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Deacidification by *Schizosaccharomyces*: interactions with *Saccharomyces*

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Abstract

The objective of this work was to study the partial consumption of malic acid in a semi-synthetic medium by *Schizosaccharomyces* yeast under wine-making conditions. The aim was to control the extent of deacidification by stopping the activity of *Schizosaccharomyces* by the addition of *Saccharomyces* yeast at different delayed intervals of time. This was thought possible because the *Schizosaccharomyces* yeasts are much more slower than the *Saccharomyces* yeasts and may be inhibited by the latter. In fact, the opposite was observed. *Schizosaccharomyces* exhibited an amensal effect against *Saccharomyces*: the *Saccharomyces* growth was inhibited by *Schizosaccharomyces*, this inhibition being proportional to the *Schizosaccharomyces* concentration, reversible, and sensitive to temperature. Such interactions similar to a killer effect have not been described before. At the same time a competition towards sugar was noted, being negative for *Schizosaccharomyces* since these yeasts have slower metabolic rates. Nevertheless, total demalication was nearly always possible because sufficient concentrations of *Schizosaccharomyces* were always reached. The delayed inoculation of *Saccharomyces* after *Schizosaccharomyces* was not a good solution to obtain partial deacidification.

Keywords: *Schizosaccharomyces*; *Saccharomyces*; Mixed culture; Inhibition; Amensalism; Competition; Deacidification

1. Introduction

Malic acid is one of the two major acids of grape musts and its removal from wines is often necessary. *Schizosaccharomyces* yeasts are microorganisms naturally occurring in grape musts flora but not developing in usual wine-making conditions. The species *pombe* is able to metabo-

lize malic acid to ethanol with a high efficiency and, therefore, can be used for biological deacidification of wines instead of the usual method that is malo-lactic fermentation (MLF) (Peynaud et al., 1964; Gallander, 1977; Munyon and Nagel, 1977; Beelman and Gallander, 1979). In order to produce high quality wine it is recommended that *Schizosaccharomyces pombe* is used for demalication and *Saccharomyces cerevisiae* for alcohol formation in a second step (Yang, 1973; Munyon and Nagel, 1977). When only partial deacidifica-

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tion is desired for organoleptic reasons the action of *Schizosaccharomyces* yeasts has to be limited (Magyar and Panyik, 1989). Two possibilities exist:

The first one is a mixed product formation by *Schizosacch. pombe* and *S. cerevisiae* at different percentages of each yeast (Ethiraj et al., 1983; Charpentier et al., 1985); this method is difficult to control in industrial conditions since the mixing of both yeasts must be done with a great accuracy, and, also, it may be difficult to predict the evolution of the percentage of both yeasts during batch operation.

The second one is a two-step inoculation procedure: a first partial sugar degradation by *Schizosacch. pombe* followed by its removal and reinoculation with *S. cerevisiae* (Snow and Gallander, 1979; Yokotsuka et al., 1993). The main disadvantage of this approach is that a filtration or centrifugation step is necessary.

The objective of the work presented here is to perform partial demalication by *Schizosacch. pombe* and alcohol formation by *S. cerevisiae*, and to try to control the extent of deacidification by *Schizosacch. pombe* without removing it from the medium by inoculating *S. cerevisiae* at different delayed time intervals. According to the literature (Gallander, 1977; Snow and Gallander, 1979; Satyanarayana et al., 1988), *Schizosacch. pombe* is inhibited by *S. cerevisiae* and thus its deacidification activity is expected to be stopped without its removal from the medium being necessary. This vinification method would be more practical. The originality of our study is the determination of the growth kinetics of both yeasts and the measurement of their viability during mixed cultures.

2. Materials and methods

2.1. Yeasts strains

The *Schizosaccharomyces pombe* strain used was the G2 one obtained from the Institut Coopératif du Vin (Montpellier, France).

The *Saccharomyces cerevisiae* strain used was the commercial Lalvin K1 yeast, provided by

Lallemand Inc. (Montréal, Canada) as dry active yeast.

2.2. Media

Conservation medium

For *Schizosacch. pombe* the maintenance medium contained per l of water: 50 g glucose, 4 g asparagine, 4 g yeast extract, 5 g KH_2PO_4 , 0.4 g MgSO_4 , 20 g agar, pH 4.

For *S. cerevisiae* the maintenance medium consisted of the following per l of water: 20 g glucose, 10 g yeast extract, 20 g agar.

Strains were maintained on agar slants at 4°C.

Culture medium

The composition of the medium for batch cultures was per l of water: 100 g glucose, 4 g yeast extract, 4 g asparagine, 5 g KH_2PO_4 , 0.4 g MgSO_4 , 8 g malic acid, pH 3.

2.3. Batch cultures

A preculture was first prepared in 250-ml Erlenmeyer flasks containing 100 ml of culture medium with only 50 g l^{-1} of glucose. It was incubated at 30°C with magnetic stirring for 15 h for *S. cerevisiae* and 24 h for *Schizosacch. pombe*.

Cultures were performed in 2-l reactors containing 1.7 l of medium autoclaved at 120°C for 20 min. The temperature was regulated at 20°C and the agitation rate was 250 rpm. Reactors were inoculated with precultures in order to obtain 3×10^6 viable cells of *Schizosacch. pombe* per ml. Inoculation of *S. cerevisiae* (also 3×10^6 viable cells per ml) was delayed at different time intervals (= *Td*) of 4, 8, 12 and 16 h. Control cultures were run for each yeast (pure cultures).

2.4. Analytical methods

Biomass determination

The concentration of cells for both yeasts was determined by numeration with a hemacytometer (Thoma slide) under a microscope. Since the yeasts are morphologically very different they could be counted separately. *S. cerevisiae* appears as round cells with buds, whereas *Schizosacch.*

pombe is oblong and multiplies by fission. The number of counted cells was between 200 and 400 for each observation, and the observations were repeated twice. The experimental error for the concentration measurement was thus 10% (Lange et al., 1993).

Viability was estimated by viable staining with methylene blue (100 mg of MB in 1 l of 2% (w/v) tri-sodium citrate). After mixing volume to volume with the MB solution and a staining time of 10 min, the cellular suspension was observed under a microscope. Dead cells appeared blue, while viable cells remained unstained. The experimental error on viability measurements was 2% (Lange et al., 1993).

Sugar determination

Glucose concentration was estimated by the dinitrosalicylic acid method for reducing sugars (Miller, 1959).

L-Malic acid determination

L-Malic acid was assayed by the enzymatic method of Boehringer Mannheim (Meyan, France).

Definitions

The percentage of viability is defined as the ratio of viable cells to total cells for each yeast.

The specific growth rate is defined as: $\mu = 1/X \cdot dX/dT$ (h^{-1}) where X is the concentration of viable cells in millions per ml and T time in h. X as a function of time is obtained by multiple regression of the experimental growth curves. The regression program is based on the Reinsch method: the regressed curve is a 3rd order function and the sum of the square of the differences with the experimental points is minimized. dX/dT is the derivative of this regressed curve. μ_{max} is the highest value of μ .

3. Results

This study was conducted on a semi-synthetic medium of known composition to avoid lack of reproductibility sometimes encountered with experiments on grape musts. The composition of

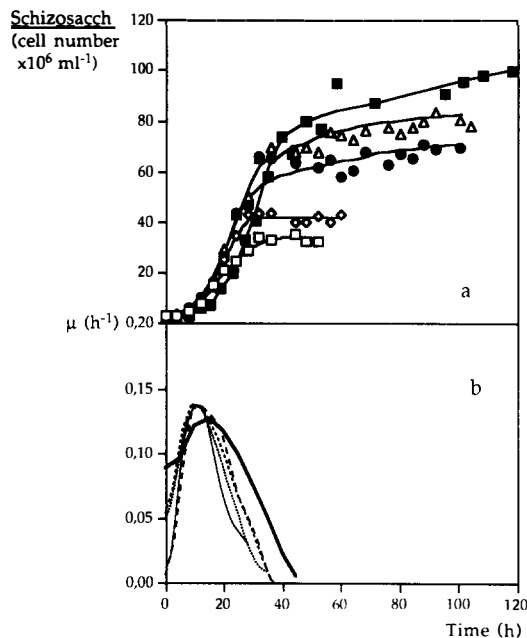


Fig. 1. (a) Growth curves of *Schizosacch. pombe*: pure (control ■) and mixed cultures: $T_d = 4$ h (■), $T_d = 8$ h (◇), $T_d = 12$ h (●), $T_d = 16$ h (▲). Experimental data (symbols) and regressed curves (lines). (b) Evolution of specific growth rate of *Schizosacch. pombe* as a function of time for the control (—) and different values of T_d : $T_d = 4$ h (---), $T_d = 8$ h (···), $T_d = 12$ h (-·-·), $T_d = 16$ h (- - -)

the synthetic medium was defined so that it gave similar kinetics as the grape must (Taillandier, 1990). During batch cultures which lasted 50 to 100 h, samples were collected at 4-h intervals to establish kinetics of growth, consumption of malic acid and glucose. The *Schizosacch. pombe* strain studied was previously shown to be very efficient for malic acid elimination (Taillandier and Strehaiano, 1991). In order to obtain only partial demalication we chose to slightly delay the inoculation by *S. cerevisiae* after the inoculation by *Schizosacch. pombe*. The time of delay (T_d) was 4 to 16 h.

3.1. Yeast growth

Schizosacch. pombe

For these yeasts growth curves are shown in Fig. 1a. Viability was always greater than 95%. The maximum biomass concentration reached de-

depends on the time of addition of *S. cerevisiae* yeasts. The shorter the T_d is, the further from control the amount of biomass produced is. For $T_d = 4$ h the maximum biomass concentration is reduced approx. three times.

From these data, specific growth rates μ were calculated as explained in the Materials and methods. They are plotted vs. time in Fig. 1b. Evolution of μ is similar for the control and for mixed cultures with different T_d : the maximum value of μ , μ_{\max} was about 0.15 h^{-1} and was reached after about 10 h. The growth rate of *Schizosacch. pombe* was not affected by *S. cerevisiae*. Thus, there was not inhibition, but, more likely, competition between these two yeasts.

S. cerevisiae

Contrary to *Schizosacch. pombe*, the viability of *S. cerevisiae* was not always good. Data can be found in Fig. 2. Growth curves considering only viable cells are established in Fig. 3a, time zero corresponding to time of inoculation of *S. cerevisiae*. For mixed cultures the amount of biomass produced was very low compared with the pure culture and decreased when T_d was increased. Two growth phases can be distinguished. Specific growth rates were calculated and plotted as a function of time for all experiments (Fig. 3b). The two growth phases are found again, each one giving a different value for μ_{\max} . The faster growth always occurred in the first phase where μ_{\max} was

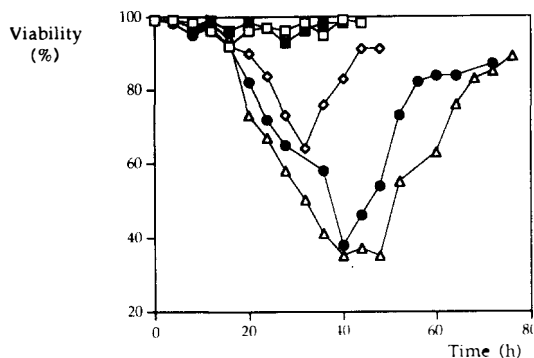


Fig. 2. Percentage of viability of *S. cerevisiae* K1 vs. time for different T_d for mixed cultures: 4 h (\square), 8 h (\diamond), 12 h (\bullet), 16 h (\circ) and for the control culture (\blacksquare). $t = 0$ corresponds to the time of inoculation of *S. cerevisiae* for each cases.

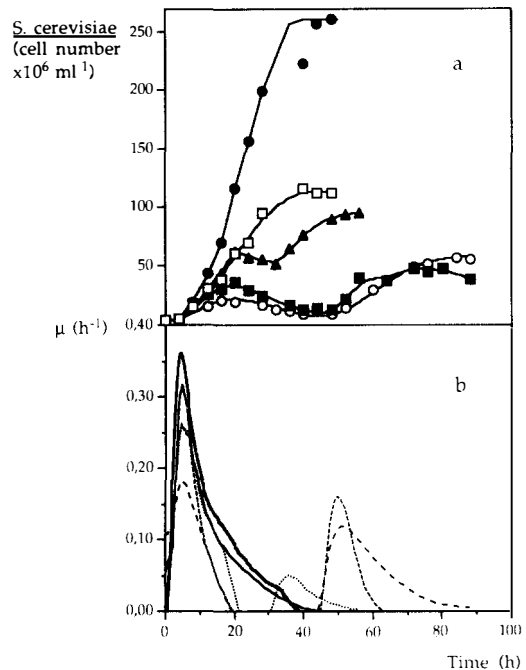


Fig. 3. (a) Growth curves of *S. cerevisiae*: pure (control \bullet) and mixed cultures: $T_d = 4$ h (\square), $T_d = 8$ h (\blacktriangle), $T_d = 12$ h (\blacksquare), $T_d = 16$ h (\circ). Experimental data (symbols) and regressed curves (lines). (b) Evolution of specific growth rate of *S. cerevisiae* as a function of time for the control (---) and different values of T_d : $T_d = 4$ h (---), $T_d = 8$ h (\cdots), $T_d = 12$ h (---), $T_d = 16$ h (---).

greater when T_d was shorter. $\mu_{\max} = 0.36 \text{ h}^{-1}$ for the control and 0.18 h^{-1} for $T_d = 16$ h. The decrease of μ_{\max} compared with the control shows that the growth of *S. cerevisiae* was greatly decreased by *Schizosacch. pombe* yeasts. Moreover, the viability, being very low for high values of T_d , could indicate that a toxin excreted by *Schizosacch. pombe* may be present in the medium, as suggested by King and Beelman (1986) in the case of interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos*.

3.2. Substrates consumption

Glucose

Glucose consumption is shown in Fig. 4a for all cultures (pure and mixed). For each experiment $t = 0$ is the time of inoculation of the

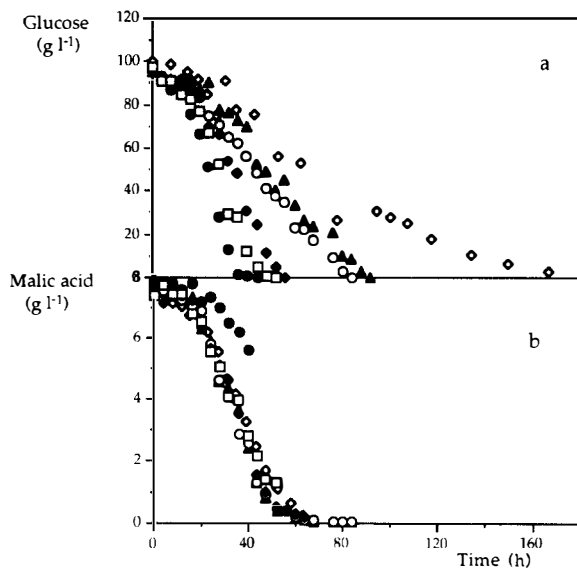


Fig. 4. (a) Kinetics of sugar consumption for pure (*S. cerevisiae*: ● and *Schizosacch.*: ◇) and mixed cultures: $T_d = 4$ h (□), $T_d = 8$ h (◆), $T_d = 12$ h (○), $T_d = 16$ h (▲). (b) Kinetics of malic acid consumption for pure (*S. cerevisiae*: ● and *Schizosacch.*: ◇) and mixed cultures: $T_d = 4$ h (□), $T_d = 8$ h (◆), $T_d = 12$ h (○), $T_d = 16$ h (▲). $t = 0$ is the time of inoculation of *Schizosacch.*

Schizosacch., that is to say, the same in all cases. The time necessary for sugar exhaustion is directly proportional to T_d for mixed cultures, the shortest time being for the *S. cerevisiae* control and the longest for the *Schizosacch. pombe* control. This time can also be correlated to the total amount of both yeasts. It is shorter when more yeast cells are present.

Malic acid

Fig. 4b represents malic acid consumption kinetics for all batch cultures. For each experiment $t = 0$ is the time of inoculation of the *Schizosacch.*, that is to say, the same in all cases. It can be observed that for the *Schizosacch. pombe* control and for all mixed cultures except for $T_d = 4$ h, malic acid was entirely consumed at the same rate, showing that *Schizosacch. pombe* played a preponderant role in deacidification activity. For the *S. cerevisiae* control only 2.6 g l^{-1} of malic acid were consumed. For $T_d = 4$ h, 6.7 g l^{-1} was consumed, corresponding to 84% of the malic

acid initially present. In this case, the concentration of *Schizosacch. pombe* was only 32×10^6 cells per ml, this not sufficient to degrade 8 g l^{-1} of malic acid. Partial demalication is thus obtained only for very small values of T_d and would not be easy to control.

4. Discussion

The marketed *S. cerevisiae* K1 strain chosen for studying interactions with *Schizosacch. pombe* in this work was a killer one, possessing the killer factor K2 as characterized by Lallemand Inc. Killer yeasts are able to secrete a proteinaceous toxin into the medium which can kill sensitive yeast strains. The killer phenomenon is widespread among laboratory and industrial (brewing, wine-making) strains of the genus *Saccharomyces* and also some other genera (Pasqual et al., 1990; Van Vuuren and Jacobs, 1992; Shimizu, 1993).

Our results show that *Schizosacch. pombe* G2 is not sensitive to *S. cerevisiae* K1, since neither its viability nor its growth rate were affected during mixed cultures. The specific growth rate was similar to the one obtained for the control but less biomass was produced. This indicates a competition towards sugar due to the low value of μ_{\max} : 0.15 h^{-1} for *Schizosacch. pombe* and 0.18 h^{-1} to 0.36 h^{-1} for *S. cerevisiae*.

On the contrary, *S. cerevisiae* K1 exhibits a sensitivity to *Schizosacch. pombe* G2 since both its viability and μ_{\max} strongly decrease during mixed cultures, indicating an action of an inhibitor produced by *Schizosaccharomyces* (King and Beelman, 1986). This sensitivity was higher when T_d was raised, indicating that the toxic effect of *Schizosacch. pombe* was linked to its biomass concentration. The inhibition of *S. cerevisiae* can not be explained either by the effect of the ethanol concentration which is never above 50 g l^{-1} , or by the effect of pH since as a consequence of the degradation of malic acid the pH of the medium is raised up to 3.8. Thus, the interaction between these two yeasts is amensalism (Bailey and Ollis, 1986) and the manifestations we observed were very similar to a killer

effect of *Schizosacch. pombe* against *S. cerevisiae*. In general, for killer yeasts the toxic protein is excreted during the growth phase (Sawant et al., 1988; Van Vuuren and Jacobs, 1992; Portugal et al., 1994) and this is why the effect against *S. cerevisiae* was stronger when *Td* was raised. Moreover, it has been shown that killer proteins generally have a high activity for pHs of about 3.5 and even 3 (Barre, 1984; Pasqual et al., 1990; Van Vuuren and Jacobs, 1992; Shimizu, 1993). Nevertheless, competition certainly exists too. The second growth phase observed for *S. cerevisiae* corresponding to the raising of viability suggests that this killer-like phenomenon was reversible. The killer protein may be denaturated by proteases present in the medium as suggested by Shimizu (1993) and Portugal et al. (1994), which allowed a second growth phase of *S. cerevisiae* after recovery.

Killer activity against *S. cerevisiae* has not been previously reported in the genus *Schizosaccharomyces* (Philliskirk and Young, 1975; Pasqual et al., 1990; Van Vuuren and Jacobs, 1992). The same experiments (data not reported) were also performed at 30° C and, with another *S. cerevisiae* strain 522 Da which is a K1 sensitive strain, at both temperatures. 30° C is the temperature of red wine making, whereas 20° C is used for white wines. For the 522 Da strain the same phenomenon was observed: an amensal effect of *Schizosacch. pombe* against *S. cerevisiae*. At 30° C, this effect was also observed for both *S. cerevisiae* strains but was less strong, indicating a sensitivity of this effect to temperature, like the killer effect.

In order to confirm that the phenomenon we observed was a killer effect we carried out the classical method of methylene blue agar (Woods and Bevan, 1968; Somers and Bevan, 1969) at 30° C. This method did not show very clear zones of inhibition, but Portugal et al. (1994) showed that it was not a very sensitive technique. However, to be sure that the phenomenon reported was a killer effect, the killer protein must be isolated from the medium.

Partial demalication was obtained only when *Td* was very short at 20° C and was not obtained at 30° C. This method is not convenient to this aim because the *Schizosacch. pombe* we used was

very efficient for malic acid degradation contrary to others strains studied in the past (Gallander, 1977; Snow and Gallander, 1979). An alternative solution could be the use of immobilized *Schizosaccharomyces*, as has been suggested elsewhere (Taillandier et al., 1994).

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