AaP with the exception of γ-aminobutiric acid, as these amino acids would have been poor sources of nitrogen for the yeast. Comparing the NaB sample with the control sample, the behavior of AaNP followed the same tendency as that of AaP.

CONCLUSIONS

From the results obtained it may be concluded that the Na33 strain seems to be resistant to ethanol since it consumed amino acids up to the end of fermentation. This resistance to ethanol would also have been favored by the aerobic conditions where the strain was produced and reconstituted before being inoculated in the must. Consequently, active dry Na33 strain could be interesting for the fermentation of musts which are rich in sugars. In addition, the wine obtained with this strain had a lower concentration of amino nitrogen than the control wine, and thus a greater biological stability.

The difficulty which the Na33 strain presented in competing for the uptake of nitrogen compounds (which can be growth limiting) at the beginning of fermentation, could be one of the causes that prevented its implantation in all the inoculated samples. It was also observed that, in one of the inoculated samples, where this strain did not predominate, it contributed to the fermentation, as the consumption of amino acids was intermediate between those of the control sample and the samples where the strain predominated.

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