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Effect of Organic Acids and Nitrogen Source on Alcoholic Fermentation: Study of Their Buffering Capacity

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The effect of tartaric acid and other organic acids on alcoholic fermentation was studied. Organic acids in media with high sugar concentrations and ammonium as the sole nitrogen source had an enormous impact on *Saccharomyces cerevisiae* metabolism during alcoholic fermentation. The main effect on yeast metabolism was the quick acidification of the media in the absence of organic acids. All of the organic acids used in this study (tartaric, malic, citric, and succinic acids) showed a buffering capacity, but not all of the acids had the same one. However, the results suggested that buffering should not be considered the only effect of organic acids on yeast metabolism. Nitrogen source also had a great influence on media pH. Ammonium consumption by yeasts produced a greater acidification of the media than when amino acids were used.

KEYWORDS: Tartaric acid; *Saccharomyces cerevisiae*; ammonium; amino acids; pH

INTRODUCTION

Acidity is one of the most important organoleptic parameters in wine and is mainly due to the presence of weak organic acids. Practically all of these acids in wine are already present in the grapes. However, very small quantities of organic acids such as succinic and acetic acids are produced during alcoholic fermentation (*1*). The composition and concentration of each acid is essential for the quality of the final wine (*2*). The concentration of the acids depends on factors such as the nature of the grape must, the microbial activity of the yeast strain, and the enological practices involved in wine-making (*1*).

Tartaric and malic acids are the main acids in grapes (up to 90%) and, therefore, the main cause of wine acidity. Other organic acids (succinic, citric, pyruvic, lactic, gluconic acids, etc.) are in low molar concentrations and contribute little to the titratable acidity and pH of wine. The tartaric/malic acid ratio varies considerably from one variety to another. Whereas tartaric acid concentration remains practically constant during grape ripening, malic acid decreases (*3*). As a result, the grape must of most varieties contains more tartaric than malic acid. Moreover, some *Saccharomyces* strains can consume a small portion of the malic acid initially present in the grape must (*3*). Tartaric acid, on the other hand, and not another less stable acid such as malic or citric acid, is added to must grapes that are deficient in acidity because it is resistant to degradation and metabolization by wine microorganisms. Despite this microbial stability, tartaric acid concentration decreases slowly in wine because it is precipitated as potassium and calcium salts during fermentation.

However, organic acids are not important just because of their organoleptic contribution. They also play an important biotechnological role in industrial fermentations. These acids are in equilibrium with their salts; they act as a buffer and, thus, maintain the pH of wines in the range from 2.9 to 4 (*4*). Actively growing yeasts acidify their medium through a combination of differential ion uptake, proton secretion during nutrient transport, direct secretion of organic acids, and CO_2 evolution (*5*). Therefore, the buffering capacity of grape must is important to prevent alterations in the pH of the medium, which can affect the cytosolic pH of yeast and its metabolism during wine fermentation; that is, ethanol production appears to be particularly sensitive to alterations in the pH of the medium.

Finally, it should be taken into account that many yeasts are able to use certain carboxylic acids for growth. For this reason, an understanding of organic acid transport in yeasts is important not only because yeasts use organic acids as sources of carbon for growth but also because they could control the intracellular pH and contribute to the intracellular charge balance by enhancing K^+ ion uptake (*5*, *6*). In *Saccharomyces cerevisiae*, two different uptake systems for monocarboxylic acids are known: one is shared by acetic, propionic, and formic acids, and the other transports lactic, pyruvic, acetic, and propionic acids (*7*−*9*). On the other hand, no permeases for dicarboxylic acids have been described in *S. cerevisiae*, and these acids are assumed to enter the cell in the undissociated form. Therefore, the undissociated acid traverses the cell membrane and then dissociates in the higher pH environment of the cytosol, causing both a cytoplasmic acidification and intracellular accumulation

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of the membrane-impermeant acid anion (*10*). In other yeast species, however, some dicarboxylic acid permeases have been identified, and they are usually related to malic acid transport. The uptake system is usually a malate−proton symport that normally accepts other dicarboxylic acids such as D-malic, succinic, and fumaric acids but has never been described as accepting tartaric acid. This kind of permease occurred in *Kluyveromyces marxianus* (*11*), *Schizosaccharomyces pombe* (*12*), *Candida utilis* (*13*), and *Hansenula anomala* (*14*). In *H. anomala* (*14*) and *C. utilis* (*15*), a similar permease for citric acid has also been described, but it is not permeable to tartaric acid.

The aim of the present work was to study how the concentration of tartaric acid and other organic acids in wine affects the evolution of the pH of the growth medium and the *Saccharomyces* metabolism during alcoholic fermentation. Because grape musts can vary, we mainly used a defined medium, the sugar concentration of which was similar to that of grape must. As well as ammonia, the compound that accounts for 40% of all available nitrogen in must, we used amino acids as the source of nitrogen. Amino acids contain both a weak acid and a weak base functional group, so they may act as buffers in the same way as organic acids.

MATERIALS AND METHODS

Fermentation Experiments. *Effect of Organic Acids.* The fermentations were carried out in a defined medium that consisted of 100 g L^{-1} glucose, 100 g L^{-1} fructose, and 1.7 g L^{-1} yeast nitrogen base without amino acids and ammonium sulfate (Difco, Detroit, MI). The nitrogen source used was 5 g L^{-1} of ammonium sulfate.

Several organic acids were added to the defined medium to determine the effect they had on fermentation: increasing concentrations of 1, 2.5, 5, 10, and 20 g L^{-1} of tartaric acid were tested; malic, citric, and succinic acid were tested at concentrations of 1 and 5 g L^{-1}.

All fermentations were carried out with an initial pH of 3.5 adjusted with 0.1 N NaOH.

Effect of Medium pH. The effect of the pH on fermentation was studied with and without tartaric acid (5 g L^{-1}). Initial pH values were 2.5, 3.5, 4.5, and 5.5, adjusted with 0.1 N NaOH or 0.1 N HCl.

Effect of Nitrogen Source. The effect of the nitrogen source, with and without tartaric acid (5 g L^{-1}), was analyzed under the following conditions:

(a) 5 g L^{-1} of Ammonium Sulfate. The total assimilable nitrogen [TAN; it includes α-amino acids (except proline) and ammonia] analyzed by the formol index method (*16*) was 1 g L^{-1}.

(b) 0.5 g L^{-1} of Ammonium Sulfate. TAN was 0.1 g L^{-1}.

(c) A solution (1.53 g L^{-1}) containing the following amino acids was used: 0.4 g L^{-1} of arginine and proline; 0.15 g L^{-1} of glutamic acid; 0.1 g L^{-1} of glutamine, threonine, and serine; 0.05 g L^{-1} of alanine; 0.04 g L^{-1} of aspartic acid and leucine; 0.02 g L^{-1} of lysine, phenylalanine, histidine, isoleucine, valine, glycine, and tyrosine; 0.005 g L^{-1} of tryptophan and methionine. The TAN in the fermentor was 0.1 g L^{-1}.

(d) 1.53 g L^{-1} of Amino Acids and 4.5 g L^{-1} of Ammonium Sulfate. TAN was 1 g L^{-1}.

All of these experiments were carried out with an initial pH of 3.5 adjusted with 0.1 N NaOH.

The fermentations were performed in 500-mL bottles filled with 450 mL of the defined medium and covered with a cotton cap. A commercial *S. cerevisiae* wine strain QA23 (Lallemand S.A., Toulouse, France) was inoculated at the initial population of 2×10^6 cells mL^{-1}. All fermentations were carried out at 25 °C.

Every day the CO_2 released was assessed by measuring the weight loss. In the latter stages of the fermentation, the sugar consumption was assayed by enzymatic kits (Boehringer Mannheim, Germany). Fermentation was considered to be over when the residual sugars were below 2 g L^{-1}. The pH was monitored throughout the fermentation with a Crison micro pH-meter (Crison Instruments, S.A., Barcelona, Spain). Consumption of ammonium was also assayed by enzymatic kits (Boehringer).

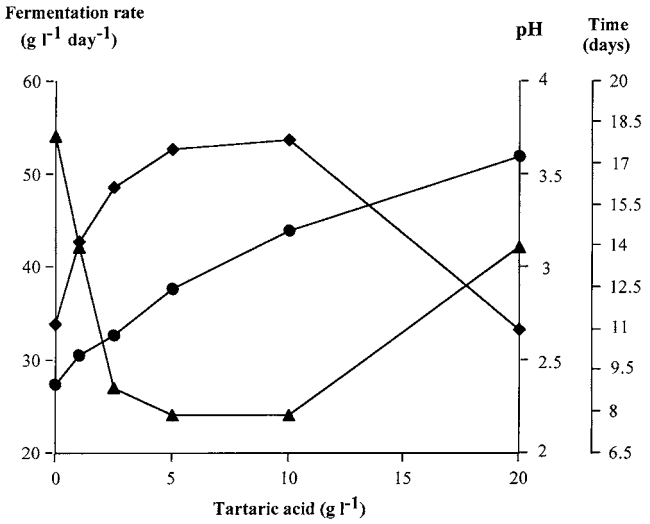

Figure 1. Changes in maximal fermentation rate (◆), final fermentation day (▲), and final pH (●) as a function of tartaric acid concentration.

Maximal fermentation rate was the maximal slope obtained from the plot of sugar consumption versus fermentation day and was expressed as the concentration of sugar consumed (in grams per liter) per day.

Fermentations with Grape Must. The medium was prepared from concentrated white must (Concentrados Palleja, Riudoms, Spain), which was diluted with water to obtain a final sugar concentration of 220 g L^{-1} (1:4). The titratable acidity in must was 2.1 g L^{-1} (expressed as tartaric acid), and the main acids were tartaric (1.47 g L^{-1}), malic (0.95 g L^{-1}), and citric (0.34 g L^{-1}) acids. Fermentations were performed in 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, but excluded atmospheric oxygen. The inoculated population of yeast was 2×10^6 cells mL^{-1}.

The fermentations were incubated at 25 °C without shaking.

Intracellular Metabolites. Membrane cells were permeabilized and the intracellular metabolites extracted using boiling buffered ethanol, as described by Gonzalez et al. (*17*). Analytical HPLC was carried out on a Hewlett-Packard HP 1050 connected to a Hewlett-Packard Integrator 3395A equipped with an HP 1047 RI detector (Agilent Technologies, Wilmington, DE). The extract (25 μL) was injected into a 300 × 7.8 mm Aminex HPX-87H column (Bio-Rad, Hercules, CA). The solvent used was 2.5 mM H_2SO_4 at 0.5 mL min^{-1}. The analysis temperature was 60 °C. The concentration of each metabolite was calculated using external standards and expressed as milligrams per gram of dry cell weight. Dry cell weight determination was performed as previously described by Sierkstra et al. (*18*).

RESULTS

Effect of Tartaric Acid Concentration on Alcoholic Fermentation and Changes in pH. The yeast fermentation rate depends on various physical, chemical, and biological factors in the environment such as the temperature, pH, and nutritional compounds. A defined medium with different concentrations of tartaric acid (0, 1, 2.5, 5, 10, and 20 g L^{-1}) was inoculated with a commercial *S. cerevisiae* wine strain. Fermentations were carried out at 25 °C, and the initial pH was adjusted to 3.5. Sugar consumption was monitored daily. Tartaric acid concentrations of 5 and 10 g L^{-1} gave the highest fermentation rate and the shortest fermentation time (**Figure 1**). Below and above these optimal concentrations, the maximal rate decreased. Fermentation halted after 18 days when no tartaric acid was present in the medium, leaving 32 g L^{-1} of unfermented sugars. On the other hand, the start of sugar consumption was delayed

Table 1. Kinetic Parameters and Final pH of Fermentations with Different Initial pH Values in the Absence or Presence of Tartaric Acid

pH		day of FF[a]	MFR[b]	$[sugar]_f$	final pH
2.5	control	21[c]	25.91	50.61	2.29
	5 g L^{-1} TA[d]	28	31.16	1.95	2.54
3.5	control	21[c]	30.20	19.86	2.39
	5 g L^{-1} TA	9	45.19	0.95	3.04
4.5	control	21[c]	31.90	20.17	2.45
	5 g L^{-1} TA	7	54.21	1.9	3.62
5.5	control	21[c]	33.61	12.52	2.48
	5 g L^{-1} TA	9	51.97	1.03	3.77

[a] Final fermentation. [b] Maximal fermentation rate. [c] Suspended fermentation. [d] Tartaric acid.

in the medium that had the highest tartaric acid concentration (20 g L^{-1}), but fermentation was completed (data not shown). The buffering capacity of the tartaric acid was tested by measuring the final fermentation pH. The initial pH decrease was inversely proportional to the concentration of tartaric acid in the medium. When there was no acid in the medium, the final pH was minimum (~2.4), whereas the final pH of the medium with the highest concentration of acid (20 g L^{-1}) had the same initial value (3.5) (**Figure 1**).

The effect of different initial pH values was analyzed in fermentations with (5 g L^{-1}) or without tartaric acid. The most striking result was that none of the controls (fermentations without tartaric acid) were able to ferment all sugars (**Table 1**). However, the addition of tartaric acid was enough for the yeast to complete all of the fermentations whatever the initial pH. Fermentations with tartaric acid at pH values of 3.5, 4.5, and 5.5 lasted about the same time, whereas the fermentation at pH 2.5 took longer and had a lower rate. All of the control fermentations (without acid) had similar final pH values (ranging from 2.2 to 2.5) regardless of the initial pH. Fermentation with acid at pH 2.5 also had a similar final value (2.5) but, as mentioned above, sugars were consumed.

The evolution of the pH during the above fermentations was also monitored during the process (**Figure 2A**). The minimum was reached after 2 days of fermentation and remained unchanged. Moreover, to check the initial pH changes, it was also monitored during the first hours of fermentations with and without tartaric acid in the media (**Figure 2B**). The acidification of the medium without acid started in the first hours of fermentation, and the minimal pH was almost reached after 24 h.

Analysis of the Intracellular Concentration of Organic Acids. The intracellular concentration of tartaric acid was studied in the fermentations with different concentrations of tartaric acid in the media. Tartaric acid accumulated inside the cells throughout the fermentations and reached maximal concentration at the end of the process (**Figure 3**). There was a good relationship between the extracellular and intracellular concentrations of the acid with the exception of the medium with 20 g L^{-1}, the cells of which accumulated a large amount of tartaric acid at the end of the process. The intracellular tartaric acid concentration was also determined in fermentations at different pH values and was found to be similar regardless of the initial pH (data not shown).

A fermentation with grape must was carried out, and the intracellular organic acids were quantified to analyze this accumulation in vinification conditions (**Figure 4**). A similar intracellular concentration of the total organic acids was detected in all of the samples analyzed (~8−13 mg g^{-1} of dry cell weight). Malic, succinic, and lactic acids were the main organic

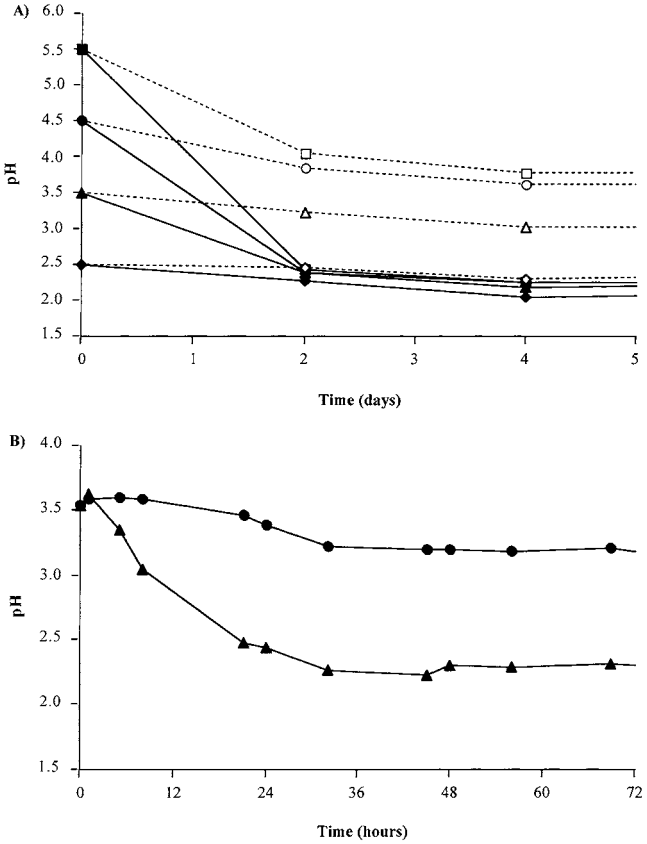

Figure 2. Evolution of pH throughout the fermentation: (A) evolution of pH starting from different initial pH values in fermentations with (dotted line and open symbol) and without tartaric acid (solid line and solid symbol) [initial pH: 2.5 (◇), 3.5 (△), 4.5 (○), and 5.5 (□)]; (B) evolution of pH in a fermentation with (●) and without (▲) tartaric acid in the first hours of the process.

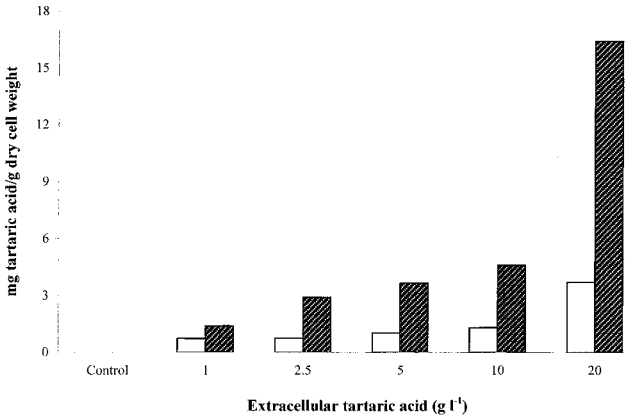

Figure 3. Intracellular concentration of tartaric acid during fermentations with different concentrations of tartaric acid in the media: (white bars) midfermentation (MF); (shaded bars) final fermentation (FF).

acids in dry yeast. Citric acid was the main intracellular acid in the middle stage of fermentation. However, tartaric acid, which was almost undetectable until midfermentation, was clearly accumulated in the latter phases of the process.

Effect of Other Wine Organic Acids on Alcoholic Fermentation and pH Evolution. Other wine organic acids were also tested in fermentations with the defined medium because they appeared to be important in fermentations performed with grape must. Malic and succinic acids were used as dicarboxylic

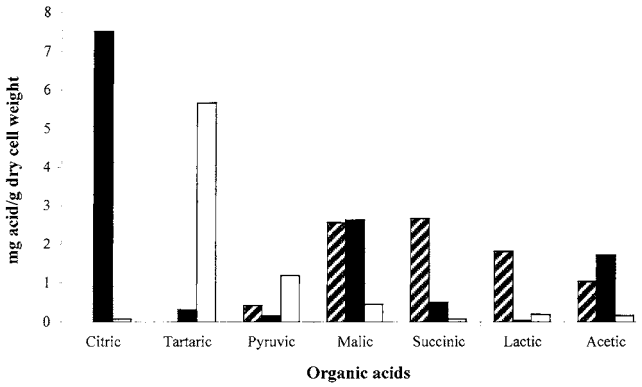

Figure 4. Intracellular concentration of the main organic acids throughout an alcoholic fermentation of grape must at 25 °C: (shaded bars) dry yeast; (black bars) midfermentation (MF); (white bars) final fermentation (FF).

Table 2. Kinetic Parameters and Final pH of Fermentations with the Addition of Organic Acids at Different Concentrations

acid	[acid], g L^{-1}	day of FF[a]	MFR[b]	final pH
control		18[c]	33.93	2.55
tartaric	1	14	42.77	2.60
malic		14	40.79	2.54
citric		22	35.29	2.58
succinic		22[c]	35.41	2.48
tartaric	5	9	52.63	3.15
malic		10	52.17	3.01
citric		9	43.90	2.98
succinic		9	44.06	2.85
tartaric + malic	1 + 1	12	45.33	2.67
	5 + 5	10	49.16	3.32
tartaric + citric	5 + 0.5	9	51.37	3.20

[a] Final fermentation. [b] Maximal fermentation rate. [c] Suspended fermentation.

acids and citric acid was used as the tricarboxylic acid at different concentrations (**Table 2**).

At low concentrations (1 g L^{-1}), media with tartaric or malic acid had the highest fermentation rate and the shortest fermentation. With citric acid, the end of fermentation was delayed but all sugars were exhausted, whereas fermentation halted when succinic acid was used. It should be taken into account that although the concentrations (in grams per liter) were equal for all of the acids studied, the molarities were obviously different. Problems in fermentation with succinic acid, however, cannot be explained in this way because the fermentation with succinic acid was the one with the highest molarity. The pH of these fermentations (**Figure 5A**) evolved in a similar way to the control fermentation with differences of <0.2 unit. Anyway, the pH of control and succinic acid fermentations decreased the most.

All of the fermentations finished in similar times when 5 g L^{-1} of acid was used in the media (**Table 2**). Fermentations with tartaric or malic acids again gave the highest maximal fermentation rates. In these conditions, there were clear differences in the pH changes (**Figure 5B**). The pH of all fermentations with an organic acid in the medium was clearly higher than that of the control. However, there were also considerable differences among the various acids tested. The minimal and maximal decreases in pH were for tartaric and succinic acids, respectively.

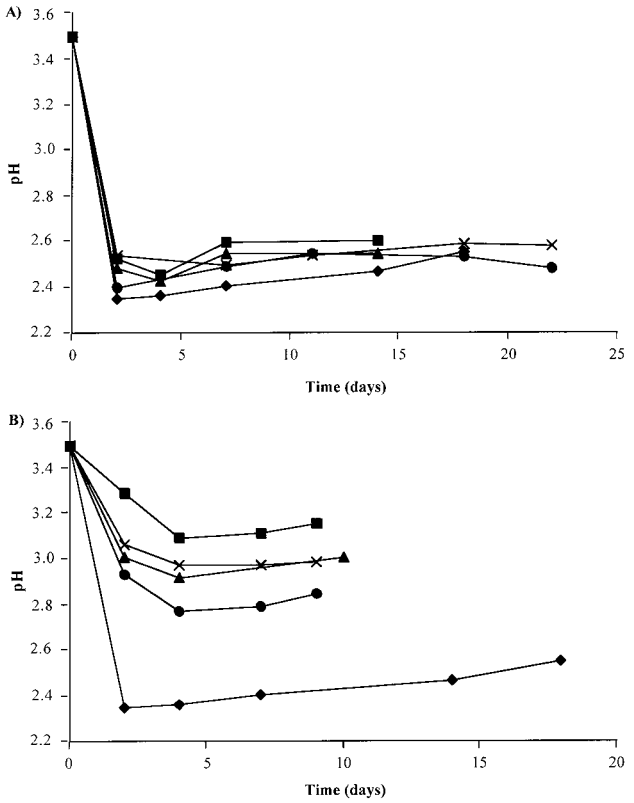

Figure 5. Evolution of pH in fermentations with different organic acids at (A) 1 g L^{-1} of acid and (B) 5 g L^{-1} of acid: (◆) control; (■) tartaric acid; (▲) malic acid; (×) citric acid; (●) succinic acid.

Table 3. Kinetic Parameters and Final pH of Fermentations with Different Nitrogen Sources in the Absence or Presence of Tartaric Acid

N source	TAN,[a] g L^{-1}		day of FF[b]	MFR[c]	final pH
NH_4^+	1	control	18[d]	33.93	2.38
		5 g L^{-1} TA[e]	9	52.63	3.15
	0.1	control	22[d]	25.81	2.31
		5 g L^{-1} TA	15	33.51	3.28
amino acids	0.1	control	10	38.87	2.73
		5 g L^{-1} TA	11	45.15	3.52
amino acids + NH_4^+	1	control	14	36.69	2.46
		5 g L^{-1} TA	11	38.61	3.23

[a] Total assimilable nitrogen. [b] Final fermentation. [c] Maximal fermentation rate. [d] Suspended fermentation. [e] Tartaric acid.

Combinations of acids affected neither the development of the fermentation nor the buffering capacity (in comparison with the same concentration of tartaric acid only) (**Table 2**).

Effect of Nitrogen Source on Alcoholic Fermentation and pH Evolution. Up to now all fermentations have been carried out with ammonia as the sole nitrogen source, but amino acids are also important sources of nitrogen for yeast during wine fermentations. **Table 3** shows how alcoholic fermentation and pH are affected when amino acids are the only nitrogen source or when they are mixed with ammonia. Ammonia and amino acid concentrations are expressed as total assimilable nitrogen.

When tartaric acid was not present in the media, changes in the ammonia concentration were not enough to prevent suspended fermentations. All of the ammonia was consumed in the first 2 days of fermentation when 0.1 g L^{-1} of assimilable nitrogen was present. However, a large amount of ammonia

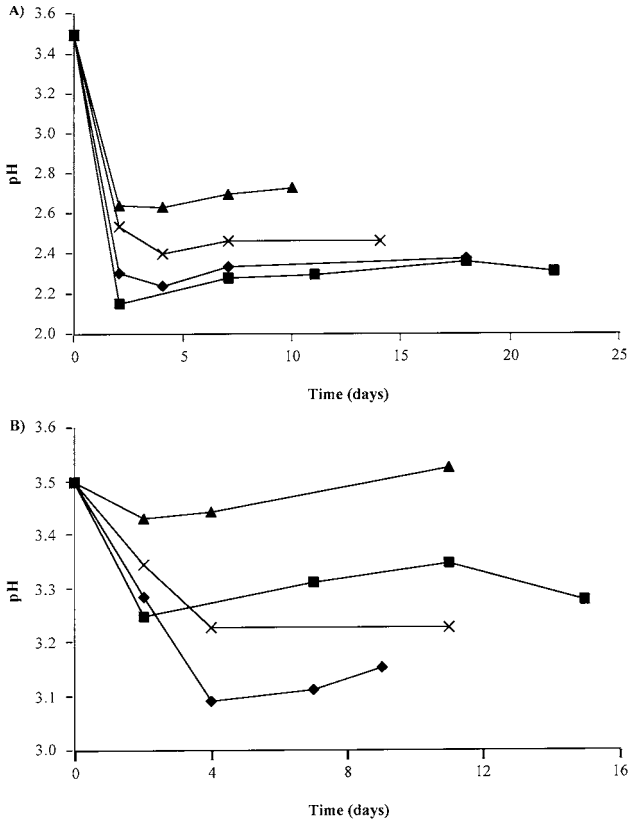

Figure 6. Evolution of pH in fermentations with different nitrogen sources (A) without and (B) with tartaric acid (5 g L^{-1}): (◆) 5 g L^{-1} of ammonium sulfate (1 g L^{-1} TAN); (■) 0.5 g L^{-1} of ammonium sulfate (0.1 g L^{-1} TAN); (▲) 1.53 g L^{-1} of amino acids (0.1 g L^{-1} TAN); (×) 1.53 g L^{-1} of amino acids + 4.5 g L^{-1} of ammonium sulfate (1 g L^{-1} TAN).

remained in the medium when 1 g L^{-1} was used (data not shown), but the fermentation ended when this minimal concentration of assimilable nitrogen (0.1 g L^{-1}) consisted of amino acids. Oddly enough, fermentation with a mixture of ammonium and amino acids was slower than when only amino acids were used (**Table 3**).

The pH decreased the least when amino acids were the sole nitrogen source and decreased the most when fermentations had only ammonium (**Figure 6A**). Surprisingly, the medium with ammonium and amino acids reached a pH value lower than that of the medium with only amino acids. As expected, pH values did not change as much when these fermentations were carried out with tartaric acid (**Figure 6B**). The pH values changed only very slightly in the fermentation with amino acid and tartaric acid, but the same medium plus ammonium registered a considerable pH decrease. Acidification was maximal in the medium with 1 g L^{-1} of assimilable nitrogen as ammonium.

DISCUSSION

Tartaric and malic acids represent an average of 90% of the total acids present in grapes. The grape is the only cultivated fruit of European origin that accumulates significant quantities of tartaric acid (*3*), and it is absent in other fermented foods. Titratable acidity plays an important role in the stability, color, taste, and aroma of wine but, at the same time, is involved in the buffering capacity of wine. Our goal was to study the effect of tartaric acid and other wine organic acids on alcoholic fermentation, that is to say, on yeast metabolism. The plot of the maximal fermentation rate versus tartaric acid concentration showed a bell-shaped curve that is similar to the influence of other physicochemical factors on microbial growth. The optimal tartaric acid concentration was between 5 and 10 g L^{-1}. Concentrations below or above this optimal range caused slower fermentations and fermentation halted when no tartaric acid was present in the initial medium. Organic acids should not be a problem in wine industrial fermentations because their concentration in grape musts is usually in the optimal range. However, sluggish and suspended fermentations have been associated with vintages of high ripeness. It has been claimed that the high sugar and the low nitrogen contents of these musts are responsible for these fermentations. However, low acidity is another feature of the musts from overripe grapes. The titratable acidity of these musts is usually lower than the optimal values presented in this study and, thus, they can contribute to the appearance of problematic fermentations.

In our hands, tartaric acid helped to prevent sluggish or suspended fermentations because of its buffering capacity and because it kept the pH within the optimal values for yeast development. When this acid was not present, fermentations had the lowest final pH values. However, this does not appear to be quite so clear when we analyze the fermentations with different initial pH values. Regardless of the initial pH, the pH of all the control fermentations decreased to values of ~2.2−2.5 and sugar was not completely consumed. However, when the initial medium was adjusted to this low pH (2.5), the presence of tartaric acid was enough to complete the fermentation successfully, and the differences in final pH between this fermentation and control ones were not very important. Therefore, the differences between fermentation with and without tartaric acid could not be due only to the effect of the pH and, somehow, the acid enables yeasts to complete fermentations. Carmelo et al. (*19*) reported that the ability of the cells to grow or maintain viability at high external hydrogen ion concentration reflected their capacity to maintain control over their intracellular pH by excluding protons. However, the same authors proved that the rapid activation of *S. cerevisiae* plasma membrana H^+-ATPase, which is associated with internal pH acidification, was not in fact caused by the low external pH itself but was induced by the weak organic acid used as the acidulant.

Tartaric acid is mostly present as undissociated acid (~75% of the total concentration) at a pH of 2.5 (pK_a values = 3.01 and 4.37). This protonated form of the acid is freely permeable to the cell membrane and then dissociates in the higher pH environment of the cytosol, causing both a cytoplasmic acidification and intracellular accumulation of the membrane-impermeant acid anion (*10*). Analysis of intracellular metabolites showed that tartaric acid entered and accumulated during the fermentation. As far as we know, tartaric acid is not metabolized by *S. cerevisiae*, and the advantage for this yeast remains elusive. As mentioned above, passive diffusion might account for the uptake of undissociated organic acids because no permeases are known for dicarboxylic acids in *S. cerevisiae*. In fact, no permease has been identified that accepts tartaric acid in any yeast. Nevertheless, it is striking that the intracellular concentration of tartaric acid was the same despite the differences in the extracellular pH, which were >1.5 units. These pH changes also involved important differences in the percentage of the undissociated form (only 10% at pH 4). A higher proportion of the undissociated acid could mean that the intracellular concentration of tartaric acid should be higher unless tartaric acid transport is regulated. Thus, our results suggest that there is a regulated system for tartaric acid transport.

Other wine organic acids were tested in conditions that were similar to those of tartaric acid, and the same conclusions could

be reached. All of the organic acids showed buffering capacity, but not all of the acids had the same capacity. Tartaric and succinic acids had the highest and lowest buffering capacities, which confirmed the results of Dartiguenave et al. (*4*). In fact, the only fermentation to become suspended was in the medium with 1 g L^{-1} of succinic acid (control fermentation not included). This may be because succinic acid was mainly in the undissociated form (~83%) at a wine pH of 3.5. Thus, its buffering capacity was lower than that of other acids such as tartaric, which was mainly in dissociated form. However, tartaric acid is a good buffer only at some pH values. Therefore, because of its pK_a, tartaric acid cannot maintain pH values as high as 5.5 or 4.5. In fact, tartaric acid could not prevent a decrease of almost 2 units in fermentations at an initial pH of 5.5, although the fermentation finished without problems. This might suggest that the pH must decrease to a range more critical for the yeast to endanger the process (below pH 3).

The medium acidified rapidly in the first hours of fermentation when ammonium was used as the sole nitrogen source (and no organic acid was present). Ammonium can be concentrated ~200-fold by cells, and its transport produced an increased rate of H^+ extrusion and, consequently, an acidification of the external media (*20*). On the other hand, the decrease in pH was not so sharp when a mixture of amino acids was used. Amino acids can also buffer the medium. However, the decrease in the pH of the medium that contained both nitrogen sources (amino acids and ammonium) was similar to when ammonium was the only nitrogen source. Therefore, amino acids did not buffer at the pH of wine, as Dartiguenave et al. previously reported (*21*), but their consumption produced a lower acidification of the medium than when ammonium was used. The differences between the two nitrogen sources are mainly due to how they are transported across the cell membrane. Ammonium crosses this barrier by a uniport system coupled to the extrusion of a proton by the ATPase pump (*22*). The entry of the ammonium to the cell causes its dissociation in the higher pH environment of the cytosol. To prevent this, the proton produced is excreted to the medium, which acidifies the extracellular medium. Amino acids, on the other hand, cross the membrane using a symport with one or more protons. This system is also usually coupled with an ATPase pump (*22*), but in this case, the protons are extruded to compensate for their entry from the extracellular medium; therefore, acidification outside the membrane is reduced. Ammonium salts are often added in industrial wine fermentation to increase the nitrogen content of the must. In light of our results, the effect of this practice on the final pH of the wine and fermentation rate should be tested.

In conclusion, organic acids in media with high sugar concentrations and ammonium as the sole nitrogen source had an enormous impact on *S. cerevisiae* metabolism during alcoholic fermentation. When there were no organic acids present, the main effect on yeast metabolism was that the media acidified quickly. However, the role of these organic acids inside the cell remains unclear. Jennings (*6*) reported that the transport of organic acids is important to regulate the intracellular pH and balance the intracellular charge. The constant intracellular concentration of organic acids throughout a grape must fermentation supported this hypothesis. Ammonium transport increased the rate of H^+ extrusion and therefore produced an intracellular alkalanization (*20*). The entry of organic acid may supply the cell with H^+ to prevent alkalinization and enable the transport of ammonium coupled with protons. However, it is still not known how organic acids are transported. As mentioned above, our results suggested a regulated transport, which implies that there are specific permeases for these acids. These transporters may regulate the entry of the acid to the cell or the anion extrusion from the cytoplasm so that dicarboxylic acids do not accumulate, as suggested previously for monocarboxylic acids (*23*). In *S. cerevisiae*, the Dal family of proteins, whose function is still unknown, are thought to be related to dicarboxylic acid permeases, because they presented similarities with tartrate permeases of bacteria (*24*). Anyway, the intracellular effect of organic acids on the growth and performance of yeast during fermentation is still far from being fully understood.

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