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Kinetics of Production and Consumption of Organic Acids During Alcoholic Fermentation by *Saccharomyces cerevisae*

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Summary

This paper presents a study dealing with the production and consumption kinetics of the main organic acids during alcoholic fermentation carried out by a Saccharomyces cerevisiae strain normally used for winemaking. The experiments were carried out using a synthetic medium in which the initial malic acid concentration and the initial pH value were the parameters studied. The kinetics of malic acid consumption and of some organic acids production was then quantified. The results show that a decrease in pH value favors malic acid consumption, while an increase of the initial malic acid concentration increases the consumed amount. The specific malic acid consumption rate shows that its assimilation was microbial growth independent, while the other organic acids specific production rates show that their excretion was strongly associated with the microbial growth. The pH evolution during the fermentation was followed and partially explained by the evolution of the global organic acid production.

Key words: Saccharomyces, organic acid production, alcoholic fermentation, malic acid consumption

Introduction

Acidity is one of the most important organoleptic parameters in wine. Normally, wine acidity is mainly due to some organic acids such as tartaric, malic, acetic, lactic acid and other organic acids which can be found at low concentrations, like succinic and pyruvic acid. The concentration of each of these acids depends on some factors, such as the nature of the grape must, the microbial activity of the yeast strain, as well as the operational conditions in which the winemaking is carried out. Tartaric acid, which is normally present in grape must, is partially eliminated by precipitation. Some *Saccharomyces cerevisiae* strains are able to consume a little proportion of the malic acid initially present in the grape must (*1,2*). The malic acid consumption during the alcoholic fermentation strongly contributes to a decrease in the acidity of the grape must. The acidity value obtained at the end of alcoholic fermentation is a very important parameter for the development of the malolactic fermentation (*1*). This second fermentation which is carried out by lactic acid bacteria transforms the malic acid into lactic acid. The yeast producers propose two kinds of *Sacch. cerevisiae* strains: firstly, yeast which barely modifies the acidity (it is commonly called yeast for acidity preservation), and secondly, yeast able to partially consume the malic acid in grape must (they are commonly called deacidifying yeasts). In spite of the difference in the acidity value which is obtained by either first or second types of yeast, very little scientific research has been published on kinetic behaviors of these different types of yeast and the production of minor organic acids.

In this paper we present the results obtained using an enological *Sacch. cerevisiae* strain (FA1). This strain was expected to preserve acidity, that is to say, to con-

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sume a small proportion of malic acid. In order to avoid the variability of grape must, the experiments were carried out using a synthetic medium in which the composition was similar to grape must. A balance of the main organic acids was established. Finally, a kinetic analysis of the pH and the production and consumption of the main organic acids was carried out.

Material and Methods

Microorganisms. An acidity preservation strain of *Saccharomyces cerevisiae* (FA1) was used. This yeast is commonly used in winemaking and is produced and marketed by Lallemand S. A. (Montreal, Canada). The strain was stored at 4 °C in a solid medium culture. The solid medium composition in gram per liter was the following: glucose, 20; yeast extract, 10; agar-agar, 10; in order to avoid some contamination by bacteria 0.1 gram per liter of chloramphenicol was added. Then, the solid medium was sterilized at 121 °C for 10 minutes.

Preculture. Initially, the yeast strain was activated in a liquid medium whose composition in grams per liter was the following: glucose, 20; yeast extract, 10; and casein peptone, 2. The pH value was adjusted to 4.5 using a H_3PO_4 solution (85 % v/v). Then, the culture medium was sterilized at 121 °C for 15 minutes. The preculture was carried out in a 250 mL Erlenmeyer flask with 150 mL of liquid medium and it was stirred magnetically at 150 rpm. After inoculation, each Erlenmeyer flask was incubated at 30 °C for 16 hours.

Culture conditions. The fermentations were carried out in 2 L fermenters (Setric Instruments, France) with 1.5 L liquid medium whose composition in grams per liter was the following: glucose, 180; yeast extract, 1; KH_2PO_4, 5; $(NH_4)_2SO_4$, 2; $MgSO_4$, 0.4; citric acid, 0.4; tartaric acid, 4. Two malic acid concentrations were studied at 5 and 8 g/L, respectively. Then, the culture medium was sterilized at 121 °C for 20 minutes. After sterilization, the pH value was adjusted using a NaOH (5 M) solution. Three levels of initial pH values were studied: 3.2, 3.4 and 3.6. Each fermenter was inoculated in order to obtain 4 million of viable yeasts per milliliter. Before inoculation the yeast cell suspension used as inoculum was centrifuged and washed twice with a NaCl (8 g/L) solution to eliminate the metabolic products obtained during the preculture phase. After inoculation, each fermenter was incubated at 30 °C and mechanically agitated at 250 rpm.

Analytical methods. Five mL samples were withdrawn at regular time intervals. The total concentration of yeasts in the culture medium was determined using an optical density - dry weight correlation. The percentage of viable yeasts was measured using a methylene blue staining method (4). At the same time, a 4 mL sample was centrifuged at 15 000 rpm for 10 minutes. After centrifugation, the glucose, ethanol, glycerol and some organic acid concentrations in the liquid phase were determined. The glucose concentration was evaluated using an enzymatic analyzer (Yellow Spring Instruments, Ohio, USA). Ethanol was quantified by gas chromatography (Chrompack 437A) using a Chrompack Poraplot Q wide-bore column (0.53 mm x 25 m). The temperature of the oven was 170 °C and the injector and detector were heated to 200 °C. The carrier gas was nitrogen. An isopropanol solution (1 % v/v) was used as an internal standard (2 mL of isopropanol: 1 mL of sample). Gas chromatographic data were collected with a Spectra-Physics SP 4290 integrator. Glycerol and succinic, malic, lactic and acetic acid were determined by a high performance liquid chromatographic method (Thermo Separation Products, Freemont, CA, USA) using a BIORAD AMINEX HPX-87X column. A sulfuric acid (0.005 M) solution was used as a carrier phase. The carrier solution flow rate was 0.4 mL/min, the column temperature was controlled at 40 °C. The detection was carried out by a refractometer. Liquid chromatographic data were collected with a personal computer and the calculations were carried out automatically.

Numerical calculation. Because some organic acids are dicarboxylic acids (malic, succinic) and others are monocarboxylic acids (lactic and acetic), the amounts of produced and consumed COOH functions were calculated using equations 1 and 2:

$$\gamma(\mathrm{COOH})_{\mathrm{produced}} = \sum \frac{\gamma(\mathrm{OA})_{\mathrm{p}}}{M} N_{\mathrm{a.f.}} \qquad /1/$$

$$\gamma(\mathrm{COOH})_{\mathrm{consumed}} = \sum \frac{\gamma(\mathrm{OA})_{\mathrm{c}}}{M} N_{\mathrm{a.f.}} \qquad /2/$$

where: $(OA)_c$ = consumed organic acid (g/L); $(OA)_p$ = produced organic acid (g/L); M = molecular mass (g/mol) and $N_{a.f.}$ = number of acid functions in the molecule.

The equations 3, 4 and 5 were used to calculate the profile of microbial specific growth rate and the specific rates of COOH consumption and COOH production:

$$\mu_x = \frac{1}{X}\frac{dX}{dt} \qquad /3/$$

$$\mu_{(\mathrm{COOH})\mathrm{P}} = \frac{1}{X}\frac{d\gamma(\mathrm{COOH})_{\mathrm{produced}}}{\mathrm{dt}} \qquad /4/$$

$$\mu_{(\mathrm{COOH})\mathrm{C}} = \frac{1}{X}\frac{d\gamma(\mathrm{COOH})_{\mathrm{consumed}}}{dt} \qquad /5/$$

where: μ_x = specific growth rate (1/h); $\mu_{(COOH)P}$ = specific produced organic acid rate (mmol/g/h); $\mu_{(COOH)c}$ = specific consumed organic acid rate (mmol/g/h); X = biomass concentration (g/L) at t and t = time (h).

A computer program was used to calculate each derivative expression in equations 3, 4 and 5.

Results

Balance sheet for organic acids

The *S. cerevisiae* strain FA1 is commonly used for winemaking. The studied parameters were the initial malic acid concentration and the initial pH value. The levels of each of these variables were indicated in the Materials and Methods section. In order to visualize the different experimental data obtained in function of the two variables (malic acid and pH), a multiple linear correlation was carried out. The polynomial equation is:

$$Y = b_0 + b_1(\mathrm{pH}_i) + b_2(Mal_i) + b_{12}(\mathrm{pH}_i)(Mal_i) + b_{11}(\mathrm{pH}_i)^2 \qquad /6/$$

This equation relates the final concentration of the different organic acids at the end of the alcoholic fermentation (Y) to the initial pH value pH_i and to the initial malic acid concentration Mal_i. The constants in equation 6 were determined using a multiple linear regression program (STATIT-CF, Paris). Table 1 shows the values of the constants as well as the correlation coefficients. In all the cases, it is observed that the correlation coefficients are very near to 1. The correlation coefficients indicate a good correlation between the experimental data and the calculated values using equation 6. Thus, equation 1 will be used to visualize the obtained results.

Table 1. Equation 1 constants and correlation coefficient values

	Consumed malate	Produced acetate	Produced lactate	Produced succinate
b0	75.22	–73.71	12.20	–10.43
b1	–39.94	42.90	–8.52	5.96
b2	–1.22	0.42	0.63	0.15
b12	0.37	–0.10	–0.17	–0.03
b11	5.31	–6.18	1.46	–0.84
Correlation coefficients	0.98	0.99	0.97	0.95

Fig. 1 shows the amount of consumed malic acid at the end of alcoholic fermentation versus both initial pH value and the initial malic acid concentration. The amount of consumed malic acid was calculated by the difference between the malic acid at the beginning of the fermentation and the residual concentration obtained at the end of alcoholic fermentation. The end of alcoholic fermentation was defined as the moment when the residual glucose concentration was zero. The profile in Fig. 1 shows that an increase in the initial malic acid concentration raises the malic acid consumption. On the other hand, an increase in the initial pH value lowers the cell capacity to metabolize the malic acid which is present in the culture medium.

Fig. 2 shows that the amount of produced acetic acid was particularly affected by the pH value at the beginning of alcoholic fermentation. However, the acetic acid profile shows that low and high pH values inhibit acetic acid production. The highest acetic acid concentration was observed at pH values around 3.4. An increase in the initial malic acid concentration has a slight positive effect on acetic acid production.

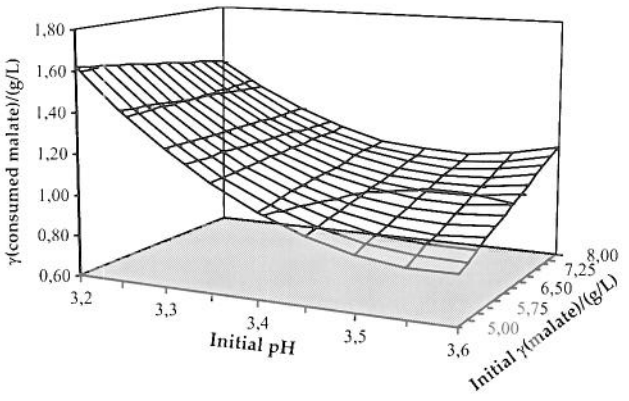

Fig. 1. Consumed malate versus initial values of pH and malate concentrations

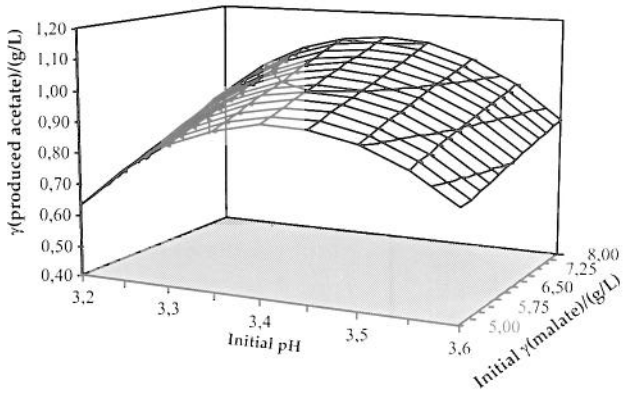

Fig. 2. Produced acetate versus initial values of pH and malate concentrations

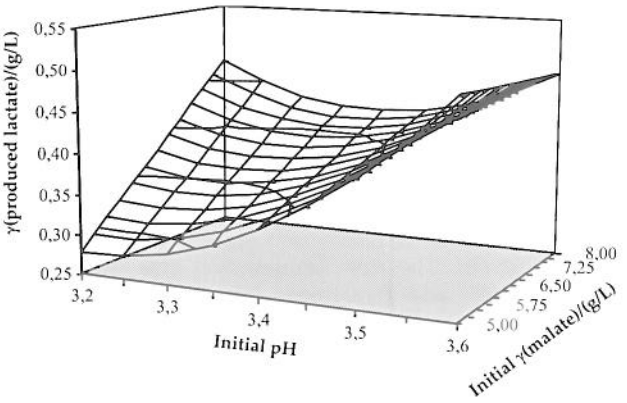

Fig. 3. Produced lactate versus initial values of pH and malate concentrations

Fig. 3 shows the profile of the lactic acid concentration which was obtained at the end of the alcoholic fermentation versus both studied parameters. This profile shows that for high pH values, the final lactic acid concentration is independent of the initial malic acid concentration. Whereas, for low pH values, an increase of the initial malic acid concentration induces more lactic acid production. At low malic acid concentrations an increase of the pH value in the culture medium induces malic acid consumption; however, this phenomenon is less noticeable at higher initial concentrations of malic acid.

The amount of succinic acid was mainly affected by the presence of malic acid (Fig. 4). It can be observed that an increase in the malic acid concentration induces more succinic acid production. However, the pH influence was lower. As well as for acetic acid production,

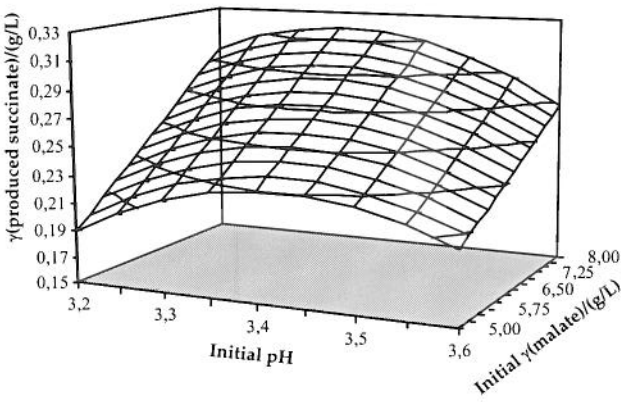

Fig. 4. Produced succinate versus initial values of pH and malate concentrations

high and low pH values slightly inhibit succinic acid production. The highest succinic acid concentration was observed around the pH value of 3.35.

pH evolution and organic acid evolution during alcoholic fermentation

The total acidity value is an important parameter at the end of the grape must fermentation process with respect to wine quality. The total acidity value of the medium or of the wine can be expressed by the pH value that is directly linked to organic acid and mineral salt concentrations in the fermented must. As indicated previously, malic acid is the organic acid which varies most during grape must fermentation. In order to try to explain the contribution of malic acid to the final pH value, two alcoholic fermentations were carried out using the FA1 strain. The first fermentation was carried out without malic acid (»reference fermentation«) and the second one was carried out adding 5 grams per liter of malic acid at the beginning of alcoholic fermentation. Before inoculation, in both cases, the pH value was adjusted to 3.2.

The concentrations of produced and consumed COOH functions (mmol/L) were calculated as indicated in the Material and Methods sections and their profiles are shown in Fig. 5.

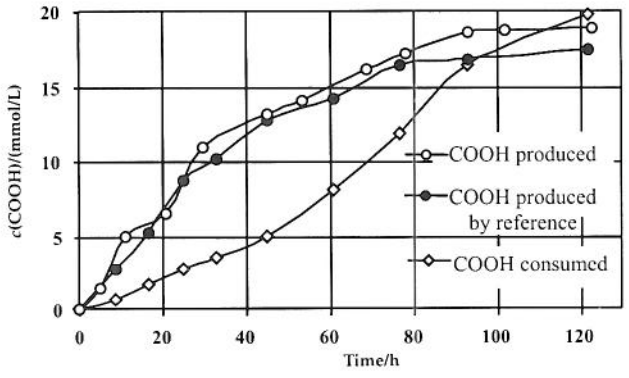

Fig. 5. Consumed and produced COOH throughout the alcoholic fermentation

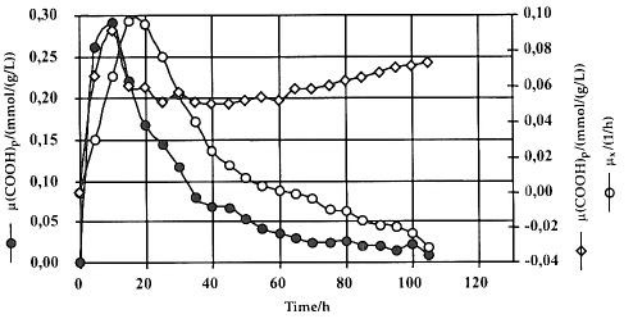

Fig. 6. Specific growth rate and specific consumed and produced COOH rate for the reference fermentation and the fermentation carried out with 5 g/L of malic acid, pH = 3.2

The profiles of produced COOH (sum of the produced organic acids) for the reference and for the test fermentation carried out with 5 grams per liter of malic acid were almost the same, but a little greater in the presence of malic acid. Both profiles show a high COOH production rate during the first 40 hours of cultivation. Later, the COOH production rate decreases until reaching a constant value. The profile of consumed COOH shows a constant tendency all throughout the alcoholic fermentation. These experimental data indicate that the COOH consumption (malic acid consumption) takes place throughout the alcoholic fermentation.

In order to best demonstrate this relation in metabolic activities, Fig. 6 shows that the profiles of the specific growth rate and specific COOH production rate were very similar. These two profiles indicate that the production of organic acids during the alcoholic fermentation is strongly associated with microbial growth. On the other hand, the profile of the specific COOH consumption rate shows that consumption of malic acid was only associated with the microbial growth at the beginning of the fermentation. However, after 20-hour incubation, the COOH consumption rate remained constant and it increased slightly at the end of the alcoholic fermentation. These results show that the malic acid consumption is independent of the physiological state of the yeast so the consumption rate of malic acid should be similar if the cell is in the exponential or stationary phase.

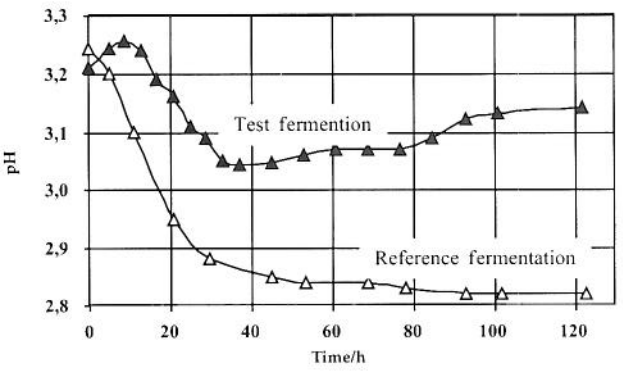

Fig. 7. pH evolution for the reference fermentation and the fermentation carried out with 5 g/L of malic acid

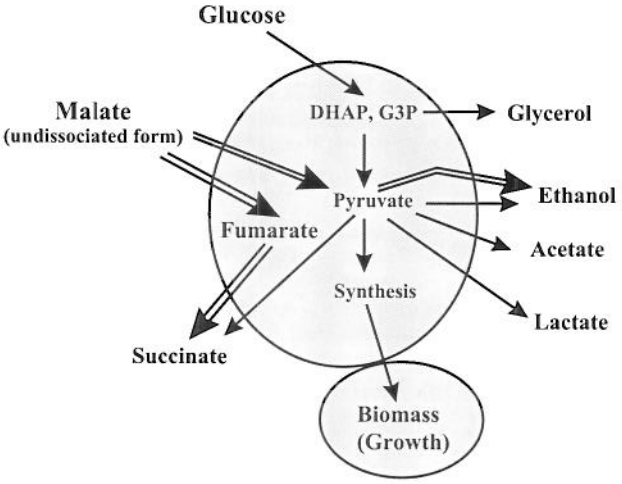

Fig. 8. General metabolism of malic acid (====) and glucose (-------) by *Sacch. cerevisiae* FA1

Fig. 7 shows the two pH profiles for the reference and for the fermentation carried out with 5 g/L of malic acid. The pH evolution for the reference fermentation (without malic acid) shows a decrease in the pH value all throughout the alcoholic fermentation. A strong decrease in the pH was observed during the first 40 hours of incubation (from 3.2 to 2.85). This coincides with the profile of produced COOH (Fig. 5). The profile of the pH corresponding to the fermentation carried out with 5 g/L of malic acid shows a slight increase in the pH value during the first 10 hours of incubation. Later, the pH value diminishes from 3.25 to 3.05. After 40 hours of incubation, the pH value was stabilized and it increased slightly from 3.08 to 3.14 at the end of the alcoholic fermentation. This slight increase in the pH value coincides with the decrease in organic acid production (Fig. 5).

Discussion

The results obtained in this experiment using the FA1 strain show that a little modification of the initial malic acid concentration can change the amount of consumed malic acid; but for a fixed pH the percentage of consumed malic acid is constant: from 16 to 32 % when the pH varies from 3.2 to 3.6. In winemaking, the malic acid concentration in grape must can vary from 2 to 10 g/L (*5*). This value will mainly depend on the climatic conditions where the grapes have been cultivated and on the grape variety. Moreover, pH values for grape must range between 2.8 and 3.6. These differences would modify the malic acid consumption initially present in the grape must. Several experiments have demonstrated (*1, 5, 6*) that the *Sacch. cerevisiae* yeast is able to consume an amount of malic acid which varies according to the strains. The positive effect of low pH on malic acid production has already been reported (*7, 8*) and can be explained as follows. The transport of malic acid inside the cell is mainly achieved by passive diffusion through the cellular membrane. In these conditions, only the undissociated form is able to go through the cellular membrane (*9*). Malic acid has two acidic functions with two different pK_A values (5.13 and 3.46, respectively). When the pH level becomes higher than the pK_A value, the dissociated form progressively replaces the protoned form. Thus, when the initial pH value in the culture medium increases, the protoned form disappears. The protoned form is in equilibrium with the dissociated form. This means that when the malic acid concentration increases, the equilibrium remains constant with an increase in the protoned form. This is why the consumed amount increases but the percentage remains constant.

Then, the profiles of produced COOH functions for the reference and for the fermentation carried out with 5 g/L of malic acid were compared. These results should indicate that malic acid degradation for this pH (3.2) slightly affects the production of the other organic acids. In fact, only the succinate production was increased in the presence of malic acid from 0.12 to 0.19 g/L at 5 g/L and 0.3 g/L at 8 g/L. Malic acid only had an influence on the production of succinate and not on the production of acetate or lactate. This could be explained by the metabolic pathways of this substrate in *Sacch. cerevisiae*. Two pathways are proposed in the literature (*6,10*). They are probably both used during alcoholic fermentation. According to our data, succinate formation by the citric acid cycle should be more active at the beginning of the alcoholic fermentation, whereas in the second part malic acid should more likely be converted into ethanol independently from growth (Fig. 8). Nevertheless, the increase in the ethanol concentration at the end of alcoholic fermentation should be too weak to be detected.

The effect of pH on the production of lactate and acetate remains unexplained. Both were produced during the growth phase only. The very different evolution of pH between the reference and the culture with 5 g/L of malic acid shows the importance of this substrate on the final medium acidity. Without malic acid consumption, the pH drops drastically during alcoholic fermentation, probably due to the consumption of ammonium and the production of organic acids. In spite of the malic acid consumption of 32 % at 3.2 of initial pH, the pH variation after alcoholic fermentation is only –0.08 pH, which explains why this strain is considered as an acidic preservation strain.

Conclusion

These experiments show that the initial pH value and the initial malic acid concentration in the culture medium can strongly affect the malic acid consumption and the main organic acid production. The malic acid consumption is largely responsible for the pH variation during alcoholic fermentation. The initial presence of malic acid in the culture medium is very important to the auto-regulation of the pH during alcoholic fermentation and particularly at the end (stationary growth phase). Initial pH values affect lactate and acetate production, whereas malic acid mainly affects the final succinate concentration but not the amount of produced biomass (data not shown). Moreover the malate consumption is not associated with microbial growth. The whole body of data validates the hypothesis that malic acid

consumption serves either the ethanol or the succinate production along two possible pathways, but not the biomass production. The global production of organic acids shows that production and microbial growth are strongly related. The obtained kinetic data will be very important in explaining malic acid consumption under real winemaking conditions and in predicting the intensity of malic acid degradation using this strain. The FA1 strain is considered as an acidity-preservation yeast because the final pH is always inferior to the initial pH (data not shown) in spite of the considerable malic acid consumption. This observation shows that, from the enological point of view, minor products of alcoholic fermentation, such as organic acids, should be considered and not only malate assimilation.

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Kinetika proizvodnje i utroška organskih kiselina tijekom alkoholnog vrenja s pomoću *Saccharomyces cerevisiae*

Sažetak

U radu je prikazana kinetika proizvodnje i utroška glavnih organskih kiselina tijekom alkoholnog vrenja provedenog sa sojem Saccharomyces cerevisiae koji se normalno koristi u proizvodnji vina. Ispitivanja su provedena koristeći sintetsku podlogu u kojoj su praćeni početna koncentracija jabučne kiseline i početna vrijednost pH. Pri tome je kvantitativno utvrđena kinetika utroška jabučne kiseline i proizvodnja nekih organskih kiselina. Rezultati potvrđuju da snizivanje vrijednosti pH potpomaže utrošak jabučne kiseline, dok povišenje početne koncentracije jabučne kiseline povećava njezin utrošak. Specifična brzina utroška jabučne kiseline pokazuje da je njezina asimilacija neovisna o mikrobnom rastu, dok je specifična brzina proizvodnje ostalih organskih kiselina neposredno povezana s rastom mikroorganizama. Tijekom fermentacije praćena je promjena pH te je djelomično objašnjena porastom proizvodnje ukupnih organskih kiselina.