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F. Delcourt, Patricia Taillandier, F. Vidal, Pierre Strehaiano. Influence of pH, malic acid and glucose concentrations on malic acid consumption by *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, 1995, 43 (2), pp.321-324. 10.1007/BF00172832 . hal-02143212

HAL Id: hal-02143212

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Submitted on 29 May 2019

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To cite this version :

Delcourt, F. and Taillandier, Patricia  and Vidal, F. and Strehaiano, Pierre  *Influence of pH, malic acid and glucose concentrations on malic acid consumption by Saccharomyces cerevisiae.* (1995) *Applied Microbiology and Biotechnology*, 43 (2). 321-324. ISSN 0175-7598

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Influence of pH, malic acid and glucose concentrations on malic acid consumption by *Saccharomyces cerevisiae*

Abstract Malic acid consumption by *Saccharomyces cerevisiae* was studied in a synthetic medium. The extent of malic acid degradation is affected by its initial concentration, the extent and the rate of deacidification increased with initial malate concentration up to 10 g/l. For malic acid consumption, an optimal pH range of 3–3.5 was found, confirming that non-dissociated organic acids enter *S. cerevisiae* cells by simple diffusion. A full factorial design has been employed to describe a statistical model of the effect of sugar and malic acid on the quantity of malate degraded (g/l) by a given amount of biomass (g/l). The results indicated that the initial malic acid concentration is very important for the ratio of malate consumption to quantity of biomass. The yeast was found to be most efficient at higher levels of malate.

Introduction

Malic acid is one of the main organic acids in grapes, and generally it is necessary to remove it from wines in order to improve their organic qualities and to ensure their biological stability. The malic acid concentration usually decreases slightly during alcoholic fermentation, but the consumption of this organic acid depends on the genus and species of the yeasts used. The yeast *Schizosaccharomyces pombe* has been reported to metabolize malic acid efficiently (100%) (Ribereau-Gayon and Peynaud 1962; Mayer and Temperli 1963; Taillandier et al. 1988). The intensity of malic degradation was not affected by the concentration of malic acid in the medium (Taillandier et al. 1988) unlike the situation in other yeast genera such as *Saccharomyces*.

The ability of *Saccharomyces cerevisiae* to use malic acid during the fermentation of a synthetic medium simulating grape must has been known for many years (Vezeinhet and Barre 1982), but few studies deal with the kinetic aspects of malate degradation. It should be noted that, with *S. cerevisiae*, the malic acid is never completely consumed (Usseglio-Tomasset 1989). The extent of malic acid degradation depends on the medium composition. It is generally admitted that non-dissociated organic acids enter yeast cells by simple diffusion (Salmon 1987a). Baranowski and Radler (1984) have discovered a specific proteic uptake system of malic acid in *Saccharomyces bailii*, but not in *S. cerevisiae*. Salmon (1987b) showed that the malic enzyme of *S. cerevisiae* catalyses the NAD(P)⁺ dependent-decarboxylation of L-malate into pyruvate. This enzyme is characterised by its ability to decarboxylate oxaloacetate and its strong dependence on Mn²⁺ ions (Fück and Radler 1974; Kuckzynski and Radler, 1982). Malic acid could play a role in supplying the C4 carbon skeleton through malate dehydrogenase and the oxidative reactions of the tricarboxylic acid cycle when the usual pathway for carbon skeleton synthesis is deficient (Salmon et al. 1987).

The objective of this work was to increase the level of deacidification of an enological strain of *S. cerevisiae*. On the one hand we tried to elucidate the effect of initial malic acid and glucose concentrations on the level of acid utilisation by the yeast. We were also interested in the effect of pH on malic acid metabolism (deacidification) during alcoholic fermentation. The pH range of enological conditions is 3–4, we therefore studied the effect of pH on deacidification activity in this range.

On the other hand, we studied the linkage between the ratio of malic acid consumption (g/l) to the amount of biomass (g/l) and initial concentrations of glucose and malic acid. Experimental design techniques have been used to measure the effect of each factor and the interactions with a relatively small number of experiments.

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Materials and methods

Microorganism and culture medium

The strain of *Saccharomyces cerevisiae* 236 employed in this study was supplied by National Institute of Agronomic Research (INRA) of Guadeloupe. The standard culture medium contained KH_2PO_4 5 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4 g/l, $(\text{NH}_4)_2\text{SO}_4$ 2 g/l, yeast extract 2 g/l, glucose 100 g/l, malate 4.5 g/l, pH 3.4. For some experiments the glucose, the malate and pH were varied as described in Results.

Culture conditions

Batch experiments were performed in 2-l fermenters (Setric Genie Industriel, Toulouse). The volume of the medium in the fermenter was 1.2 l, the initial concentration of biomass was 3×10^6 cells/ml. The inoculum was prepared in 250-ml conical flasks with 150 ml standard culture medium. The temperature was regulated at 30°C, the pH was not regulated. Agitation rate was 150 rpm.

Analytical determinations

Biomass was estimated gravimetrically. The cell dry weight was obtained by filtration of a known sample volume through a cellulose acetate membrane filter (Sartorius 0.45 μm), washed with distilled water and dried to constant weight. Cell viability was determined by methylene blue staining.

L Malic acid concentrations were measured using an enzymatic procedure from Boehringer Mannheim no 648357. Glucose concentrations were estimated by immobilized glucose oxidase (Yellow Springs Instrument) and ethanol concentrations were determined by gas chromatography (Intersmat, Pavillon sous bois, France) with a Porapak-Q column, using 1-propanol (1% v/v) as the internal standard and a flame ionisation detector. The carrier gas was nitrogen (1.8 bars; 30 ml min⁻¹). The temperature of the column was 170°C, that of the detector 250°C and that of the injector 250°C.

Experimental design

A full factorial design of experiments was used (Goupy 1988). A linear model was used to show the influence of two factors, glucose and malate concentrations, on the ratio of malate consumption (g/l) to the quantity of biomass (g/l) in the culture. This biological characteristic (y) is represented on this model by Eq. 1, where y is the estimated response and x_1 (glucose) and x_2 (malic acid) are the factor levels:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 \quad (1)$$

x_1 and x_2 are the coded values of each variable, and b_0 , b_1 , b_{ij} are the coefficients of the polynomial estimated by the least-squares method of fitting with a Statitcf multivariable linear regression analysis with constant. The coefficient of multiple correlation (r multiple) indicated the quality of multivariable linear regression. This full factorial design needed seven experiments to be carried out, defined by the coded values of the two parameters (Table 1): three mid points of the coded values (0,0,0) allowing the estimation of the experimental variance (tests 5-7, Table 1), and four factorial points (tests 1-4, Table 1). The experimental design was applied to the expression of the variable y =ratio of malate consumption (g/l) to the amount of biomass (g/l).

Table 1 Experimental design defining glucose (x_1) and malic acid (x_2) initial concentration conditions. Glucose: $-1=50$ g/l and $+1=100$ g/l; malic acid: $-1=2.5$ g/l and $+1=7$ g/l

Test number	Design matrix			Work matrix y
	X_1 glucose	x_2 malic acid	$x_1 x_2$ interaction	
1	-1	-1	+1	0.29
2	+1	-1	-1	0.12
3	-1	+1	-1	0.30
4	+1	+1	+1	0.28
5	0	0	0	0.25
6	0	0	0	0.25
7	0	0	0	0.25

Results

Effect of initial concentration of malic acid

Experiments were conducted in fermenters with standard medium except that the malate concentration varied from 2.5 g/l to 11 g/l. Figure 1 clearly demonstrates that the concentration of malic acid (2.5–11 g/l) does not affect the specific rate of growth (μ). For the concentrations studied, malic acid does not restrict the yeast's production of biomass.

The quantity of malate consumed increased with the initial malate concentration up to 9.5 g/l (Fig. 2). Malate was not entirely consumed by the yeasts, but with high initial levels of malate (9.5 g/l), 2.2 g/l malic acid was degraded and with low levels (2.5 g/l) only 0.8 g/l was degraded, that is to say about 60% less.

Effect of initial concentration of glucose

The degradation of 4.5 g/l malic acid was studied for different initial glucose concentrations. The sugar concentration was varied from 50 g/l to 200 g/l and, all cases, glucose was exhausted. We noted that both the

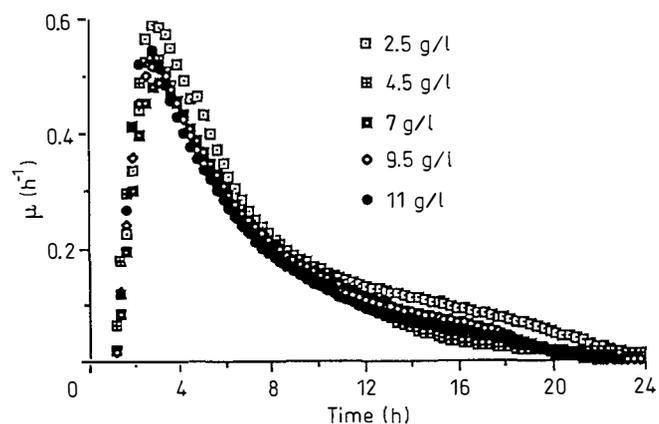


Fig. 1 Specific rate of growth [$\mu = (1/x)(dx/dt)$] for different initial malic acid concentrations

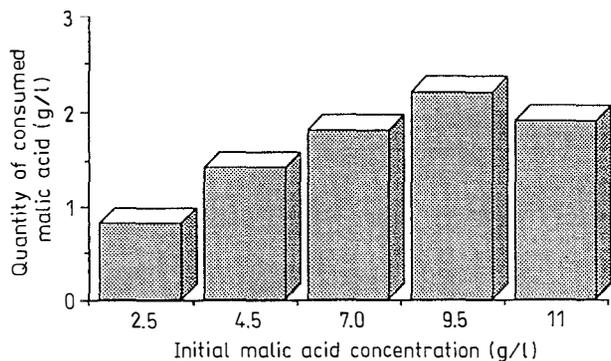


Fig. 2 Influence of initial malic acid concentration on the total quantity of malic acid consumed

specific rate of deacidification [ν_M , g acid consumed (g biomass) $^{-1}$] and malic acid consumption (g/l) were found constant above a sugar concentration of 50 g/l (Table 2). Below this concentration the initial sugar concentration essentially affects ν_M and the total quantity of consumed malic acid but not the ratio of malic acid consumption to the amount of biomass.

Influence of pH

The initial pH of the medium was varied between 3 and 4. For the different pH values tested the specific deacidification rates (ν_M) are shown versus the time for the three fermentations in Fig. 3. A marked decrease of the maximal value of ν_M is observed for the higher pH value. Malic acid consumption was respectively 1.2, 1.4 and 0.9 g/l for the three pH values assayed. Malic acid has pK_a values of 3.45 and 5.10 for the monoanionic and dianionic species respectively. These results seem to be consistent with the generally accepted assertion that non-dissociated organic acids enter yeast cells by simple diffusion (Salmon 1987a).

Experimental design

The values of y for each experiment are listed in Table 1. The coefficients of the polynomials (b_0 , b_i , b_{ij}) de-

Table 2 Influence of the initial glucose concentration on the deacidification activity of yeast

Parameter	Initial glucose concentration			
	50 g/l	100 g/l	170 g/l	200 g/l
ν_M^a (g g $^{-1}$ h $^{-1}$)	0.2	0.5	0.5	0.5
Malic acid consumption (g/l)	0.6	1.4	1.3	1.3
Ratio of malic acid consumption (g/l)/amount of biomass (g/l)	0.21	0.23	0.23	0.22

^a ν_M is the specific rate of malate consumption, g acid consumed (g biomass) $^{-1}$ h $^{-1}$ = (1/x) (d[malate]/dt)

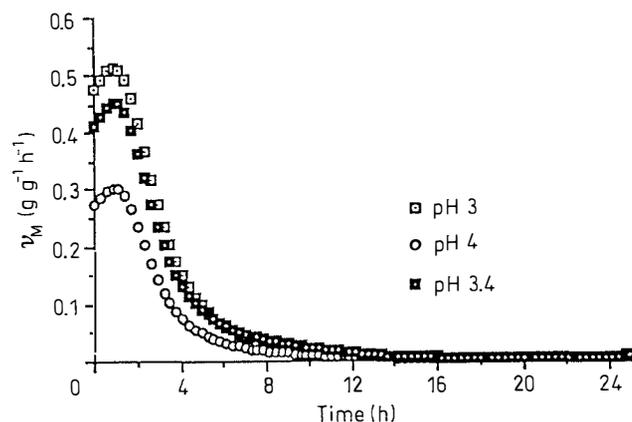


Fig. 3 Specific deacidification rate versus time for different initial pH. ν_M is the specific rate of malate consumption [g acid consumed (g biomass) $^{-1}$ h $^{-1}$]

scribing the quantity of malic acid consumed by a given quantity of biomass are listed in Table 3. The coefficient of multiple correlation (r multiple) is higher than 90%. Here the simple Eq. 2 is obtained:

$$y = 0.249 (0.0475 \pm 0.0021)x_1 + (0.0425 \pm 0.0021)x_2 + (0.0375 \pm 0.0021)x_1x_2 \quad (2)$$

Discussion

For the first series of experiments, when the initial malate concentration was below 9.5 g/l, the malate consumption was greater since its concentration was important. Biomass production remained constant (data not shown) whatever the initial malic acid concentration. So, we can conclude that the deacidification activity of the yeast increases with the initial level of malic acid up to 9.5 g/l, the critical malate concentration for saturation kinetics.

The formalism of Luedeking and Piret in Fig. 4 for standard medium with 4.5 g/l malate, shows that, with *S. cerevisiae*, growth and deacidification activities are strictly linked. This linear correlation means that the cessation of malic acid degradation is simultaneous with the ending of glucose consumption. This model can explain the fact that, with low concentrations of glucose, malic acid was less consumed. Similar observations were made by Peynaud et al. (1964).

Table 3 Coefficients for the calculation of y from Eq. 1

Variable	Regression coefficient
x_1	-0.0475
x_2	0.0425
x_1x_2	0.0375
Constant	0.2486
Population standard deviation	0.0009
r multiple	0.99

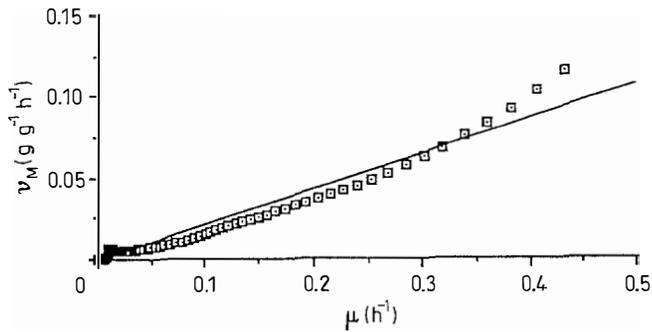


Fig. 4 Formalism of Luedeking and Piret: $\nu_M = f(\mu)$ for standard culture conditions

According to Eq. 2 obtained from the experimental design, the two factors x_1 and x_2 have opposite effects on the ratio malate consumption to quantity of biomass: glucose concentration has a negative effect whereas malate concentration has a positive effect. This polynomial enables the calculation of the optimal conditions in order to maximize the activity of deacidification by a given quantity of biomass. As a consequence, it would be better to work with a high level of malic acid and with a low level of sugar. This means that the cells of *S. cerevisiae* are more efficient at deacidification when there is not too much, but enough, glucose and a lot of malate up to 7 g/l.

The results presented in this communication show that the yeast *S. cerevisiae* 236, selected for rum fermentation in the French West Indies, is quite efficient for malic acid consumption. Because of this it could also be used to make wine from tropical fruits that contain malic acid but in a lesser quantity than in grape must, about 4.6 g/l.

Acknowledgements We thank A. Parfait and the Centre Régional d'Innovation et de Transfert de Technologie-Biotechnologie et Agro Industrie de la Caraïbe (CRITT BAC) for their financial support based on European program (STRIDE). Thanks are also due to INRA for having put the *Saccharomyces cerevisiae* 236 at CRITT-BAC's disposal.

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