The role of malic acid in the metabolism of *Schizosaccharomyces pombe*: substrate consumption and cell growth

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Summary. The effect of initial concentrations of malate varying from 0 to 28.6 g/l was studied. The acid was found to be inhibitory for growth of *Schizosaccharomyces pombe* but not for its deacidification activity. Malate was never integrated into biomass but partly transformed into ethanol if the aeration rate was weak (oxygen limitation). In the absence of glucose, resting cells of *S. pombe* were able to degrade malic acid if their concentration was sufficient, but their viability gradually decreased. However, for 0.15 g/l of growing cells (inoculum) 6 g/l of glucose was necessary to consume 8 g/l of malate. When the medium did not contain sugar no growth was observed despite the partial consumption of malate, showing that the acid was neither a carbon source nor an energy source.

Introduction

Because of their ability to degrade malic acid into ethanol (well known as maloalcoholic fermentation), *Schizosaccharomyces pombe* yeasts can be used in winemaking to deacidify grape musts and wines. Some studies have been undertaken in order to improve this technology for the field of enology (Charpentier et al. 1985; Benda 1973; Snow and Gallander 1979). From a metabolic point of view, despite some investigations (Maconi et al. 1984; Mayer and Temperli 1963; Osothsilp and Subden 1986), the metabolism of malate by the yeast is not well established and many contradictions have appeared in the literature (Taillandier et al. 1988a). In particular, points not yet elucidated are the following:

1. The metabolic pathway of malic acid by *S. pombe*.
2. Its mode of transport into the cells.
3. Its role as a substrate or co-substrate.

We previously showed that the consumption of malate is neither linked to cell growth nor to the consumption of sugar (Taillandier et al. 1988b). Magyar and Panyik (1989) reported similar results. In this work we present complementary experiments.

Materials and methods

Microorganism and culture medium. The yeast strain *S. pombe* G2 was isolated and supplied by the Institut Coopératif du Vin (Montpellier, France). The standard culture medium contained: glucose, 100 g/l; yeast extract, 5 g/l; asparagine, 4 g/l; KH_2PO_4, 5 g/l; $MgSO_4$, 0.4 g/l; malate, 8 g/l; pH = 3.0. For some experiments the glucose and malate concentrations were varied as described in Results and discussion.

Culture conditions. The cultures were performed either in erlen meyer flasks or in fermentors. The erlenmeyer flasks were magnetically stirred at 200 rpm and placed in a water bath regulated at 30° C.

The fermentors were 2 l total volume (Setric Génie Industriel, Toulouse, France), instrumented and equipped with a flat-bladed impeller. The vessel was round-bottomed, with air supplied through a sparger located in the bottom. The temperature was set at 30° C, the aeration rate was 0.15 vvm and the agitation rate 280 rpm. Under these conditions, the volumetric mass transfer coefficient value (K_La) was 10.1 h^{-1} and the dissolved oxygen concentration (pO_2) during the fermentation was never zero (excess of oxygen). The gas stream exited through a condenser to avoid ethanol evaporation.

Analytical determinations. Biomass was measured turbidimetrically at 640 nm and calibrated to cell dry weight determinations. Cell viability was estimated by methylene blue staining.

Glucose and malic acid concentrations were determined by HPLC using an Ion-300 Interaction column (Interaction Chemicals, Mountain View, Calif., USA) and 0.025 M H_2SO_4 as eluant with a flow rate of 0.4 ml/min. The detector (differential refractometer) and the pump were obtained from Spectra-Physics, Les Ulis, France.

Ethanol was measured by gas chromatography (Intersmat, Pavillon-sous-bois, France) with a Porapak-Q column, using 1 propanol (1% v/v) as internal standard and a flame ionisation detector. The carrier gas was nitrogen (1.8 bars; 30 ml/min). The temperature of the column was 170° C, of the detector 250° C and of the injector 250° C.

Results and discussion

Effect of the initial concentration of malic acid

Experiments were conducted in fermentors on standard medium except that the concentration of malate varyied from 0 to 28.6 g/l. In all these cases, malate was entirely consumed by the yeasts. The specific rates of growth (μ) and deacidification (ν_M), rate of deacidification (dM/dt) and yield of conversion of sugar to ethanol ($Y_{P/S}$) and of sugar to biomass ($Y_{X/S}$) are shown in Table 1.

These data confirm the high efficiency of *S. pombe* for deacidification. In very acid grape musts, the malate concentration is about 10–15 g/l (Benda 1973). Since dM/dt was found to be quite constant and maximal when the malate concentration was greater than 18.6 g/l, the yeasts should always be able to deacidify any grape must. The specific growth rate strongly decreased when the malate concentration was greater than 2.2 g/l and seemed to stabilize for higher concentrations of the acid, indicating the inhibitory effect of malic acid on growth.

It has been known for a long time that *S. pombe* yeasts convert malic acid into ethanol and not into biomass. Dittrich (1963) and Mayer and Temperli (1963) established the stoichiometry of the reaction by measurement of gaseous exchange in a Warburg apparatus under strictly anaerobic and also aerobic conditions. They also measured the ethanol produced in each case and proposed the following stoichiometry for the reaction:

$$1\ \text{malate} + 3\,O_2 \rightarrow 4\,CO_2 + 3\,H_2O \quad \text{(aerobiosis)}$$
$$1\ \text{malate} \rightarrow 2\,CO_2 + 1\ \text{ethanol} \quad \text{(anaerobiosis)}$$

Thus, the theoretical yield of malate conversion into ethanol is 0.343 g/g (46 g/134 g) for anaerobiosis.

According to our results (Table 1), the yield of sugar conversion into biomass was 0.033 g/g when the medium did not contain malic acid and was never more 0.025 g/g when malic acid was added to the medium. Contrary to De Queiros and Pareilleux (1990), we conclude that malic acid is not assimilated into biomass since $Y_{X/S}$ was decreased by the addition of malic acid at all concentrations.

The yield of sugar conversion into ethanol did not change with initial concentration of malate. For our conditions of culture, the pO_2 was about 50% of saturation during the time course of the culture. Therefore the malate should be entirely metabolized into CO_2 and H_2O by the yeasts, in agreement with the information in the literature.

To confirm these results, experiments were performed in erlenmeyer flasks for two concentrations of glucose, in the presence or absence of malate (Table 2). For the four conditions both substrates (malate and sugar) were totally removed. Production of biomass was slightly decreased in the presence of malate showing that this substrate was not a source of carbon integrated in the cellular materials. Consumption of 8 g/l of malic acid led to an additional production of 2 g/l of ethanol independent of the concentration of glucose. It is generally acknowledged that for cultures of microorganisms in erlenmeyer flasks the pO_2 near saturation at the beginning rapidly falls to zero. In anaerobiosis, the maximum theorical yield is 2.75 g ethanol produced for 8 g malate consumed (Mayer and Temperli 1963). So, we can assume that for this experiment most of the acid was transformed into ethanol because of the very weak aeration (oxygen limitation). Comparing both previous experiments we can confirm that the yield of malate conversion into ethanol depends on the intensity of aeration.

Table 1. Effect on growth, consumption of malate, ethanol and biomass yield of initial malate concentration

Initial malate conc (g/l)	μ (h^{-1})	dM/dt (g/l/h)	ν_M (g/g/h)	$Y_{P/S}$ (%)	$Y_{X/S}$ (%)
0.0	0.27	—	—	43.4	3.3
1.2	0.27	0.08	0.12	44.0	2.4
2.2	0.18	0.12	0.19	45.0	2.3
8.0	0.17	0.40	0.74	42.0	2.2
18.6	0.09	0.74	1.55	44.0	2.4
23.0	0.10	0.82	1.64	44.0	2.5
28.6	0.09	0.68	2.17	43.0	2.5

μ, specific growth rate; dM/dt, rate of malate consumption; ν_M, specific rate of malate consumption (g of acid consumed/g of biomass per hour); $Y_{P/S}$, concentration of ethanol produced/concentration of glucose consumed; $Y_{X/S}$, concentration of biomass produced/concentration of glucose consumed

Table 2. Effect of malic acid on production of biomass and ethanol

Initial glucose conc (g/l)		Difference in ethanol produced (g/l)	Difference in biomass produced (g/l)
41.0	without malate	Control	Control
41.0	with 8 g/l of malate	+2.3	−0.27
21.5	without malate	Control	Control
18.5	with 8 g/l of malate	+1.9	−0.23

The differences of ethanol and biomass produced relative to controls lacking malate are given

Relationship between deacidification and consumption of glucose

We previously showed that 8 g/l of malate added four times to a culture of *S. pombe* during the stationary phase after sugar exhaustion was rapidly eliminated (Taillandier et al. 1988b). At the same time, the viability of the yeast cells was continuously declining.

In a new experiment, we measured the rates of deacidification (global rate, dM/dt, and specific rate, ν_M) in erlenmeyer flasks containing standard medium without glucose (Fig. 1). The flasks were inoculated with different concentrations of resting cells of *S. pombe* taken from a culture during the stationary phase. Dur-

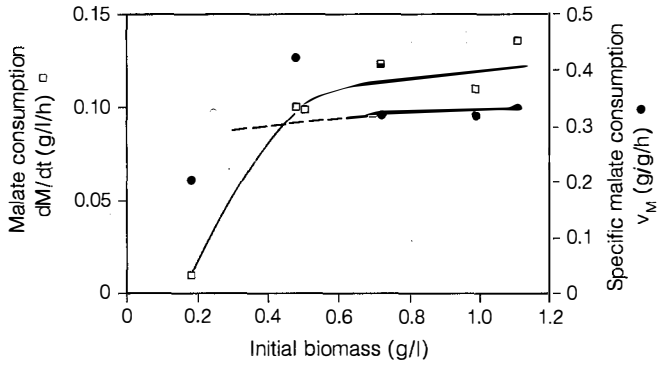

Fig. 1. Rates of deacidification for different concentrations of initial viable biomass

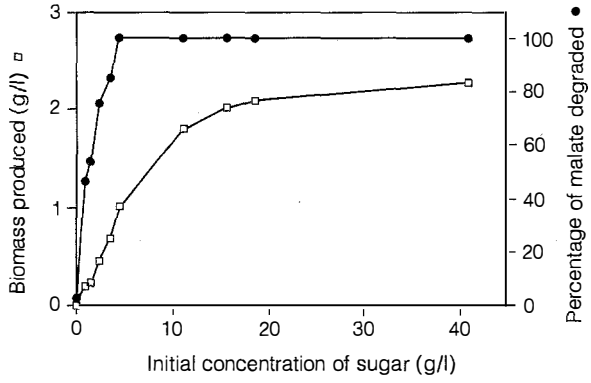

Fig. 2. Culture of *Schizosaccharomyces pombe* on synthetic medium containing 8 g/l of malate and variable concentrations of glucose

ing malate consumption, cell viability gradually decreased and, after 30 h, all the cells were dead. For the culture remaining in the stationary phase without addition of malate, the viability stayed at about 95% for 70 h after exhaustion of the sugar.

From 0.7 g/l of biomass, the rates of deacidification were maximal and constant. We can deduce from this experiment that the degradation of malic acid by non-growing cells without concomitant consumption of sugar is possible but induces loss of viability of *S. pombe*. We can suppose that to consume more than 8 g/l of malic acid in the absence of sugar with the same maximum rate a higher concentration of biomass would be necessary.

Degradation of 8 g/l of malic acid was then studied for different initial concentrations of glucose, varying from 0 to 41 g/l (Fig. 2). Experiments were carried out in erlenmeyer flasks inoculated with 0.15 g/l of biomass each from the same culture. In all cases, glucose was completely consumed. We noted that no biomass was produced in the absence of glucose but a small amount of the acid was degraded. According to Kunkee (1967), malate is neither a carbon source nor an energy source. We observed that the deacidification activity is not directly linked to the amount of sugar metabolized but to the concentration of viable biomass.

In conclusion, in opposition to De Queiros and Pareilleux (1990), and in agreement with Mayer and Temperli (1963), Peynaud et al. (1964), Vezinhet and Barre (1982) we showed that malic acid is not integrated into biomass after its catabolism by *S. pombe*. Yield of conversion into ethanol of this substrate depends on the intensity of aeration. Our results show that consumption of malate without consumption of sugar is possible but affects cell viability, suggesting that the metabolism of the acid by *S. pombe* requires energy (maybe for the transport of the acid into the cells). We propose the hypothesis that in the case of non-growing cells lacking an energy source, deacidification activity consumes energy reserves thereby limiting maintenance processes and decreasing the cell viability. Despite this new data, information about *S. pombe* metabolism is still lacking and we continue investigations in this field, in particular the stoichiometry and transport of malic acid using radioactive substrates.

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