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Influence of the Timing of Nitrogen Additions during Synthetic Grape Must Fermentations on Fermentation Kinetics and Nitrogen Consumption

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Nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentations. In the present study, we have supplemented nitrogen-deficient fermentations with a mixture of ammonium and amino acids at various stages throughout the alcoholic fermentation. The timing of the nitrogen additions influenced the biomass yield, the fermentation performance, the patterns of ammonium and amino acid consumption, and the production of secondary metabolites. These nitrogen additions induced a nitrogen-repressed situation in the cells, and this situation determined which nitrogen sources were selected. Glutamine and tryptophan were the main amino acids consumed in all the fermentations. Ammonium is the preferred nitrogen source for biomass production but was hardly consumed when it was added in the final stages of the fermentation. The higher ammonium consumption in some fermentations correlated with a greater synthesis of glycerol, acetate, and acetaldehyde but with a lower synthesis of higher alcohols.

KEYWORDS: ***Saccharomyces cerevisiae*; Alcoholic Fermentation; Amino acids; Ammonium; GAP1; MEP2**

INTRODUCTION

The nitrogen composition of grape musts affects the growth and metabolism of yeast, the fermentation rate, and the completion of fermentation (*1*). Nitrogen deficiencies are one of the main causes of stuck or sluggish fermentations. One way of avoiding these problems is to add nutritional supplements, usually inorganic forms of nitrogen such as ammonium salts, to grape must prior to fermentation (*2*−*4*). These additions are generally made empirically in wine cellars, and the initial nitrogen concentration in the must or the nitrogen requirements of the usual yeast strain used in the cellar are not determined. Yeasts respond metabolically to differences in nitrogen availability, so this lack of control of nitrogen leads to differences in wine composition.

Nitrogen affects yeast cells in two ways: it increases biomass production and stimulates the rate of sugar utilization. Nitrogen additions during the period of cell growth have resulted in maximum cell populations. Later additions during the stationary phase have had no effect on the cell population but have increased the specific fermentation rate, thus reducing the length of the fermentation (*2*, *5*, *6*).

Nitrogen supplementation affects the pattern of nitrogen uptake. Ammonium is a preferred yeast nitrogen source, and when plentiful, it represses the expression of catabolic pathways by degrading other nitrogenous compounds (*7*, *8*). This mechanism, called nitrogen catabolite repression (NCR), has recently been studied during wine fermentations (*9*). It inhibits the uptake of arginine and alanine and stimulates the consumption of branched-chain and aromatic amino acids. Changes in the nitrogen uptake patterns influence the production of aroma and spoilage compounds (particularly hydrogen sulfide) and the amount of urea, the major precursor of the carcinogen ethyl carbamate (*10*−*13*).

The volatiles identified in wines are usually dominated by fermentation products. Organic acids, higher alcohols, and esters are the main group of flavor compounds coming from yeast metabolism (*12*). Higher alcohols can be produced either by the catabolic conversion of the branched-chain amino acids (via Ehrlich) or by the anabolic formation of these amino acids de novo from a sugar substrate (*14*). An excess of higher alcohols (above 400 mg L^{-1}) can be regarded as a negative influence on the quality of wine, but at the concentrations generally found in wines (below 300 mg L^{-1}), they usually contribute to the desirable complexity of wine. Furthermore, these alcohols, together with the acids in wine, are substrates for ester formation. Most esters, with the exception of ethyl acetate, impart a pleasant smell of fruits and flower notes in the wine (*15*).

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As mentioned, in winemaking, most of the nitrogen additions are made empirically and do not take into account the different nitrogen needs of the cell during wine fermentation, the proper timing of these additions, or the nitrogen source added. In this study, we supplemented nitrogen-deficient fermentations with a mixture of ammonium and amino acids at different stages of the alcoholic fermentation. We then studied the effect of these additions on the fermentation kinetics, the consumption of organic and inorganic nitrogen throughout the fermentation, and the influence of this consumption on the organoleptic profile of the wines. We also monitored the effect of the nitrogen supplementations on the NCR system and the effect of the nitrogen-repressed situation on nitrogen uptake.

MATERIALS AND METHODS

Strain, Fermentations, and Sampling. A commercial *Saccharomyces cerevisiae var. bayanus* wine strain QA23 (Lallemand S. A., Toulouse, France) was used in this study. Fermentations were carried out in a synthetic grape must (pH 3.3) as described by Riou et al. (*16*) but with 200 g L^{-1} of reducing sugars (100 g L^{-1} Glucose and 100 g L^{-1} Fructose) and without anaerobic factors. Only the nitrogen content changed in the different fermentations.

The yeast assimilable nitrogen (YAN) content in the control synthetic grape must was 300 mg N L^{-1}, ammoniacal nitrogen (NH_4Cl) 120 mg N L^{-1}, and amino acids 180 mg N L^{-1} (Table 2). This medium also contained 426 mg L^{-1} of Proline, but it should not be considered as assimilable nitrogen (*17*). The proportions of the different amino acids and ammonium were maintained in all the fermentations. Nitrogen-limited fermentations were carried out with 60 mg L^{-1} of YAN (24 mg L^{-1} of ammoniacal nitrogen and 36 mg L^{-1} of amino acid nitrogen), and 240 mg L^{-1} of YAN nitrogen was added at different fermentation points (96 mg L^{-1} of ammoniacal nitrogen and 144 mg L^{-1} of amino acid nitrogen).

The supplementation points were chosen by monitoring the decrease in density of the media. Density was measured throughout the fermentation by weighing 5 mL, and nitrogen was added when the density of the must was 1060 g L^{-1} (30 h after inoculation), 1040 g L^{-1} (72 h), 1020 g L^{-1} (144 h), and 1000 g L^{-1} (240 h).

Fermentations took place at room temperature (22−28 °C) in laboratory-scale fermenters: 2 L bottles filled with 1.8 L of medium and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Fermentations were in semi-anaerobic conditions since limited aeration was necessary in order to harvest samples for the subsequent analysis. The population inoculated in every flask was 2×10^6 cell mL^{-1} from dry yeast rehydrated in water at 37 °C.

In the latter stages of the fermentation, the sugar consumption was assayed by enzymatic kits (Roche Applied Science, Germany). Fermentation was considered to be complete when the residual sugars were below 2 g L^{-1}. Cell growth was determined by absorbance at 600 nm. Absorbance values were corrected for the initial absorbance reading obtained for juice.

Cells were harvested at different points during the fermentation so that mRNA could be analyzed. Flasks were magnetically stirred to resuspend settled biomass, transferred to centrifuge tubes, and centrifuged at 5000 rpm for 5 min at room temperature to prevent temperature shock. Cell pellets were transferred to 1.5 mL Eppendorf tubes and frozen immediately in liquid nitrogen. They were kept at −80 °C until they were analyzed. The supernatant of these samples was stored at −20 °C for extracellular metabolites and nitrogen content analysis.

Nitrogen Content Analysis. YAN was analyzed by the formol index method (*18*), and the ammonium content was quantified using an enzymatic method (Roche Applied Science, Germany). The individual amino and imino acids were analyzed by OPA and FMOC derivatizations, respectively, using the Agilent 1100 Series HPLC equipped with a low-pressure gradient quaternary pump, a thermostated autosampler, a DAD ultraviolet detector, and a fluorescence detector (Agilent Technologies, Germany). The sample (2 μL) was injected into a 4.6 mm × 250 mm × 5 μm Hypersil ODS column (Agilent Technologies, Germany). The gradient solvent system was: solvent A (16 mM sodium acetate and 0.022% triethylamine, adjusted to pH 7.2 with 1−2% acetic acid, and 0.6% tetrahydrofuran) from 100% at t = 0 to 0% at t = 18 min, and solvent B (20% of 66 mM sodium acetate, adjusted to pH 7.2 with 1−2% acetic acid, 40% acetonitrile and 40% methanol) from 0% at t = 0 to 100% at t = 18 min. The analysis temperature was 40 °C, and the flow rate was 1.5 mL min^{-1}. Several dilutions of each sample were analyzed and averaged using the analysis software. The concentration of each amino acid was calculated using external and internal standards and expressed as mg L^{-1}. The software used was Agilent ChemStation Plus (Agilent Technologies, Germany).

Ethanol, Glycerol, and Organic Acid Analysis. Ethanol, glycerol, and organic acids were analyzed in all the samples at the end of the fermentation process. Analytical HPLC was carried out on a Hewlett-Packard HP 1050 connected to a Hewlett-Packard Integrator 3395 equipped with an HP 1047 RI detector (Agilent Technologies, Wilmington, DE) (*19*). The wine sample (450 μL) was mixed with 50 μL of formic acid (internal standard), and 25 μL was injected into a 300 mm × 7.8 mm AMINEX HPX-87H column (BioRad, Hercules, CA). The solvent used was sulfuric acid 2.5 mM at 0.5 mL min^{-1}. The analysis temperature was 60 °C. The concentration of each metabolite was calculated using external and internal standards.

Fatty Acid Analysis. Fatty acids were extracted using the method published by López et al. (*20*). Analytical GC was carried out on a Hewlett-Packard 6890N connected to a computer with the ChemStation software (Agilent Technologies, Wilmington, DE). The extract (2 μL) was injected (splitless, 0.75 min) into a Tracer TR column of 60 m × 250 μm and 0.25 μm phase thickness with an HP automatic injector (Agilent). The temperature program was 40 °C for 5 min followed by 2 °C min^{-1} to 240 °C (15 min). Injector and detector temperatures were 220 and 240 °C, respectively. The carrier gas was hydrogen at 60 mL min^{-1}. 2-Ethylphenol (0.2 mg L^{-1}) was added as internal standard. Internal patterns were used to estimate the quantity of the different compounds.

Analysis of Higher Alcohols and Esters. Higher alcohols and esters were extracted by liquid/liquid extraction (wine 10 mL, 200 μL 1,1,2-trichlorotrifluoroethane, 0.5 g NaCl), with *n*-decanol (0.2 mg L^{-1}) as internal standard (*21*). After agitation for 2 min and centrifugation, the organic phase was extracted and 2 μL was injected. The chromatographic program used is the same as that used for the fatty acid analysis. Quantification was conducted by comparison with known quantities of different products in a hydro alcoholic solution.

RNA Extraction and cDNA Synthesis. Total RNA was isolated from yeast samples as described by Sierkstra et al. (*22*) and resuspended in 50 μL of DEPC-treated water. Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany) following the protocol provided by the manufacturer. RNA concentrations were determined using a GenQuant spectrophotometer (Pharmacia, Canada), and the quality of RNA was verified electrophoretically on 0.8% agarose gels. Solutions and equipment were treated so that they were RNase free, as outlined in Sambrook et al. (*23*).

Total RNA was reverse-transcripted with Superscript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA) in a GenAmp PCR System 2700 (Applied Biosystems, Foster City, CA). Oligo $(dT)_{12-18}$ primer (0.5 μg, Invitrogen) was used with 0.8 μg of total RNA as template in a reaction volume of 20 μL. Following the protocol provided by the manufacturer, after denaturation at 70 °C for 10 min, cDNA was synthesized at 42 °C for 50 min. Finally, the reaction was inactivated at 70 °C for 15 min.

Real-Time Quantitative PCR. The PCR primers used in this study are *ACT-F*, TGGATTCCGGTGATGGTGTT, and *ACT-R*, CGGCCAAATCGATTCTCAA (*ACT*, for actine gene); *GAP1−F*, CTGTGGATGCTGCTGCTTCA, and *GAP1−R*, CAACACTTGGCAAACCCTTGA (*GAP1*, for general amino acid permease gene); and *MEP2−F*, GGTATCATCGCTGGCCTAGTG, and *MEP2−R*, ACAACGGCTGACCAGATTGG (*MEP2*, for ammonium permease gene) (*9*).They were all designed with the available GenBank sequence data and the Primer Express software (Applied Biosystems) in accordance with the Applied Biosystems guidelines for designing PCR primers for quantitative PCR. All amplicons were short, which ensured maximal PCR efficiency and, therefore, the most precise quantification.

Table 1. Determination of Yeast Assimilable Nitrogen (YAN) in the Fermentation Media, Represented by the Amino Acid Fraction (YAN aas) and by the Ammonium fraction (YAN NH_4^+)

	control fermentation			N addition at $\rho = 1060$			N addition at $\rho = 1040$		
density (ρ)	time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})	time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})	time (h)	YAN NH_4^{2+} (mg N L^{-1})	YAN aas (mg N L^{-1})
1080	0	120	168	0	25	41	0	25	40
1060	30	70	98	36	0.5 (90[a])	4 (145[a])	36	0.1	4
1040	56	52	95	62	50	105	78	0.1 (92[a])	4 (136[a])
1020	96	36	98	96	45	104	120	68	104
1000	168	35	98	168	42	108	192	65	110
990 (end[b])	312	41	102	312	50	110	336	66	114

	N addition at $\rho = 1020$			N addition at $\rho = 1000$			no N addition		
density (ρ)	time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})	time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})	time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})
1080	0	25	41	0	26	40	0	26	40
1060	36	0	7	36	0	7	36	0	5
1040	78	0	7	78	0	4	78	0	2
1020	150	0 (92[a])	4 (143[a])	150	0	4	150	0	1
1000	240	81	109	264	0 (99[a])	2 (147[a])	264	1	1
990 (end[b])	408	88	118	456	100	130	504	1	1

[a] NH_4^+ and aas YAN content just after the nitrogen addition. [b] End of fermentation.

For each gene, a standard curve was made with yeast genomic DNA. DNA extraction was performed as described by Querol et al. (*24*), digested by RNase, and isolated by 2-fold phenol−chloroform extractions and ethanol precipitation. Concentration was determined using a GeneQuant spectrophotometer (Pharmacia, Canada). Serial 10-fold dilutions of DNA were carried out to yield DNA concentrations from 4 to 4×10^{-5} ng μL^{-1}. These dilution series were amplified (in duplicate) by SYBR PCR for each gene to obtain standard curves (see above). The standard curve displays the C_t value vs $\log_{10}$ of each standard's starting quantity. The starting quantity of the unknown samples was calculated against the standard curve by interpolation, and gene expression levels are shown as the concentration of the studied gene normalized with the concentration of the housekeeping *ACT* gene.

The real-time quantitative PCR reaction was performed using SYBR Green I PCR (Applied Biosystems). In SYBR PCR, amplification is monitored by the gain in fluorescence of the double-strand-specific DNA-binding dye SYBR green. The 25 μL SYBR PCR reactions contained 300 nM of each PCR primer, together with 1 μL cDNA (or 5 μL of each DNA serial dilution for standard tubes) and one time SYBR master mix (Applied Biosystems).

All PCR reactions were mixed in 96-well optical plates (Applied Biosystems) and cycled in a PE Applied Biosystems 5700 thermal cycler under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60 °C for 60 s.

The PE5700 cycler provided cycle-by-cycle measurement of the fluorescence emission from each PCR reaction. Analysis resulted in the assignation of a threshold cycle (C_t) value to each PCR reaction. The C_t value is the cycle number at which an increase in reporter fluorescence above a baseline signal can first be detected. The threshold was positioned to intersect the exponential part of the amplification curve of positive reactions, as recommended by Applied Biosystems. The C_t value is inversely proportional to the log of the amount of template in the PCR reaction; the lower the C_t value, the higher the concentration of template in the PCR reaction. Assuming a 100% effective PCR amplification, a difference of one C_t value corresponds to a $2^1 = 2$-fold difference in the amount of template. All samples were analyzed in duplicate, and the expression values were averaged by the analysis software (Applied Biosystems). The coefficient of variation in all samples analyzed was less than 10%.

RESULTS

Effect of Nitrogen Addition on Fermentation Kinetics and Nitrogen Consumption. Five fermentations started with a nitrogen content of 60 mg L^{-1}, which is low enough for a fermentation to be sluggish but high enough for it to finish. Four of these nitrogen-deficient fermentations were supplemented at different points with 240 mg L^{-1} of YAN; the first one at a density of 1060 g L^{-1}, and the second, third, and fourth at 1040, 1020, and 1000 g L^{-1}, respectively. The remaining fermentation was not supplemented, but subjected to nitrogen deficiency throughout the process. As a fermentation control, we used the same medium with a nondeficient amount of nitrogen (300 mgN L^{-1}) (*9*).

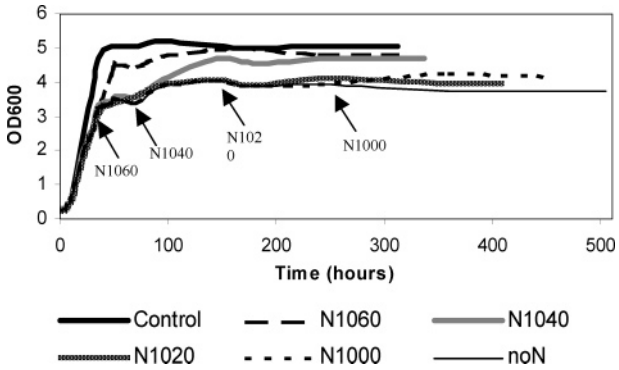

Figure 1. Effect of nitrogen additions on O. D. measures ($\lambda = 600$ nm) throughout synthetic grape must fermentations. The arrows indicate the time of addition.

Figure 1 shows the effect of nitrogen additions on O. D. measures throughout the fermentations studied. The nitrogen-deficient fermentations had lower O. D. values than the control fermentation. When nitrogen was added in the first half of the fermentations (density of 1060 and 1040), these effects were almost overcome, and the O. D. values were similar to those of the control fermentation. Additions at densities of 1020 and 1000, however, had minimal effects on O. D. measures.

Table 1 summarizes the evolution of the fermentation and nitrogen consumption, measured as ammonium and amino acid nitrogen. Unlike their effect on the O. D. values, the nitrogen additions clearly stimulated the fermentation regardless of when they were made. In the nitrogen-deficient fermentation, yeast consumed the total YAN after the first day (data not shown). However, nitrogen was not completely consumed in the control fermentation. The nitrogen additions were all carried out when

Table 2. Total Consumption of Amino Acids and Ammonia at the End of Each Fermentation Expressed as mg N L^{-1}[a]

amino acids	full N media content	control consumption	N1060 consumption		N1040 consumption		N1020 consumption		N1000 consumption		no N consumption
		total	total	post-add	total	post-add	total	post-add	total	post-add	total
Gln	47.4	25.5	34.9	22.1	24.5	11.8	29.0	16.2	23.1	10.3	12.8
Trp	10.3	6.6	6.8	4.2	7.7	4.1	8.1	4.5	7.8	4.1	3.6
Thr	9.9	9.4	3.6	1.4	2.6	0.7	2.0	0.2	1.9	0.1	1.8
His	3.8	3.7	1.5	1.5	1.2	1.2	0.8	0.8	0.4	0.4	–
Leu	4.7	4.2	2.6	1.1	1.8	0.8	1.4	0.4	1.2	0.2	1.0
Ile	3.8	3.2	1.8	0.6	1.4	0.7	0.8	0.2	0.7	0.1	0.6
Phe	2.8	2.4	1.2	0.4	1.3	0.5	1.2	0.4	0.9	0.1	0.8
Val	5.1	3.0	1.6	0.4	1.1	0.1	1.0	–	1.1	–	1.0
Ser	7.5	2.8	2.9	0.8	1.7	–	1.7	–	1.9	0.1	1.8
Met	1.8	1.8	1.1	0.5	1.0	0.6	0.6	0.3	0.5	0.2	0.3
Lys	1.9	1.1	0.7	–	1.0	0.9	1.0	0.9	0.1	–	0.2
Arg	47.9	0.6	5.9	–	9.0	0.9	8.8	0.7	8.2	–	8.2
Tyr	1.4	0.4	0.2	–	0.3	–	0.3	–	0.3	–	0.3
Glu	11.8	0.5	2.3	–	2.0	–	2.7	0.2	2.5	–	2.5
Gly	3.1	0.2	–	–	–	–	–	–	0.3	–	0.4
Ala	12.1	–	1.2	–	1.7	–	2.7	–	3.1	–	3.2
Asp	4.2	–	–	–	–	–	–	–	–	–	0.1
YAN aas	179.5	65.5	68.4	34.5	58.2	22.2	62.1	24.6	53.9	15.7	38.6
YAN NH_4^+	120.0	79.2	64.9	39.5	51.4	26.0	29.5	4.1	25.4	–	25.4

[a] In the fermentations with nitrogen additions, post-add represents the nitrogen taken up after this addition.

the initial YAN had already been depleted, and the later the nitrogen was added, the lower the amount of YAN was consumed (**Table 2**). The ammonium consumed was 54% of the total YAN consumed in the control fermentation (Table 2), but this proportion decreased when nitrogen was added later in the fermentation. Ammonium was proportionally preferred as the nitrogen source when the additions were made in the first half of the fermentations (N1060 and N1040). In later additions (N1020 and N1000), the small amount of nitrogen consumed was mostly from amino acids.

The consumption of amino acids was monitored at different points during the fermentations. The yeast's pattern of amino acid utilization changes with the time of YAN supplementation (**Table 2**). The amino acids can be grouped in different sets according to the preference of the cell in the different conditions. The amino acids that are most consumed are glutamine and tryptophan. Together they represented 32% of the total assimilable amino acids of the synthetic grape must (**Table 2**), and regardless of the fermentation conditions, their consumption accounted for 50% to 65% of the total amino acids consumed. On the other hand, the consumption of arginine, glutamate, glycine, alanine, and aspartate, which were approximately 44% of the total assimilable amino acids in the medium (**Table 2**), was together hardly 2% of the total amino acids consumed in the control fermentation. The consumption of these amino acids was much higher in the fermentations supplemented with nitrogen. However, yeast cells only consumed these amino acids before the nitrogen addition: that is, when the fermentations were nitrogen-deficient. Last, there is one other set of amino acids, consisting of threonine, histidine, leucine, isoleucine, phenylalanine, valine, and methionine, which was consumed proportionally more in the control fermentation than in the supplemented fermentations.

***GAP1* and *MEP2* Gene Expression.** The expression of the nitrogen transporters *GAP1* and *MEP2* was analyzed and quantified relative to the expression of the housekeeping actine gene. Time zero was the expression of yeast before inoculation (and after rehydration). Both genes were repressed in the first hours after inoculation in the must-like medium (**Figure 2**). In the nitrogen-deficient fermentation, these genes started to be activated/de-repressed after 30 h, when nitrogen was almost depleted. The expression of both genes increased continuously during the first days of fermentation and peaked after 4 and 6 days for *GAP1* and *MEP2*, respectively. The expression of the genes decreased in the last days of fermentation, after several days without a nitrogen source. On the other hand, the presence of residual nitrogen in the control fermentation repressed these genes throughout.

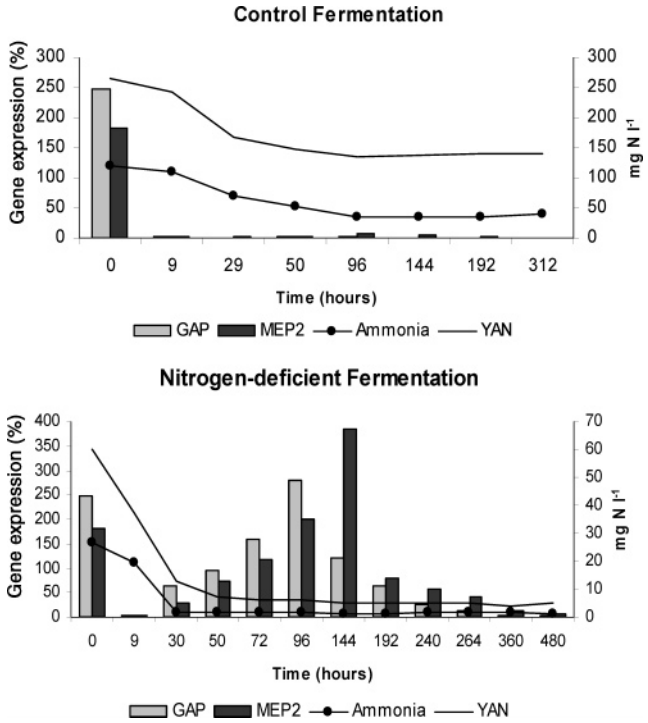

Figure 2. Gene expression of ammonium permease (*MEP2)* and general amino acid permease (*GAP1)* at time zero (before inoculation) and at different points during the control fermentation and the nitrogen-deficient fermentation (without nitrogen addition). The data were quantified by calculating the ratio between the concentration of the studied genes normalized with the concentration of the housekeeping *ACT* gene, and expressed as a percentage (the quantity ratio 1 was set as 100%). YAN and ammonia consumption throughout the fermentations are also indicated.

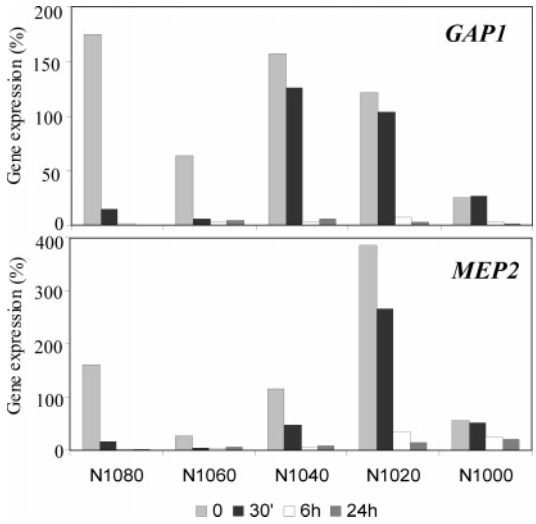

Figure 3. Relative gene expression of *GAP1* and *MEP2* at different points in the first 24 h after the nitrogen addition. Time zero represents the point just before this addition. The data were calculated as in Figure 2.

Figure 3 shows the gene expression of *GAP1* and *MEP2* in the first 24 h after the nitrogen addition. They were both repressed in all the fermentations. However, the later the addition took place in the fermentation process, the longer it took for the genes to be repressed. When nitrogen was added at the end of the fermentation (density 1000), the effect was negligible because of the low expression at this point.

Analytical Profile. We analyzed the residual sugars, ethanol, glycerol, and acids in the wines obtained from the different fermentations and such flavor compounds as higher alcohols, volatile fatty acids, and esters, which arose from yeast metabolism (**Table 3**). The later the nitrogen addition was, the lower the concentration of glycerol, acetic acid, and acetaldehyde was. The higher alcohol content was lower when excess nitrogen was available at the beginning of the fermentation (control fermentation and N1060). These different concentrations were accounted for by the increase in isoamyl alcohol and 2-phenylethanol. The concentration of these compounds increased considerably in the fermentations with nitrogen additions in the later phases (or no addition), and were approximately 2 and 5 times higher than in the control fermentation. The increase in isoamyl alcohol did not lead to a corresponding clear increase in its ester (isoamyl acetate) in these fermentations, and the phenyl-2-ethanol acetate ester only increased slightly. In fact, the differences in the concentration of the total acetate esters between the fermentations were due to the concentration of ethyl acetate, which was more than 95% of the total acetate esters. Its concentration was higher in the control fermentation and the N1060 and 1040 fermentations, which correlated with a higher acetate concentration. The differences in the concentration of fatty acids and their esters were smaller in the final products of the fermentations.

DISCUSSION

The addition of nitrogen to grape musts, especially in the form of ammoniacal nitrogen, is a common winemaking practice that prevents nitrogen-related fermentation problems. Several studies, in which grape musts were supplemented with diammonium phosphate, have proved that nitrogen supplements can optimize fermentation performance (*2*−*4*). In the present study, we supplemented a nitrogen-deficient synthetic must with a mixture of ammonium and amino acids at different stages of the alcoholic fermentation. Then we studied the effect of these additions on the fermentation kinetics, the consumption of organic and inorganic nitrogen throughout the fermentation, and the influence of this consumption on the aroma compound profile of the wines.

Table 3. Secondary Metabolites Produced by Yeasts during the Different Fermentations[a]

	control ferm.	N1060	N1040	N1020	N1000	no N addition
	Alcohols and Acids (g L^{-1})					
ethanol	98.7	97.2	98.0	98.0	98.7	101.1
glycerol	6.56	6.57	6.32	6.11	5.78	6.12
acetate	1.17	1.22	0.98	0.80	0.89	0.81
acetaldehyde	0.33	0.28	0.26	0.25	0.24	0.22
citrate	0.41	0.41	0.38	0.41	0.39	0.41
succinate	0.13	0.21	0.27	0.26	0.23	0.23
lactate	0.04	0.05	0.04	0.03	0.02	0.02
	Higher Alcohols (mg L^{-1})					
n-propanol	37	33	28	20	13	12
isobutanol	11	13	16	16	16	16
isoamylic alcohol	50	48	81	97	94	94
phenyl-2-ethanol	11	21	42	46	53	43
Σ	109	115	167	179	176	165
	Fatty Acids (mg L^{-1})					
isobutyric acid	0.39	0.40	0.43	0.39	0.50	0.41
butyric acid	0.63	0.73	0.67	0.72	0.59	0.60
isovaleric acid	0.63	0.37	0.30	0.44	0.80	0.60
valeric acid	0.12	0.09	0.09	0.10	0.18	0.15
hexanoic acid	2.06	1.58	1.40	1.53	1.90	1.85
octanoic acid	2.30	1.95	1.84	2.34	2.43	2.49
decanoic acid	0.39	0.37	0.21	0.19	0.14	0.46
dodecanoic acid	0.16	0.14	0.09	0.16	0.32	0.28
Σ	6.70	5.63	5.02	5.87	6.86	6.83
	Acetate Esters (mg L^{-1})					
ethyl acetate	35	35	32	25	19	28
isobutyl acetate	0.023	0.024	0.012	0.016	0.011	0.011
isoamyl acetate	0.49	0.46	0.39	0.69	0.39	0.29
hexyl acetate	0.006	0.005	–	–	–	–
phenyl-2-ethanol acetate	0.21	0.37	0.41	0.47	0.41	0.29
Σ	35.73	35.86	32.81	26.18	19.81	28.59
	Fatty Acid Esters (mg L^{-1})					
ethyl butyrate	0.224	0.220	0.163	0.232	0.164	0.124
ethyl isobutyrate	0.006	0.005	0.006	0.005	0.004	0.007
ethyl hexanoate	0.089	0.071	0.23	0.31	0.23	0.085
ethyl octanoate	0.022	0.022	0.060	0.081	0.059	0.020
ethyl decanoate	0.002	0.004	0.019	0.024	0.019	0.004
Σ	0.343	0.322	0.478	0.652	0.446	0.240

[a] Values are the average of two determinations and the coefficient of variation in all the compounds analyzed was less than 10% with the exception of decanoic acid (18%), dodecanoic acid (38%), ethyl octanoate (16%), and ethyl decanoate (29%).

We observed a reduction in the fermentation length regardless of the time of addition and, consequently, a reduction in the total fermentation time. However, the fermentation length decreased even further when nitrogen was added during the exponential phase and yeast cells probably used this nitrogen for biomass production. These results largely agree with those previously reported (*2*−*4*). However, the yeast strain QA23 used in this study seems to have low nitrogen requirements. It used only 147 mg N L^{-1} in the control fermentation and finished it with only 60 mg N L^{-1}. Agenbach (*25*) established that fermentations require a minimal amount of 140 mg N L^{-1} to avoid getting stuck. In fact, nitrogen demands and preferences are strain dependent (*26*−*28*) and, therefore, it should be taken into account that we only used one strain.

As in previous experimental studies (*2, 6*), we observed that nitrogen additions during the period of cell growth resulted in an increase in cell biomass. During the cell growth phase of

the fermentation, most carbon- and nitrogen-containing compounds are diverted to biomass production. When growth stops, however, only small amounts of these nutrients are required, primarily for cell maintenance (*3*).

The metabolism of nitrogen depends heavily on its uptake through the different nitrogen transporters. In this study, we monitored the activity of the genes encoding two important permeases in the transport of amino acids (*GAP1*) and ammonium (*MEP2*) throughout fermentation. In a previous study (*5*), we observed that both permeases were repressed in a nitrogen-rich medium by the mechanism called nitrogen catabolite repression (NCR). The present study confirms this repression because in the control fermentation their expressions were almost negligible and in the limiting nitrogen condition their expressions dropped sharply at the beginning, when nitrogen was still available, and increased continuously when it was not. The NCR of both transporters was fast and effective, as seen with the nitrogen additions, although the cell response to the excess of nitrogen in the medium was quicker when the nitrogen addition was in the first half of fermentation. During the last stages of fermentation, ethanol content is high and it is well established that the first target of ethanol toxicity is the plasma membrane (*29, 30*), which can be impaired for a long period of anaerobic growth. Therefore, the sensing system of the cell, mainly located in the plasma membrane, may be affected by both effects (*31*).

The moment of the fermentation process at which the NCR was established (by the nitrogen addition) determined the pattern of amino acid consumption. As previously reported (*9*), arginine, alanine, aspartate, glutamate, and glycine were the amino acids that were most affected by the NCR because they were hardly consumed when there was an excess of nitrogen. In fact, they were not taken up until the medium was depleted of good nitrogen sources. These amino acids must be transported mainly by the general amino acid permease (Gap1p) or by other specific permeases also subjected to NCR. A similar uptake pattern for these amino acids was previously reported in both synthetic and natural grape juices (*1, 26*). On the other hand, in brewing conditions (*32*) arginine and glutamine were rapidly consumed whereas ammonium uptake was delayed. Branched-chain and aromatic amino acids behaved in a completely different way. Except for tryptophan, they were mostly consumed in the first stages of the control fermentation: that is, when the cells were subjected to NCR from the beginning of the fermentation process. A common feature of the genes that encode the permeases of the branched-chain amino acids (*BAP1* and *BAP2*) and aromatic amino acids (*TAT1* and *TAT2*) is that they are induced in a nitrogen-rich medium (*33, 34*).

Regardless of the time of addition, glutamine and tryptophan were the main amino acids consumed after the nitrogen additions, and therefore, they may be very important for the yeast cell metabolism throughout the process.

Ammonium accounted for 40% of the total YAN of the fermentation media. However, its consumption depended on the timing of the addition. Ammonium is the preferred nitrogen source for biomass production but was hardly consumed when it was added in the final stages of the fermentation. These differences in ammonium uptake are difficult to explain in terms of permease regulation. In the present study and in our previous one (*9*), we detected that, the more nitrogen there was in the fermentation media, the more repressed the three MEP genes were. Marini et al. (*35*) have proposed two possible hypotheses to explain this paradox: either the yeast possesses additional ammonium transport systems unrelated to the Mep proteins, or highly concentrated ammonium is taken up into the cells by simple diffusion.

The timing of the nitrogen additions directly determined the likely aroma characteristics of the wines. Glycerol increased in the fermentations with higher biomass production and higher ammonium consumption. The relationship between biomass formation and glycerol synthesis has already been reported (*36*−*38*). Likewise, a higher glycerol yield was also observed on a synthetic glucose-rich medium when ammonium was used as the sole nitrogen source instead of a mixture of ammonium and amino acids (*39*). Michnick et al. (*40*) also related the production of glycerol to the accumulation of acetate and acetaldehyde.

Higher alcohols were also affected by the changes in nitrogen utilization. These compounds can be produced either by the catabolic conversion of the branched-chain amino acids (via Ehrlich) or by the anabolic formation of these amino acids de novo from a sugar substrate (*14, 15*). Our results show that the anabolic route is of greater importance because the increase in isoamyl alcohol and 2-phenyl ethanol was inversely proportional to the consumption of leucine and phenylalanine, respectively. Furthermore, the closer the nitrogen concentration is to the growth-limiting level, the higher the yield of fusel alcohols is. There is also an inverse correlation between ammonium consumption and the production of fusel alcohols (*12*). A greater concentration of higher alcohols did not seem to determine an increase in esters. In contrast, the acetate concentration seemed to determine a greater concentration of acetate esters, especially ethyl acetate.

In conclusion, our study shows the quantity and quality of the nitrogen demands of the wine strain QA23. Although further studies should be carried out with other wine strains, our data show that cell growth and fermentation have different preferred nitrogen sources. Nitrogen additions always improved fermentation performance but had a minimal effect on biomass production when added in the second half of the fermentation. These nitrogen additions subjected the cells to NCR and changed the profile of nitrogen consumption. The differences in the pattern of nitrogen consumption were related to different aroma compound compositions in the wines. In our opinion, this study is a starting point for further investigation into using an ammonium/amino acid mixture as nitrogen supplementation in the wine industry and the effect that these additions have on yeast physiology, fermentation performance, and wine quality.

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