# *In Vitro* Specific Activities of Alcohol and Aldehyde Dehydrogenases from Two Flor Yeasts during Controlled Wine Aging

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Two flor yeast strains of *Saccharomyces cerevisiae* (*S. cerevisiae* strains capensis and bayanus) which form velum on the surface of sherry wine during biological aging have been used. Aldehyde and alcohol (isoenzymes I and II) dehydrogenases were detected *in vitro* during the entire wine-aging process in the flor yeast strains. All enzymatic activities decreased during the first 155 days of wine aging, and after this period, an increase was observed. Ethanol consumption in the wine and the specific activity of alcohol dehydrogenase I were independent of the *S. cerevisiae* strain. The greater activity of alcohol dehydrogenase II is directly related to the higher acetaldehyde production by *S. cerevisiae* race bayanus in the wine. This strain has a slower and prolonged growth in the flor film, which permits a continued accumulation of acetaldehyde in the wine. The higher activity of aldehyde dehydrogenase in capensis strain during the flor formation may be related to the production and consumption of large amounts of acetic acid, which involved a more abundant and accelerated cellular growth. We suggest that part of the acetaldehyde might be converted into ethanol during wine aging by alcohol dehydrogenase I in order to maintain a normal redox balance.

**Keywords:** *Flor yeast; alcohol dehydrogenase; aldehyde dehydrogenase; biological aging; sherry wine*

## INTRODUCTION

The procedure for making fino sherry wines consists of two consecutive steps. The first step consists of grape must fermentation, which produces a "young" wine using fermentative yeasts. The next step is a post-fermentative treatment, in which this young wine is fortified with approximately 15.5% (v/v) ethanol before subsequent aging (Domecq, 1989). Wine aging occurs in the so-called solera system (different aged sets of oak casks) under a flor film of yeasts, which grow on the surface of the wine. Flor yeasts belong to the *Saccharomyces* genus although they are physiologically different from typical fermentative yeasts. These are unable to grow aerobically in wine, but by contrast, flor yeasts can grow well aerobically and have a high fermentative capability in musts (Suarez and Iñigo, 1990). The wine under flor film is submitted to some special conditions as a consequence of the oxidative metabolism of yeast and the reducing environment created in the wine. A reducing environment is produced because the dissolved oxygen is consumed by flor yeast and wine is prevented by the flor film from the uptake of new oxygen from the air (García-Maiquez, 1988). The combination of both actions causes transformations of the substances contained customarily in wines that result in the apparition of different and unique organoleptic flavors and aromas.

In the last few years, several studies have been carried out to determine the relationship between some enzymatic activities of wine yeast and their products formed during wine fermentation (Millan and Ortega, 1988; Rosi *et al.*, 1989; Millan *et al.*, 1990; Mauricio *et al.*, 1993). In the present study, we have measured and compared the specific activities of $NADP^+$-linked aldehyde dehydrogenase (ALDH; aldehyde:$NADP^+$ oxidoreductase, EC 1.2.1.4) and isoenzymes I and II of alcohol dehydrogenase (ADH; alcohol:$NAD^+$ oxidoreductase, EC 1.1.1.1) in two strains of *Saccharomyces cerevisiae* during the flor film formation on wine. In addition, we have tried to establish a relationship between the *in vitro* enzymatic activities mentioned above and their products formed in wine, with the objective to choose flor yeast strains, which accelerate the biological aging process of the fino wines. The physiological roles of the two isoenzymes (ADH I and II) and the enzyme ALDH-$NADP^+$ during wine aging are also discussed.

## MATERIALS AND METHODS

**Yeast Strains.** *S. cerevisiae* race bayanus F12 and *S. cerevisiae* race capensis G1, which are typical flor film-forming strains, were used in this study. The yeast strains were isolated from the surface of wine with 15.5% (v/v) ethanol contained in oak casks in a wine cellar in the Montilla-Moriles region. Isolated colonies were selected on YM agar plates (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, and 2.5% agar, pH 6.5) and grown to pure culture. Cells were stored in test tubes on YEPD agar (0.3% yeast extract, 0.5% peptone, 1% glucose, and 2.5% agar, pH 6.5) at 4 °C in our laboratory. These strains were identified and characterized according to Kreger-van Rij (1984) following the usual criteria for fermentation and assimilation of different carbon and nitrogen sources. On the basis of maltose fermentation, bayanus was positive and capensis was negative. These strains are representative of flor films from the Montilla-Moriles region (southern Spain). Criteria and tests for their selection have been reported by us in previous papers (Guijo *et al.*, 1986; Moreno *et al.*, 1991).

**Wine.** The wine used was obtained by spontaneous fermentation of musts from 1993 vintage in Bodegas Alvear in the Montilla-Moriles region. The wine contained 15.5 ± 0.06% (v/v) ethanol, 1.6 ± 0.1 g/L reducing sugars, 84.8 ± 3.1 mg/L acetaldehyde, and 6.03 ± 0.04 mequiv/L acetic acid. Its pH was 3.16 and its dissolved oxygen content 7.50 ± 0.17 mg/L, measured by a dissolved oxygen meter (Crison, model Oxy-

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92). The wine was sterilized by filtration through a Seitz-Supra EK filter from Seitz (D-6550, Bad Kreuznach, Germany).

**Inoculation and Biological Aging Conditions for the Wine.** In the laboratory, wine was divided into 32 batches (one for sample) that were placed in 5 L Erlenmeyer flasks with 5 L of wine, at the same surface/volume ratio as in the cellar barrels (0.016 $cm^{-1}$).

The inocula of the yeasts were grown in YM medium supplemented with 5% (w/v) glucose at 28 °C for 48 h, collected by centrifugation at 5000*g* for 5 min, and washed once with distilled sterile water. The yeasts were resuspended in a known volume of sterile wine; 24 Erlenmeyer flasks were inoculated with $1 \times 10^6$ viable yeast cells/mL of wine (12 with capensis and 12 with bayanus) and plugged with hydrophobic cotton. The remaining Erlenmeyer flasks were used as controls without inoculation. Aging processes were conducted at 18 °C in the dark.

Three independent experiments were performed for each *S. cerevisiae* strain. The results given in this study are the averages of these experiments.

**Preparation of Yeast Cell-Free Extract for Enzymatic Activities.** Cells from the flor film were collected by suction from the surface of each Erlenmeyer flask. The number of total and viable cells was obtained by counting under a light microscope in a Thoma chamber following staining of the cells with methylene blue (European Brewery Convention, 1977).

Cells were collected by centrifugation (5000*g*, 5 min) and washed once in 0.1 M phosphate buffer (pH 7), containing 0.01 M EDTA. Pellets of yeast cells were resuspended in the same buffer. Aliquots of 0.5−1 mL containing $2 \times 10^8$ cells were vortexed for 30 s with 1 g of glass beads (Sigma; 0.5 mm diameter) and cooled for 30 s on ice−water. This procedure was always repeated seven times. The cellular debris and glass beads were removed by centrifugation (5000*g*, 5 min), and the supernatant was analyzed for enzyme activities. All procedures were performed at 4 °C. Protein concentration in the supernatant was determined by the method of Bradford (1976), using bovine $\gamma$-globulin (Sigma) as standard.

**Assay of Enzyme Activities.** ADH activity was assayed spectrophotometrically according to the method of Racker (1950) as modified by Tamaki *et al.* (1977) and Johansson and Sjöström (1984). ADH activity was measured at 25 °C in cuvettes with 20 $\mu$L of crude extract and 2.98 mL of reaction mixture containing 100 mM sodium pyrophosphate buffer (pH 8.5), 2 mM $\beta$-mercaptoethanol, 70 mM semicarbazide, and 1 mM $NAD^+$. The reaction was started by addition to the experimental cuvette of 100 mM ethanol. Enzyme units are expressed as $\mu$mol of NADH formed/min, under the conditions of the assay. Differentiation between the isoenzymes ADH I and II was accomplished by thermal denaturation of the former from the crude extracts according to Schimpfessel (1968) and Fernández *et al.* (1972). The activity of each of the two isoenzymes was determined in the extracts by assaying alcohol dehydrogenase activity before (total ADH activity) and after (ADH II activity) incubation at 61 °C for 15 min. The activity of ADH I was obtained by subtracting the activity of isoenzyme II from the total activity.

For the assay of the $NADP^+$-linked aldehyde dehydrogenase (ALDH-$NADP^+$), 20 $\mu$L samples were mixed in cuvettes with 1.98 mL of reaction mixture containing 50 mM glycylglycine buffer (pH 7.7), 15 mM $MgCl_2 \cdot 6H_2O$, 3 mM pyrazole, and 65 $\mu$M $NADP^+$. The reaction was started by addition of 0.1 mL of 0.01 M acetaldehyde to the experimental cuvette.

**Quantification of Ethanol, Acetaldehyde, and Acetic Acid in the Wine.** Ethanol was determined by the method of Crowell and Ough (1979), and acetaldehyde and acetic acid were determined by the enzymatic methods developed by Boehringer Mannheim GmBH Barcelona (Spain).

## RESULTS

**Flor Yeast Growth.** The growth pattern in the flor velum differed between the two *S. cerevisiae* strains. The capensis strain produced a noticeable film about 20 days after beginning the wine aging, whereas the bayanus strain required about 35 days. The maximum number of flor yeast cells during the wine aging was higher for *S. cerevisiae* race capensis than for bayanus (Figure 1). As a result, the flor films produced were also different. Capensis formed a thick flor film (0.6 cm) containing both viable and nonviable cells that reached its maximum cell density ($96.47 \times 10^7$ cells/$cm^2$) 150 days after inoculation. On the other hand, cell density in the bayanus flor peaked at $8.15 \times 10^7$ cells/$cm^2$ at 35 days. This formed a thin flor film (0.1 cm) consisting mainly of viable cells; however, a large number of inactive cells settled in the bottom of the Erlenmeyer flasks, so the total number of cells in the flor only accounted for a small fraction of total cells in the wine. After formation of the flor film (20 and 35 days for capensis and bayanus strains), the dissolved oxygen concentration was $0.6 \pm 0.1$ mg/L for both yeast strains.

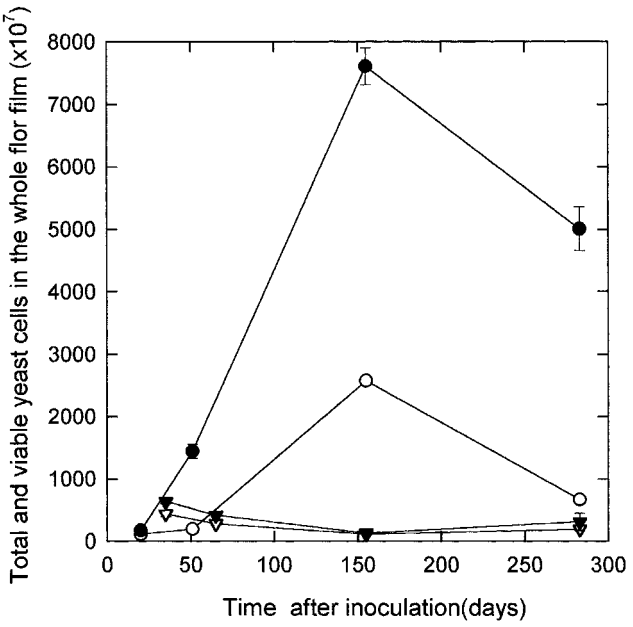


**Figure 1.** Total (●,▼) and viable (○,▽) number of yeast cells in the whole flor film during wine-aging processes with *S. cerevisiae* race capensis (●,○) and *S. cerevisiae* race bayanus (▼,▽).

***In Vitro* Specific Activity of the Isoenzymes ADH I and II during Wine Aging: Concentrations of Ethanol and Acetaldehyde in Wine.** During the first 155 days of wine aging, the specific activity of ADH I in the yeasts and the ethanol concentration in the wine decreased. A subsequent increase in ADH I activity was found after 155 days in both strains (Figure 2). Therefore, the ethanol consumption in wine and the specific activity of the isoenzyme ADH I were not strain specific.

In both strains, parallel evolution of ADH II activity was observed throughout the aging process. However, the enzyme from *S. cerevisiae* race bayanus reached higher levels, which can be directly related to the higher amount of acetaldehyde released into the wine (Figure 3).

***In Vitro* Specific Activity of $NADP^+$-Linked ALDH during Wine Aging: Concentration of Acetic Acid in Wine.** Figure 4 shows the specific activity of the ALDH-$NADP^+$ in the yeasts and the acetic acid concentration in the wine during its biological aging. The ALDH-$NADP^+$ activity was higher in capensis strain during the flor formation (20 days) than in bayanus. By contrast, when the flor formation was finished, from 35 days, ALDH activity reached higher

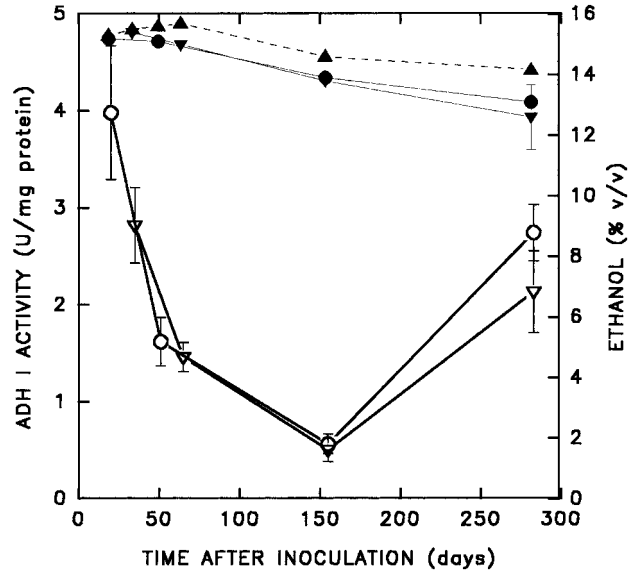


**Figure 2.** Specific activity of alcohol dehydrogenase I (ADH I, ○,▽) and ethanol concentration (●,▼) in sherry wine during aging by *S. cerevisiae* race capensis (●,○) and *S. cerevisiae* race bayanus (▼,▽). Ethanol from the noninoculated sterile-filtered control (▲).

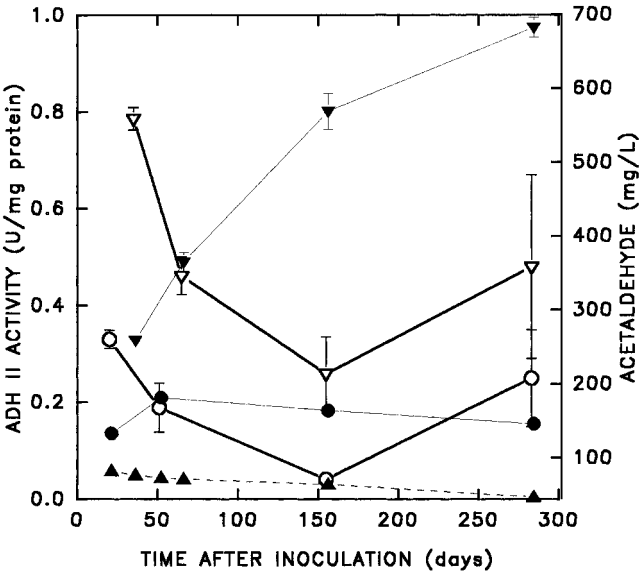


**Figure 3.** Specific activity of alcohol dehydrogenase II (ADH II, ○,▽) and acetaldehyde concentration (●,▼) in sherry wine during aging by *S. cerevisiae* race capensis (●,○) and *S. cerevisiae* race bayanus (▼,▽). Acetaldehyde from the noninoculated sterile-filtered control (▲).

levels in bayanus. Apparently there was a direct relationship between the activity observed for the ALDH and the amount of acetic acid found in the wine.

**Production and Consumption of Ethanol, Acetaldehyde, and Acetic Acid by the Two Flor Yeasts.** During flor formation, *S. cerevisiae* race capensis showed high specific rates for ethanol and acetate consumption and for acetaldehyde and acetate production (Table 1). After flor formation, rates were very low and constant during the remaining process. By contrast in bayanus, rates were very low during the flor formation, and after flor formation, they increased to a maximum value when the growth was finished (Table 1).

Both capensis and bayanus strains consumed a similar quantity of ethanol (Figure 2), but bayanus produced more acetaldehyde, a large part of which was released into the wine. This strain produced and

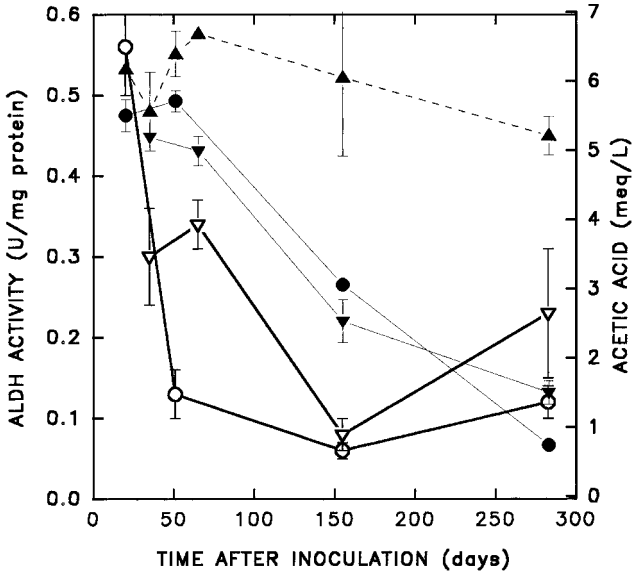


**Figure 4.** Specific activity of aldehyde dehydrogenase (ALDH-$NADP^+$, ○,▽) and acetic acid concentration (●,▼) in sherry wine during aging by *S. cerevisiae* race capensis (●,○) and *S. cerevisiae* race bayanus (▼,▽). Acetic acid from the noninoculated sterile-filtered control (▲).

consumed less acetate, especially during flor formation. *S. cerevisiae* race capensis produced little acetaldehyde, which accumulated in the wine until 50 days. However, this strain produced and consumed more acetate, especially during the flor formation step (Figures 3 and 4).

## DISCUSSION

During alcoholic fermentation, wine yeasts consume the sugars of grape must and produce the major end products ethanol and $CO_2$, as well as a quantity of flavor active secondary products. During aging of wine, the metabolic activity of flor yeasts is greatly increased as a consequence of the shift from anaerobic to aerobic metabolism. This biochemical transformation results in a partial consumption of many products of fermentation such as ethanol, glycerol, acetic acid, free amino acids, and others and in an increase of other compounds such as acetaldehyde, 2,3-butanediol, and ethyl lactate (García-Maiquez, 1988).

Because glucose is absent during wine aging, gluconeogenesis is necessary for synthesis of hexose monophosphates by flor yeasts growing under these conditions. Thus, the ethanol decrease observed in the fino dry wine during biological aging may be explained by the utilization of the ethanol by yeasts as a carbon source, following a metabolic pathway that implicates a first oxidation to acetaldehyde by alcohol dehydrogenase II with generation of NADH and a second oxidation to acetic acid catalyzed by an aldehyde dehydrogenase which is active with both $NAD^+$ and $NADP^+$ (Tamaki and Hama, 1982). Acetate metabolism proceeds by action of acetyl-CoA synthetase (Gancedo and Serrano, 1989) followed by the operation of the citrate and glyoxylate cycles. Thus, growth under these conditions depends on the operation of the tricarboxylic acid (TCA) cycle. The principal source of the cycle is acetyl-CoA, although some amino acid carbon skeletons may also enter the cycle at different points. Accordingly, utilization of amino acids by the yeasts from sterile wine during aging has been observed (Mauricio and Ortega, 1997).

**Table 1. Specific Rates for Consumption of Ethanol and Acetate and Production of Acetaldehyde and Acetate (mg × day$^{-1}$ × 10$^{-8}$ cells)**

| | interval (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *S. cerevisiae* bayanus | | | | *S. cerevisiae* capensis | | | |
| | 0−35 | 35−65 | 65−154 | 153−283 | 0−20 | 20−50 | 50−154 | 154−283 |
| ethanol consumption | 1.76 | 12.60 | 10.00 | 7.40 | 34.00 | 0.92 | 0.15 | 0.22 |
| acetaldehyde production | 1.68 | 12.00 | 9.50 | 7.00 | 32.50 | 0.88 | 0.14 | 0.21 |
| acetate production | 1.77 | 15.80 | 11.80 | 9.40 | 43.40 | 1.10 | 0.20 | 0.28 |
| acetate consumption | 1.88 | 15.90 | 12.40 | 9.50 | 43.90 | 1.10 | 0.21 | 0.29 |

In previous studies, we reported that during fermentations, ADH I from *S. cerevisiae* is synthesized during the early growth stage when the fermenting activity of the yeasts is the highest and they require the greatest enzymatic activity. After this, ADH I biosynthesis is inhibited (Millan *et al*., 1990; Salmon and Mauricio, 1994). Millan and Ortega (1988) reported that the kinetics of formation of ethanol, acetaldehyde, and acetic acid by wine yeasts do not depend directly upon the activity of ADH or ALDH enzymes but upon the relationship between their oxidized and reduced coenzymes and the sugar and acetate metabolism during fermentation. The biosynthesis of ADH I and ALDH-NADP$^+$ takes place basically during the adaptation of the yeasts to the must (first 4 h), while that of ADH II occurs immediately after exponential growth (after 12 h). From the products produced by the yeast, only the specific rate of ethanol production was found to be directly related to the specific activity of ADH I during the first 48 h of fermentation (Millan *et al*., 1990).

The reversible ethanol to acetaldehyde reaction is a generating reaction of NAD$^+$ or NADH depending on its direction. ADH I takes part in the reduction of acetaldehyde to ethanol with regeneration of NAD$^+$. In this study, we have shown that ADH I and II are active during the entire fino wine aging and may play the same physiological role, since ADH II can, under certain circumstances, play a fermentative role (Gancedo and Serrano, 1989). In this way, during biological aging, ADH I isoenzyme might operate in the same direction as ADH II isoenzyme, producing acetaldehyde from ethanol. On the other hand, during biological aging of sherry wines, these become a reducing medium as a consequence of the consumption of dissolved oxygen by the flor yeast, which also prevents the replenishing of oxygen from the outside (Suarez and Iñigo, 1990). So, it is evident that some of the acetaldehyde should be converted into ethanol by ADH I for regenerating NAD$^+$, thus maintaining the normal redox balance. This is in agreement with the high activity of ADH I observed in this study and with Williams (1989) who reported that acetaldehyde is converted to ethanol, acetic acid, and a number of sherry wine components (lactones and nitrogen components) during the second stage of the film yeast growth. In addition, the NAD$^+$/NADH ratio in flor yeasts during fermentation indicates a more balanced cellular redox process than that found in a typical fermentative strain (Mauricio *et al*., 1995).

Probably, the oxygen dissolved in wine is the limiting factor in the aerobic metabolism of flor yeasts. Nevertheless, this metabolism is different in both strains. *S. cerevisiae* race capensis behaves as a strain very well adapted to growth in wine, metabolizing the ethanol and forming a thick and abundant flor, within 20 days. As a consequence only small amounts of acetaldehyde accumulate in the wine. *S. cerevisiae* race bayanus produced and released more amounts of acetaldehyde into wine, showing a greater ADH II activity. Probably, a portion of acetaldehyde is also derived from initial substrate sources other than ethanol from the wine, such as glycerol, amino acids, etc. The consumption of acetaldehyde is slower and continues throughout the aging process, since it conserves a higher specific activity of the ALDH-NADP$^+$ and higher rates of acetate production and consumption. Consequently, this strain has a slower and prolonged growth producing a very thin flor within 35 days, which is continually renewed. This permits a continued accumulation of acetaldehyde in wine, which is characteristic of the biological aging for the production of sherry-type wine. In this way, we suggest that pure cultures of *S. cerevisiae* race bayanus added together with other indigenous yeast strains could accelerate the biological aging of fino dry wine (sherry wine).

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