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L-(−)-Malic Acid Production by *Saccharomyces* spp. during the Alcoholic Fermentation of Wine (1)

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In an attempt to increase the acidity of wine by biological means, malate-producing yeasts were selected from a collection of 282 strains isolated in different parts of Spain. Only 4% of these strains (all of which belonged to *Saccharomyces cerevisiae*) produced L-(−)-malic acid in the range of 0.5−1 g/L. This was formed between days 2 and 6 of alcoholic fermentation, reaching a maximum on days 3 and 4; the concentration remained stable from day 7. Malic acid production was favored by temperatures in the 18−25 °C range and by musts with a high pH and low concentrations of sugar, initial malic acid, and yeast-assimilable nitrogen. Oxaloacetic acid, a precursor of malic acid, had no influence on malate production. The precursors pyruvic and fumaric acid did, however, have a significant effect on the production of this acid in some strains. No direct relation between pyruvate and malate metabolism was observed.

KEYWORDS: Wine acidity; biological acidification; malic acid; alcoholic fermentation; *Saccharomyces cerevisiae*; precursors; physicochemical variables

INTRODUCTION

The acidity of a wine has a strong impact on its final quality by influencing its taste, microbiological stability, color, and whether or not malolactic fermentation takes place. Acidity is usually higher in the wines of colder regions and lower in those of warm regions. In wines showing reduced acidity, tartaric acid is commonly used as an acidulating agent since it is biologically stable and efficiently lowers the pH. However, it is expensive, and in some regions, its use is legally limited or even forbidden. An alternative is the biological acidification of the must, a process recently authorized by the Organization Internationale de la Vigne et du Vin (OIV) (*1*). This involves using yeast strains that produce D- or L-malic acid, D- or L-lactic acid, or succinic acid during alcoholic fermentation. Even though acid-forming yeasts are not all that common (*2*), strains have been isolated that produce pyruvic acid (*3*, *4*), malic acid (*4*−*10*), lactic acid (*2*, *4*, *11*, *12*), and succinic acid (*4*, *13*, *14*). The production of these acids depends largely on environmental conditions (*15*).

In grapes, malic acid is second to tartaric acid in terms of quantity. Synthesized in the chlorophyll-containing tissues, it is partly catabolized during ripening. Its concentration in the fruit depends on many factors including the microclimate (*16*, *17*), the duration of hot periods, plant vigor (*18*), the degree of insolation (*17*), and grape variety (*17*, *18*). In the grape must, its concentration ranges from 1 to 10 g/L, sometimes reaching 15 g/L in cold regions during exceptionally cool summers (*16*). During alcoholic fermentation, yeast metabolizes between 3 and 35% of the initial malic acid concentration of the must (*19*). However, some strains actually increase malic acid levels (*4*−*7*, *9*, *10*, *15*, *20*−*22*). These mainly produce the L-(−)-isomer, although traces of D-(+)-malic acid may also be made (*23*).

During alcoholic fermentation, L-(−)-malic acid is produced by yeasts via the fumarate pathway catalyzed by cytosolic or mitochondrial fumarase (*24*) or via oxaloacetic acid catalyzed by Malate dehydrogenase (MDH) (*10*, *24*). L-Malic acid acumulated by yeast cells is synthesized in the cytosol via oxaloacetic acid and not via fumaric acid (*24*). CO_2 is fixed by pyruvate to render oxalacetate, which is then reduced to malate. The enzyme pyruvate carboxylase, found in the yeast cytosol and which requires biotine as a cofactor, mediates this first reaction. MDH catalyzes the reduction of oxalacetate to malate (*10*). The biological purpose of malic acid release by yeasts remains unknown.

This paper describes the production of malic acid during alcoholic fermentation by different yeasts under different environmental conditions in an attempt to identify strains that might be of use in biological acidification.

MATERIALS AND METHODS

Isolation, Fermentation, and Selection of Strains. Malic acid production was investigated in 282 yeast strains isolated in different parts of Spain: 95 came from Madrid (central Spain), 105 came from Cordoba (southern Spain), and 45 came from La Rioja (northern Spain). The remaining 37 belonged to the collection of the Department of Food

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Table 1. Enological Properties of the Selected Malic Acid-Producing Strains

strain	C1-4	C2-9	C4-2	C9-10	M10-10	M10-7	M2-6	M2-9	M6-5	M6-7
origin[a]	C	C	C	C	M	M	M	M	M	M
malic acid (g/L)	0.53	0.49	0.48	0.48	0.63	0.63	0.59	0.56	0.59	0.63
volatile acidity (g/L acetic acid)	0.24	0.31	0.29	0.37	0.27	0.27	0.23	0.28	0.23	0.23
ethanol (% v/v)	11.3	11.2	11.1	11.3	11.1	11.1	11.1	11.2	11.1	11.1
H_2S[b]	mp	wp	np	np	mp	mp	np	wp	wp	wp
glycerol (g/L)	5.12	6.50	6.09	4.70	5.83	6.25	5.79	4.95	5.50	5.59

[a] M, Madrid; C, Cordoba. [b] np, nonproducer; wp, weak producer; and mp, medium producer.

Sciences of the Escuela Técnica Superior de Ingenieros Agrónomos-Madrid (ETSIAM). All of these strains were isolated in nonfermenting grape juice and at the beginning, middle, and end of alcoholic fermentation.

Dried yeasts were first multiplied on YPD medium (1% w/v yeast extraxt, 1% w/v peptone, and 2% w/v dextrose) solidified with 2% (w/v) agar. The growth medium was a mixed white grape must obtained from the *Vitis vinifera* varieties Gewürztraminer, Pedro Ximenez, Semillon, and Riesling. All of this must—which contained 194 g/L sugar, 115 mg/L yeast-assimilable nitrogen (YAN), and 0.6 g/L malic acid and had a pH of 3.4—was stored frozen until use.

Initial fermentations were conducted at 25 °C in 100 mL Erlenmeyer flasks containing 50 mL of the same grape must. Malic acid production did not change whether sterilized (autoclaved or filtred through 0.45 μm sieves) or nontreated must was used (*25*). Therefore, for practical reasons, all of the must was autoclaved for 15 min at 100 °C prior to inoculation (final cell count of 2×10^6 UFC/mL). These flasks were plugged with a Müller valve that allowed the CO_2 produced to pass through concentrated sulfuric acid without letting in O_2. The Erlenmeyer flasks were weighed daily until their masses remained stationary, signaling the end of alcoholic fermentation. The medium was then filtered through a 0.45 μm pore size cellulose membrane (Millipore), and the malic acid content was determined.

Changes in the concetrations of malic and pyruvic acids during alcoholic fermentation were followed in 100 mL volumetric flasks (plugged with a rubber stopper) containing 80 mL of must. These flasks were autoclaved and inoculated with yeast as above. Approximately 1 mL of must was extracted daily through the rubber stopper using a sterile syringe and filtered as described above to determine the malic and pyruvic acid concentrations.

Identification of the Best Malic Acid-Producing Strains. The ten strains that produced the most malic acid (C1-4, C2-9, C4-2, C9-10, M10-10, M10-7, M2-6, M2-9, M6-5, and M6-7) were identified according to the procedure of Yarrow (*26*). The fermenting power, the formation of ascospores, and the fermentation and assimilation of carbohydrates were used as taxonomic classification tests. Mitochondrial DNA (mtDNA) restriction analysis was performed to differentiate clones belonging to the same species (*27*); the technique used was that of Querol et al. (*28*). mtDNA was digested with the restriction endonuclease *Hinf*I (Roche), and the fragments were allowed to migrate in a 0.8% (w/v) agarose gel (5 vol per cm). DNA bands were visualized with ethidium bromide under UV light and compared to those of the λ DNA marker digested with *Eco*RI and *Hind*III (Sigma).

Changes in Malic and Pyruvic Acid Concentrations. The change in malic and pyruvic acid concentrations was monitored daily to detect any relationship between them. Enzymatic methods were used to determine the pyruvic (*29*) and malic acid concentrations (Boehringer Mannheim, catalog no. 0139068).

Oenological Aptitude of the Selected Yeasts. At the end of alcoholic fermentation, wine l-(−)-malic acid and glycerol levels were determined using an enzymospectrophotometry kit (Boehringer Mannheim, catalog nos. 0139068 and 148270), the volatile acidity was determined by steam distillation (*30*), H_2S was determined by the lead acetate method (*31*), and ethanol was determined by gravimetrically estimating the loss of CO_2 during fermentation (*32*).

Effect of Physicochemical Factors on Malic Acid Production in the Selected Strains. To reduce the amount of data to be processed, six of these 10 strains were selected (C2-9, C9-10, M10-7, M2-6, M2-9, and M6-5) according to their malic acid-producing ability and to their enological features.

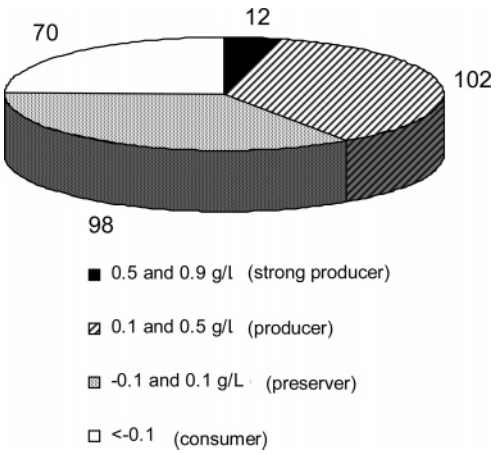

Figure 1. Distribution of the original 282 yeast strains according to their production or consumption of malic acid.

The influence of five physicochemical factors on malic acid production by the six selected strains was studied as follows: sugar concentration (194, 220, and 269 g/L), YAN concentration (115, 208, and 308 mg/L), fermentation temperature (18, 25, and 30 °C), pH (3.37, 4.02, and 4.99), and initial malic acid concentration (0.59, 2.01, and 5.24 g/L). Must pH was adjusted by adding sodium hydroxide. Fermentation tests were carried out in triplicate. The sugar concentration was estimated using a densimeter. YAN was determined by formol titration (*30*).

Effect of Three Malic Acid Precursors on Malate Production by the Six Randomly Selected Strains. The influence of three different concentrations of malic acid precursors on malate production by the six randomly selected strains was investigated as follows: pyruvic acid, original must (no additional pyruvate), must with an extra 75 mg/L pyruvate, and must with an extra 150 mg/L pruvate; oxaloacetic acid, original must (no additional oxaloacetate), must with an extra 5 mg/L oxaloacetate, and must with an extra 10 mg/L; and finally fumaric acid, original must, must with an extra 50 mg/L fumarate, and must with and extra 100 mg/L fumarate.

Statistical Analysis of the Influence of the Physicochemical Factors Studied. An additive model was used to study the influence of the different physicochemical factors (sugar concentration, YAN concentration, temperature, pH, and malic acid concentration). This model allowed an analysis not only of the effect of these factors but also of the interaction between them and the different strains. The model used is represented as follows:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

where y_{ijk} is malic acid production by individual k of strain i at the factor level j; μ represents the general mean production of malic acid; α_i is the fixed effect of the strain where $i = 1, 2, ..., 7$; β_j is the fixed effect of the factor considered (sugar concentration, YAN concentration,

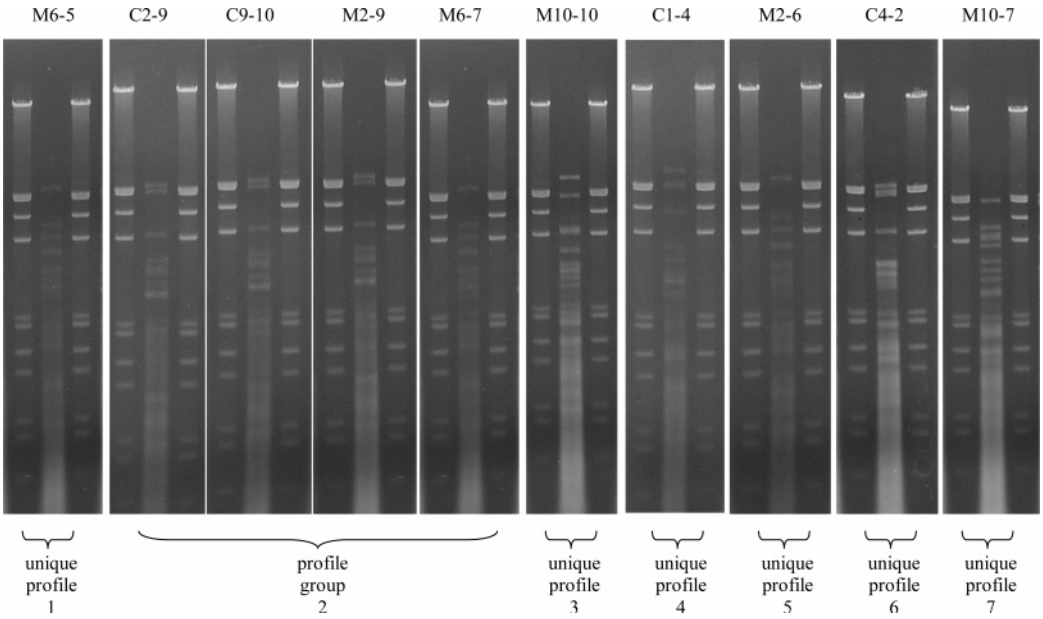

Figure 2. Electrophoretic analysis of restricted mtDNA (*Hinf*I digestion) from the ten best malic acid-producing strains. Border lane: λ DNA marker digested with *Hind*III.

Table 2. Unique Strain Profiles and Profile Groups According to Electrophoretic Analysis of Yeast mtDNA Restricted with *Hinf*I

group/profile	yeast strain
1	M6-5
2	M6-7/M2-9/C9-10/C2-9
3	M10-10
4	C1-4
5	M2-6
6	C4-2
7	M10-7

temperature, pH, or malic acid concentration) at the level j, where $j = 1, ..., 3$; $(\alpha\beta)_{ij}$ is the interaction between the yeast i and the level j of the factor considered; e_{ijk} is the residual term, for which assumptions are made as follows: $e_{ijk} \in N(0,\sigma^2)$ and e_{ijk} and $e_{ijk'}$ are uncorrelated for $k \neq k'$.

The influence of each physicochemical variable was tested at three different levels while maintaining the other variables at standard values. The experimental design was unbalanced, however, since it allowed more observations to be made at the standard levels than at the other two levels. Multiple comparisons of the means were performed for each significant variable using the Tukey−Kramer test (suitable for unbalanced designs) (*33*).

Statistical Analysis of the Effect of Precursors on Malic Acid Production. To compare the influence of the different concentrations of precursors (fumaric, oxalacetic, and pyruvic acids) on malic acid production, one way analyses of variance (ANOVA) were performed for each. Means were compared using the Fisher's LSD test. Significance was set at $p < 0.05$. All calculations were made using SAS software (SAS Institute Inc.).

RESULTS AND DISCUSSION

Consumption or Production of L-(−)-Malic Acid by the Original 282 Strains. The original 282 strains were classified into four categories according to their malic acid-producing or -consuming characteristics: strong producer, producer, preserver, and consumer (**Figure 1**). Most strains from the La Rioja region consumed malic acid, whereas those isolated in Madrid and Cordoba (warmer regions) showed a greater tendency toward either producing or preserving it. These results suggest that a relationship exists between malic acid metabolism and yeast origin.

Table 3. Effect of Physicochemical Variables on Malic Acid Production; Significance Levels

	p value				
variation	sugar	YAN	fermentation temperature	pH	malic acid
strain	0.2491	0.2034	0.3087	<0.0001	<0.0001
factor	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
strain × factor	0.9854	0.9894	0.9709	0.8312	0.2667

Five consumer strains belonging to the *Saccharomyces* (sensu stricto) group (G2-10, M7-3, M4-5, M8-2, and C2-3) were identified that, respectively, consumed 34, 38, 42, 59, and 99% of the malic acid in the must. Under the same conditions, *Schizosaccharomyces pombe*, a species widely used to degrade nearly all malic acid in wines from colder regions where malate acidity is high, metabolized 73% of the initial malate. However, *S. pombe* is a poor competitor with other species of *Saccharomyces* (*16*) and produces undesirable off-flavors (*16*, *19*, *34*). The use of *Saccharomyces cerevisiae* consumer strains instead of *S. pombe* for reducing acidity might improve a wine's fermentation kinetics and organoleptic profile.

Oenological Aptitude, Identification, and Differentiation of the Selected Malic Acid-Producing Strains. Ten malic acid-producing strains with good enological characteristics were selected (**Table 1**). According to Yarrow's classification (*26*), all belonged to *S. cerevisiae*. The profiles obtained by the restriction of their mtDNA with *Hinf*I (**Figure 2**) allowed these strains to be differentiated into one profile group and six unique profiles (**Table 2**).

Effect of the Five Physicochemical Variables Studied on Malic Acid Production. All five variables had a significant effect on malic acid production; however, no significant interactions were found between the strains and any of the factors considered (**Table 3**). The Tukey−Kramer adjustment allowed multiple comparisons of mean malic acid production at all levels. Significant differences were always found between standard and nonstandard levels for all of the physicochemical variables studied. Nevertheless, the unbalanced design of the experiment allowed no significant differences to be detected between nonstandard levels. **Tables 4−9** show the least-squares

Table 4. Effect of Strain Identity on Malic Acid Production

		p value					
estimated mean malate production (g/L)	strain	C2-9	C9-10	M10-7	M2-6	M2-9	M6-5
0.39	C2-9	×	0.98	0.17	0.66	0.00	0.83
0.34	C9-10	0.98	×	0.03	0.27	0.00	0.42
0.56	M10-7	0.17	0.03	×	0.97	0.09	0.84
0.50	M2-6	0.66	0.27	0.97	×	0.01	1.00
0.76	M2-9	0.00	0.00	0.09	0.01	×	0.00
0.47	M6-5	0.83	0.42	0.84	1.00	0.00	×

Table 5. Effect of Initial Sugar Concentration on Malic Acid Production[a]

		p value		
estimated mean malate production (g/L)	sugar (g/L)	194	220	269
0.54	194	×	0.00	0.00
0.32	220	0.00	×	0.32
0.25	269	0.00	0.32	×

[a] Estimated least-squares means and results of multiple comparisons test (Tukey–Kramer adjustment).

estimates of mean malic acid production for the different levels of the physicochemical variables analyzed, plus the adjusted significance values for pairwise differences as determined by Tukey−Kramer analysis.

Influence of Strain Identity on Malic Acid Production. The estimated means for malic acid production were in the range 0.39−0.76 g/L. Strain M2-9 produced more malic acid than any other strain; C2-9 produced the least (**Table 4**).

Effect of Initial Sugar Concentration. All of the selected strains produced malic acid at the three sugar concentrations tested (194, 220, and 269 g/L). However, the higher the initial sugar concentration, the lower the malic acid production (**Figure 3A**). The difference in malic acid production was significant between the 194 (standard) and 220 g/L sugar levels and between the 194 and 269 g/L levels (**Table 5**). These results disagree with those of Radler (*35*), who report L-(−)-malic acid production to be favored by high sugar concentrations (in the range of 20−30%). The present results might be explained by the hypothesis of Salmon (*36*), who suggested that the efflux of malic acid to the extracellular medium depends on an enzyme transporter system that is inhibited by glucose. Thus, a high sugar concentration would lead to greater concentrations of intracellular malic acid, which would then be decomposed. Moreover, the cytosolic enzyme MDH, which takes part in the formation of malic acid, is phosphorylated, inactivated, and degraded during a proteolytic process induced by glucose (*37*).

Effect of Initial Yeast-Assimilable Nitrogen Concentration. Malic acid production decreased as YAN increased (from 115 to 208 and 308 mg/L) (**Figure 3B**). In fact, malate was degraded at the highest YAN concentration. The variation in malic acid production was only statistically significant between the 115 (standard) and the 208 mg/L YAN levels and between the 115 and the 308 mg/L levels (**Table 6**). These results agree with those of Radler (*35*), who report L-(−)-malic acid formation to be favored by limiting the nitrogen supply.

Effect of Fermentation Temperature. Mean malic acid production was clearly lower at 30 °C than at 18 or 25 °C (**Figure 3C**). This variation was statistically significant between 18 (standard) and 25 °C and between 18 and 30 °C (**Table 7**).

Table 6. Effect of Initial YAN Concentration on Malic Acid Production[a]

		p value		
estimated mean malate production (g/L)	YAN (mg/L)	115	208	308
0.57	115	×	0.00	0.00
0.16	208	0.00	×	0.21
–0.02	308	0.00	0.21	×

[a] Estimated least-squares means and results of multiple comparisons test (Tukey–Kramer adjustment).

Table 7. Effect of Temperature on Malic Acid Production[a]

		p value		
estimated mean malate production (g/L)	temperature (°C)	18	25	30
0.76	18	×	0.00	0.00
0.49	25	0.00	×	0.24
0.36	30	0.00	0.24	×

[a] Estimated least-squares means and results of multiple comparisons test (Tukey–Kramer adjustment).

Table 8. Effect of pH (Section A) and Strain (Section B) on Malic Acid Production[a]

Section A

		p value		
estimated mean malate production (g/L)	pH	3.37	4.02	4.99
0.43	3.37	×	0.00	0.00
0.81	4.02	0.00	×	0.19
0.98	4.99	0.00	0.19	×

Section B

		p value					
estimated mean malate production (g/L)	strain	C2-9	C9-10	M10-7	M2-6	M2-9	M6-5
0.60	C2-9	×	0.98	0.56	0.98	0.0007	0.54
0.52	C9-10	0.98	×	0.16	0.72	<0.0001	0.15
0.79	M10-7	0.56	0.16	×	0.92		1.00
0.68	M2-6	0.98	0.72	0.92	×	0.0086	0.92
1.08	M2-9	0.0007	<0.0001	0.14	0.0086	×	0.14
0.80	M6-5	0.54	0.15	1.00	0.00	0.24	×

[a] Estimated least-squares means and results of multiple comparisons test (Tukey–Kramer adjustment).

The strain M2-9 clearly produced more malic acid at 25 that at 18 °C, M2-6 and C9-10 clearly produced less malic acid, and the other strains produced more or less the same amount of malic acid at both temperatures. The six strains seem to have distinct malic acid production capacities at 25 °C, but they all produced less malic acid at 30 °C. Nevertheless, these abilities are worth testing in different media. Shimazu et al. (*15*) reports that malic acid production is constant between 15 and 30 °C. Farris et al. (*6*) indicate that the capacity of yeasts to produce malic acid is optimal at 25 °C. The optimum temperature for most yeast enzymes is around 25 °C. The efflux of malic acid seems to be influenced by temperature (*36*).

Effect of Initial pH. Malic acid production increased with pH (test pH values: 3.37, 4.02, and 4.99). (**Figure 3D**). These results agree with those of Schwartz and Radler (*10*), who

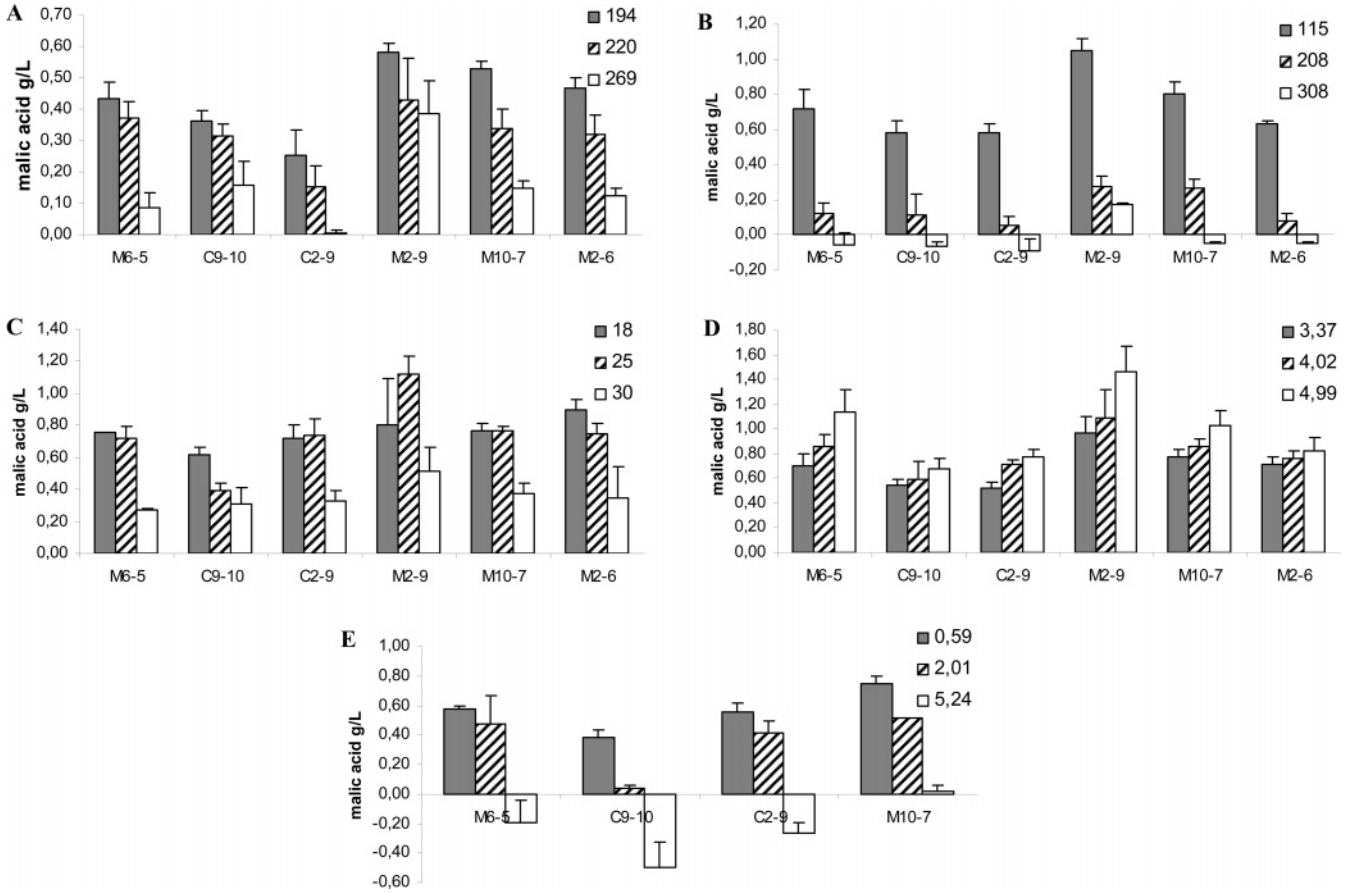

Figure 3. Effect of different physicochemical variables on malic acid production: (**A**) sugar concentration (g/L), (**B**) YAN concentration (mg/L), (**C**) fermentation temperature (°C), (**D**) pH, and (**E**) malic acid concentration (g/L).

reported the optimum pH for malic acid production to be near 5. Because both pH and strain identity had a significant effect on malic acid production, a multiple comparison of the means for both variables was performed. With respect to pH, the variation in malic acid production was statistically significant between pH 3.37 (standard) and 4.02 and between 3.37 and 4.99 (**Table 8A**). With respect to strain identity, M2-9 showed greater mean malic acid production than all of the other strains. The differences in malic acid production were significant for the following pairwise comparisons: M2-9 vs C9-10, M2-9 vs C2-9, and M2-9 vs M2-6 (**Table 8B**). No significant interaction was found between strain identity and pH.

According to Salmon (*36*), malic acid seems to enter by simple diffusion when in a nondissociated form, and its efflux mechanism seems to be a form of active transport very dependent on temperature. Malic acid dissociation depends on pH ($pk_1 = 3.50$ and $pk_2 = 5.11$), so the amount that enters the yeast depends on the pH of the extracellular medium. When the pH is <3.50, malic acid is in a nondissociated form and enters the cell entirely by simple diffusion. When the pH is between 3.5 and <5.11, it is partially dissociated and only partially enters the cell. When the pH is >5.11, malic acid is completely dissociated and it cannot enter the cell. The malic acid intracellular concentration would be higher at lower pH, and this would induce greater degradation of this end product in the yeast cell.

Effect of Initial Malic Acid Concentration. Both the initial malic acid concentration (tested levels of 0.59, 2.01, and 5.24 g/L) and the strain identity had significant effects on malic acid production (**Figure 3E**). These results agree with those of Taillandier et al. (*38*) and Streihaiano et al. (*39*). Multiple comparisons of the means of both variables were performed. However, given the smaller amount of data available on the effect of initial malic concentration (four strains were tested rather than six due to a lack of must), the statistical model used to analyze the other physicochemical factors could not be used for every pairwise comparison. Furthermore, the least-squares means of malic acid production for some of the levels could

Table 9. Effect of Initial Malic Acid Concentration (Section A) and Strain (Section B) on Malic Acid Production[a]

Section A

estimated mean malate production (g/L)	malic acid (g/L)
0.53	0.59
not estimated	2.01
not estimated	5.24

Section B

		p value					
estimated mean malate production (g/L)	strain	C2-9	C9-10	M10-7	M2-6	M2-9	M6-5
0.18	C2-9	×	0.46	0.64			0.91
–0.02	C9-10	0.46	×	0.08			0.14
0.37	M10-7	0.64	0.08	×			0.90
not estimated	M2-6				×		
not estimated	M2-9					×	
0.27	M6–5	0.91	0.14	0.90	-	-	X

[a] Estimated least-squares means and results of multiple comparisons test (Tukey–Kramer adjustment).

Table 10. Effect of Precursors on Malic Acid Production: ANOVA

		strain					
precursor	ANOVA	M6-5	C9-10	C2-9	M2-9	M10-7	M2-6
pyruvate	*F* ratio	3.82	20.86	16.15	3.60	0.98	18.22
	P value	0.09	0.002	0.012	0.09	0.43	0.003
fumarate	*F* ratio	1.13	24.36	13.90	4.69	0.74	1.11
	P value	0.38	0.001	0.016	0.06	0.51	0.39
oxaloacetate	*F* ratio	0.19	0.41	1.80	2.22	0.60	
	P value	0.83	0.68	0.24	0.19	0.58	

Table 11. Effect of Pyruvic Acid Concentration on Malic Acid Production for the Statistically Significant Strains[a]

	malic acid production (g/L)		
media	C9-10	C2-9	M2-6
must	0.52[b]	0.63[b]	0.67[b]
must + 75 mg/L	0.41	0.39	0.49
must + 150 mg/L	0.40	0.47	0.49

[a] Estimated means of malic acid production. [b] Significant difference at the 95.0% confidence level.

not be determined (**Table 9A**). A difference was found between strains M10-7 and C9-10, although the adjusted significance level is rather high (0.08); this result should be read with caution (**Table 9B**). Nevertheless, malic acid production seems to decrease when the malic acid concentration of the medium is higher (**Figure 3E**). At pH 3.4 and 5.24 g/L of malic acid, much more malic acid can enter the cell by simple diffusion; this is therefore degraded.

Malic acid can be degraded via the pyruvate pathway catalyzed by malic enzyme (ME) (*40*) or via the oxaloacetic acid pathway catalyzed by MDH (*41*). In the first of these degradation pathways, ME is a mitochondrial constitutive enzyme, which shows low affinity for malic acid. However, neither malic acid nor any other substrate can induce its production (*40*, *42*). The second degradation pathway occurs when the usual pathway for carbon skeleton synthesis is deficient (*36*).

MDH is inhibited by high glucose concentrations and by high concentrations of L-malic acid, its own substrate (*41*). Malic acid-producing strains would produce less malic acid or even consume it following the inhibition of their MDH activity originated by high L-malic acid concentrations.

Effect of the Three Malic Acid Precursors on Malic Acid Production. The ANOVA of the precursors shows that pyruvic and fumaric acid influence malate production in some assayed strains, whereas oxaloacetic acid had no influence (**Table 10**).

Effect of Pyruvic Acid Concentration. The pyruvic acid concentration influenced malic acid production (**Table 11**). All six strains produced L-(−)-malic acid at the three concentrations tested (**Figure 4A**). The fermentation of must with no added pyruvic acid produced slightly more malic acid than did the fermentation of enriched musts with strains C9-10, C2-9, and M2-6 (**Figure 4A** and **Table 11**).

Effect of Fumaric Acid Concentration. The fumaric acid concentration influenced malic acid production (**Table 12**). All six strains produced L-(−)-malic acid at the three concentrations tested (**Figure 4B**). The addition of 50 mg/L fumarate induced a slight although statistically significant increase in malic acid production with strain C2-9. A significant effect of the addition of 50 or 100 mg/L of fumarate was found with strain C9-10 (**Figure 4B** and **Table 12**), but in general, the differences between the three test concentrations were unimportant.

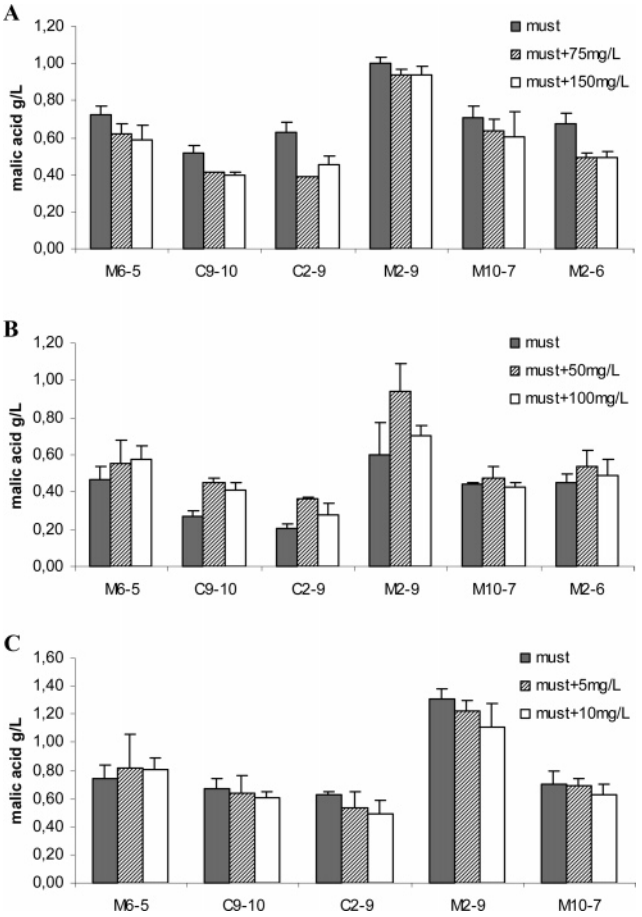

Figure 4. Effect on malic acid production of (**A**) pyruvic acid, (**B**) fumaric acid, and (**C**) oxaloacetic acid.

Table 12. Effect of Fumaric Acid Concentration on Malic Acid Production for the Statistically Significant Strains[a]

	malic acid production g/L	
media	C9-10	C2-9
must	0.27[b]	0.22
must + 100 mg/L	0.41	0.28
must + 50 mg/L	0.45	0.36[b]

[a] Estimated means of malic acid production. [b] Significant difference at the 95.0% confidence level.

Effect of Oxaloacetic Acid Concentration. The oxaloacetic acid concentration had no effect on malic acid production (**Table 10**); this was true for all of the concentrations tested (**Figure 4C** and **Table 10**).

Daily Changes in Malic and Pyruvic Acid Concentrations during Alcoholic Fermentation. Malic acid production occurred between days 2 and 6 of alcoholic fermentation, with a peak on days 3 and 4; from day 7 onward, concentrations remained stable (**Figure 5**). The pyruvic acid concentration reached a maximum on day 2 (100−200 mg/L) and fell (to 20−60 mg/L) by the end of fermentation (**Figure 5**). No direct relationship was observed between the changes in the concentrations of malic and pyruvic acids.

The best malic acid-producing yeasts examined in this work could be used to ferment grape must with low acidity. The amount of malic acid produced by the selected yeast is not very great, but it reduces the need to acidulate the wine, has an

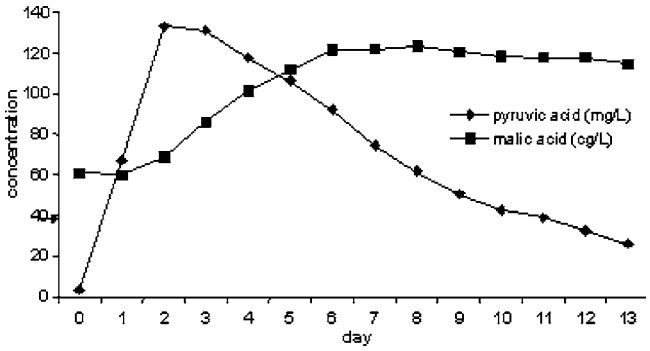

Figure 5. Mean daily changes in malic and pyruvic acid concentrations during alcoholic fermentation (data are the means of results for the initial 10 selected strains).

organoleptic effect, and is even more important when it is remembered that most yeasts partially consume malic acid during alcoholic fermentation. Malate production, however, is very dependent on the fermentation conditions since it increases with lower temperatures, high must pH, and low sugar, malic acid, and YAN concentrations. The concentrations of pyruvate and fumarate influence malic acid production in fermentations with strains C9-10 and C2-9; significant differences in malic acid production were found at the three concentrations assayed. With strain M2-6, only the addition of pyruvate had a significant influence. For the other strains, the production of malic acid was not influenced by the addition of pyruvate or fumarate. Finally, at the concentrations assayed, oxalacetate had no influence on the production of malic acid by any of the yeast strains. From a practical point of view, some viticultural and enological practices can be adapted to favor the production of malic acid, e.g., reducing the YAN in musts through planned viticulture, if necessary, by acidulating musts at the end of alcoholic fermentation, not fermenting at high temperatures (such as 30 °C), and not harvesting when grapes have very high sugar concentrations.

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LITERATURE CITED

(1) Organisation Internationale de la Vigne et du Vin. Résolution 04/2002.

(2) Radler, F. The formation of nonvolatile acids by strains of *Saccharomyces* during fermentation. Proceedings of the first specialized international symposium on yeats, Smolenice, 1972; pp 519−529.

(3) Lafon-Lafourcade, S.; Peynaud, E. Sur les taux des acides cétoniques formés au cours de la fermentation alcoolique. *Ann. Inst. Pasteur*. **1966**, *110*, 766−778.

(4) Whiting, G. C. Organic acid metabolism of yeats during fermentation of alcoholic beverages—A review. *J. Inst. Brew*. **1976**, *82*, 84−92.

(5) Dakin, H. D. The formation of L-malic acid as a product of alcoholic fermentation by yeast. *J. Biol. Chem.* **1924**, *61*, 139−145.

(6) Farris, G. A.; Fatichenti, F.; Deiana, P. Incidence de la température et du pH sur la production d'acide malique par *Saccharomyces cerevisiae*. *Connaiss. Vigne Vin*. **1989**, *23*, 89−93.

(7) Fatichenti, F.; Farris, G. A.; Deiana, P.; Cecarelli, S. Malic acid production and consumption by selected of *Saccharomyces cerevisiae* under anaerobic and aerobic conditions. *Appl. Microbiol. Biotechnol.* **1984**, *19*, 427−429.

(8) Heerde, E.; Radler, F. Metabolism of the anaerobic formation of succinic acid by *Saccharomyces cerevisiae*. *Arch. Microbiol*. **1978**, *117*, 269−276.

(9) Pronk, J.; Steensma H.; Van Dijken, J. Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **1996**, *12*, 1607−1633.

(10) Schwartz, H.; Radler, F. Formation of L-(−) malate by *Saccharomyces cerevisiae* during fermentation. *Appl. Microbiol. Biotechnol*. **1988**, *27*, 553−560.

(11) Peynaud, E.; Lafon-Lafourcade, J.; Guimberteau, G. Nature de l'acide lactique formé par les levures—Un caractère spécifique de *Saccharomyces veronae* Lodder et van Rij. *Antonie van Leeuwenhoek* **1967**, *33*, 49−55.

(12) Suárez Lepe, J. A.; Colomo, B.; Callejo, M. J. Acidogénèse lactique dans les vins d'élevage biologique de l'appellation d'origine Rueda. Influence des facteurs physiques. *I. Aliment. Agric*. **1994**, *6*, 326−332.

(13) Radler, F. The metabolism of organic acid by *Saccharomyces*. *Symposium Proceedings*; University of California: Davis, CA, 1981; pp 103−108.

(14) Thoukis, G.; Ueda, M.; Wright, D. The formation of succinic acid during alcoholic fermentation. *Am. J. Enol. Vitic*., **1965**, *16*, 1−8.

(15) Shimazu, Y.; Watanabe, M. Effects of yeast strains and environmental conditions on formation of organic acids in must during fermentation. *J. Ferment. Technol.* **1981**, *59*, 27−32.

(16) Gallander, J. F. Deacidification of eastern table wines with *Schizosaccharomyces pombe*. *Am. J. Enol. Vitic*. **1977**, *28*, 65−68.

(17) Kliewer, W.; Howarth, L.; Omori, M. Concentrations of tartaric acid and malic acid and their salts in *Vitis vinifera* grapes. *Am. J. Enol. Vitic.* **1967**, *18*, 42−54.

(18) Champagnol, F. Croissance et développement des baies. In *Elements de Physiologie de la Vigne et de Viticulture Générale*; Champagnol, F., Ed.; Saint-Gely du-Fesc: France, 1984; pp 102−110.

(19) Rankine, B. C. Decomposition of L-malic acid by wine yeasts. *J. Sci. Food Agric.* **1966**, *17*, 312−316.

(20) Battacharajee, J. K.; Maragoudakis, M. E.; Strassmorn, M. Identification of malic acid from yeast. *J. Bacteriol*. **1968**, *95*, 495−497.

(21) Calderón, F.; Varela, F.; Suárez, J. A. Influence of pH and temperature in the biosynthesis of malic acid by *Saccharomyces cerevisiae*. *Bull. OIV* **2001**, *74*, 474−478.

(22) Radler, F.; Lang, E. Malatbildung bei hefen. *Wein-Wissenschaft* **1982**, *37*, 391−399.

(23) Delfini, C.; Gaetano, G.; Gaia, P.; Piangerelli, M. G.; Cocito, C. Production of D(+) malic acid by wine yeasts. *Riv. Vitic. Enol.* **1995**, *1*, 75−76.

(24) Pines, O.; Even-Ram, S.; Elnathan, N.; Battat, E.; Aharonov, O.; Gibson, D.; Goldberg, I. The cytosolic pathway of L-malic acid synthesis in *Saccharomyces cerevisiae*: The role of fumarase. *Appl. Microbiol. Biotechnol.* **1996**, *46*, 393−399.

(25) Yéramian, N. Acidificación biológica de mostos en zonas cálidas. Ph.D. thesis, ETSIA-UPM, Spain, 2004.

(26) Yarrow, D. Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts. A Taxonomic Study*, 4th ed.; Lodder, J., Ed.; North Holland Publishing Co.: Amsterdam, London, 1999; pp 77−100.

(27) Aigle, M.; Erbs, D.; Moll, M. Some molecular structures in the genome of lager brewing yeast. *Am. Soc. Brew. Chem*. **1984**, *43*, 1.

(28) Querol, A.; Barrio, E.; Ramon, D. A comparative study of different methods of yeast strain characterization. *Syst. Appl. Microbiol.* **1992**, *15*, 439−446.

(29) Blouin, J. Application des méthodes enzymatiques optiques au dosage de certains constituants du vin. *Chim. Anal*. **1964**, *46*, 7−11.

(30) Organisation Internationale de la Vigne et du Vin. *Recueil des Méthodes Internationales d'Analyse des Vins et des Mouts*; OIV: Paris, 1995.

(31) Suárez Lepe, J. A.; Iñigo Leal, B. Taxonomía y sistemática microbianas. *Microbiología Enológica-Fundamentos de Vinificación*, 3rd ed.; Mundi Prensa: Madrid, Spain, 2004; pp 127−153.
(32) Vaughan-Martini, A.; Martini, A. Determination of ethanol production. In *The Yeasts. A Taxonomic Study*, 4th ed.; Lodder, J., Ed.; North Holland Publishing Co.: Amsterdam, London, 1999; p 107.
(33) SAS Institute Inc. *SAS/STAT*^{R} *User's Guide*, Version 8; SAS Institute Inc.: Cary, NC, 1999; 3884 pp.
(34) Dharmadhikari, M. R.; Wilker, K. L. Deacidification of high malate must with *Schizosaccharomyces pombe*. *Am. J. Enol. Vitic.* **1998**, *49*, 408−412.
(35) Radler, F. Yeasts−Metabolism of organic acids. In *Wine Microbiology and Biotechnology*; Fleet, G. H., Ed.; Harvard Academic Publishers: 1993; pp 165−182.
(36) Salmon, J. M. L-Malic acid permeation in resting cells of anaerobically grown *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1987**, *901*, 30−34.
(37) Minard Karyl, I.; McAlister-Henn, L. Glucose-induced phosphorylation of the MDH2 Isoenzyme of malate dehydrogenase in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **1994**, *315*, 302−309.
(38) Taillandier, P.; Ramon-Portugal, F.; Seiller, I.; Favarel, J. L.; Nepveu, F.; Strehaiano, P. Effet de cinq souches de levures sur l'évolution de l'acidité pendent la fermentation alcoolique. *Revue Francaise d'Œnologie*, **1999**, *178*, 18−24.
(39) Strehaiano, P.; Ramon Portugal, F.; Taillandier, P.; Silva, S.; Nepveu, F. Levures et maîtrise de l'acidité des moûts et des vins. *Revue des Œnologues* **2002**, *105s*, 23−27.
(40) Fuck, E.; Radler, F. Apfelsaurestoffwechsel bei *Saccharomyces* 1-Der anaerobe apfelsaureabbau bei *Saccharomyces cerevisiae*. *Arch. Mikrobiol*. **1972**, *87*, 149−164.
(41) Salmon, J. M; Vezinhet, F; Barre, P. Anabolic role of L-malic acid in *Saccharomyces cerevisiae* in anaerobiosis during alcoholic fermentation. *FEMS Microbiol. Lett.* **1987**, *42*, 213−220.
(42) Kuczynski, J. T.; Radler, F. The anaerobic metabolism of malate of *Saccharomyces bailii* and the partial purification and characterization of malic enzyme. *Arch. Microbiol.* **1982**, *131*, 266−270.

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