Ester Formation and Specific Activities of *in Vitro* Alcohol Acetyltransferase and Esterase by *Saccharomyces cerevisiae* during Grape Must Fermentation

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We have investigated the relationship between the alcohol acetyltransferase (EC 2.3.1.84) and esterase activities and the extracellular and cellular concentration of acetate esters in two *Saccharomyces cerevisiae* varieties during fermentation of grape must. *S. cerevisiae* var. *cerevisiae* is a typical fermentative yeast, and *S. cerevisiae* var. *capensis* is a flor veil-forming yeast. After 24 h of fermentation, a strong synthesis of ethyl, isoamyl, and hexyl acetate esters was detected, which correlates with an increase of alcohol acetyltransferase (AAT) activity. This activity decayed afterward and underwent a slow increase throughout the stationary phase. After 10 days of fermentation, a diminution of these esters was observed, being coincidental with an increase in the esterase activity measured as the hydrolysis of isoamyl acetate. After 134 days, the AAT activity and the synthesis-related activity of the esterases were higher in the nonviable yeast cells of var. *cerevisiae* found in the sediment than in the viable yeast cells of var. *capensis* found in the veil. It is suggested that these activities could be used to get rid of toxic compounds from the medium. The esters so synthetized could then be utilized by the flor veil-forming yeasts during the aerobic metabolism established when the sugar content is exhausted.

INTRODUCTION

Esters are secondary products produced by wine yeasts during anaerobic metabolism of grape sugars (Soles *et al.*, 1982; Rosi *et al.*, 1989) and constitute one of the largest and main groups of compounds affecting the quality of wine flavor. As a consequence, the control of their production is very important during wine making.

Many esters can be formed in wine, the acetate esters being most important: ethyl acetate, isoamyl acetate, isobutyl acetate, 2-phenethyl acetate, hexyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, just to mention a few. The major factors affecting the ester content of wine are the medium composition and the procedure of fermentation. However, when these factors are held constant, yeast species and strains become important (Soles *et al.*, 1982; Rosi *et al.*, 1989).

It is believed that acetate esters are synthesized through alcohol acetyltransferase (AAT), an enzyme which uses an alcohol and acetyl coenzyme A as substrates. This enzyme has been partially purified and characterized by Yoshioka and Hashimoto (1982a). However, esters can also be synthesized by esterases working through the reverse reaction in the absence of coenzyme A. Esterases mainly function by hydrolyzing esters, but in *Saccharomyces cerevisiae*, it is still uncertain whether these enzymes are mainly involved in the hydrolysis of esters or in their synthesis (Peddie, 1990).

In the last few years, several studies have been carried out to determine the relationship between some enzymatic activities of wine yeasts and their products formed in wine (Rosi *et al.*, 1989; Millan *et al.*, 1990). In this study, we measured and compared esterase (hydrolysis and synthesis of esters) and AAT specific activities in two varieties of *S. cerevisiae* (var. *cerevisiae*, nonflor veil-forming, and var. *capensis*, flor veil-forming) during fermentation of "Pedro Ximenez" grape must and subsequent flor veil formation in the wine. In addition, we have tried to establish a relationship between the enzymatic activities mentioned above and the formation of aroma compounds.

Flor veil-forming yeasts, also called "film"-forming yeasts, are very important because they are eventually involved in the aging process (in oak barrels) of dry sherry-type wines in the south of Spain. During this wine aging, the metabolic activity of these yeasts is greatly increased as a consequence of the change from an anaerobic to an aerobic metabolism. This biochemical transformation results as a partial consumption in many products of fermentation such as ethanol, glycerine, acetic acid, and free amino acids as well as others.

MATERIALS AND METHODS

Yeasts. *S. cerevisiae* var. *cerevisiae* (typical fermentative strain) and *S. cerevisiae* var. *capensis* (flor veil-forming strain) (Kreger-van Rij, 1984) were isolated from wine and from the flor veil of the dry sherry-type wine from the Montilla–Moriles region, respectively, as described by Mauricio *et al.* (1986). They were preserved in our wine yeast collection on YEPD agar (0.3% yeast extract, 0.5% peptone, 1.0% glucose, and 2.5% agar, pH 6.5).

Culture Medium and Fermentation Conditions. Must from *Vitis vinifera* grapes of the Pedro Ximenez variety with a fermentable sugar content of 232 g/L was used for culture medium. After adjusting the must pH to 3.2 with tartaric acid, we added 75 mg/L of sulfur dioxide, after which the must was sterilized through filtration.

Fermentations were conducted in 10-L fermentors thermostated at 25 °C by water circulation through a double jacket. Each fermentor was inoculated with an initial cell density of 1×10^6 viable cells per milliliter of must, obtained from a 48-h-old starting culture in the same medium.

Samples were collected at the beginning (time 0) and after 1, 2, 3, 10, 31, and 134 days of fermentation. At 134 days, cells of var. *capensis* were harvested from the flor veil, whereas those of var. *cerevisiae* (nonflor veil-forming) were harvested from the bottom of the fermentor. The veil was spontaneously formed by var. *capensis* in the wine surface after 31 days.

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0021-8561/93/1441-2086$04.00/0

Three independent fermentations with each yeast strain were carried out. All results reported herein are the average of all three experiments.

Experimental Methods. Total and viable cell numbers were measured by counting in a Thoma chamber under a light microscope after staining with methylene blue.

The analytical procedures for determination of ethanol, and volatile acidity and reducing sugars in musts and wines were those recommended by C. E. E. (1990).

Preparation of the Yeast-Cell-Free Extract for Enzymatic Activities. All procedures were performed at 4 °C. Cells were collected by centrifugation (5000*g*, 5 min) and washed once in 0.1 M phosphate buffer, pH 7. Pelletized yeast was resuspended in the same buffer. Aliquots of 1.5 mL of the cell suspensions were vortexed for 30 s with 2 g/mL of glass beads (Sigma, 0.5-mm diameter) and cooled for 30 s on ice. This procedure was repeated seven times. The homogenized cell extract was harvested with a syringe and so was the supernatant, obtained from rinsing the glass beads with the same buffer. Triton X-100 (10% v/v) was then added to the homogenate (7.5 μL/mL). The cellular debris was removed by centrifugation (2000*g*, 1 min) and the supernatant analyzed for enzyme activities. Protein concentration in the homogenized extract was determined by the Bradford (1976) method.

Assay of Enzyme Activities. The alcohol acetyltransferase (EC 2.3.1.84) activity levels and the synthesis-related activity of the esterases were monitored as previously described by Yoshioka and Hashimoto (1981) with a few modifications. The hydrolysis-related activity of the esterases was assayed using the same reaction medium but replacing the isoamyl alcohol with 7 mM of isoamyl acetate.

In this study, ethanol or isoamyl alcohol was used to measure AAT activity separately, in agreement with Nordström (1966), since both alcohols act as competitive inhibitors to each other. Reaction products were extracted with 0.5 mL of pentane. As an internal standard, 0.04 mM of decane was added.

Two microliters of sample was injected into a Perkin-Elmer 8310B gas chromatograph equipped with a flame ionization detector. A stainless-steel column, 6-m × 3-mm i.d., packed with 10% FFAP on Chromosorb W 80–100 was used. Oven temperatures were programmed as follows: 75 °C for initial temperature and 120 °C for final temperature (gradient slope, 2 °C/min). Injector and detector temperatures were 250 °C. The carrier gas was N_2 (20 mL/min).

Preparation of the Yeast Cells for Quantification of the Esters. Yeast cells were collected from the must by filtering through Millipore filters (1.2 μm), and 1×10^{10} cells were suspended in 2–3 mL of 12% (v/v) ethanol. Cells were then broken by stirring in a Vibromatic device with an identical volume of glass beads (0.5-mm diameter). After the solution was centrifuged at 5000*g* for 5 min, the supernatant was gathered along with the content, obtained from rinsing the cell debris several times with 12% (v/v) ethanol. The mixture was brought up to a final volume of 50 mL, and the pH was adjusted to 3.5. As an internal standard, 2-octanol was added. To extract esters and alcohols, freon-11 was used in a continuous extractor for 24 h. Musts and wine samples were also extracted with freon-11 under the same conditions as those of the yeast extracts.

Quantification of Esters and Alcohols. The isoamyl alcohols, 2-phenylethanol, 1-hexanol, their acetates, and ethyl acetate were determined by gas chromatography after concentrating the freon extracts to 0.2 mL in a Kuderna-Danish microconcentrator. Five-microliter volumes were injected in a gas chromatograph (Perkin-Elmer Sigma 3) equipped with an injector with a splitter, a flame ionization detector, and a capillary column (SP-1000, 60-m × 0.32-mm i.d.). The oven temperature program was as follows: 10 min at 50 °C, 1.5 °C per minute up to 180 °C, and 60 min at 180 °C. Injector and detector temperatures were 275 °C. The carrier gas was helium at 18 psi, split 1:100.

Ester content from the yeast cells was expressed as milligrams per liter of cellular volume, to be able to compare this concentration with that found in the cell-free must or in the wine.

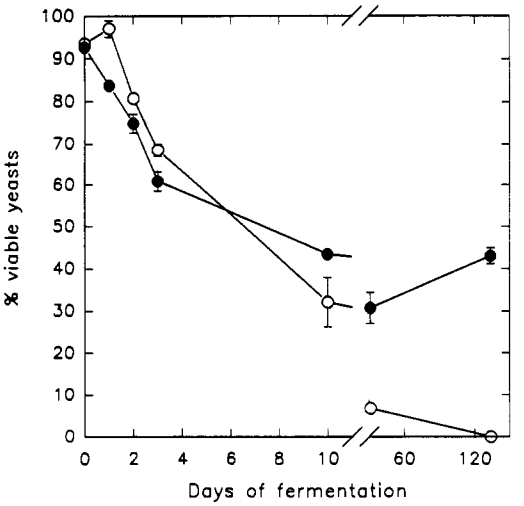

Figure 1. Percentage of viable yeast cells during wine fermentation and flor veil formation by *S. cerevisiae* var. *cerevisiae* (○) and *S. cerevisiae* var. *capensis* (●).

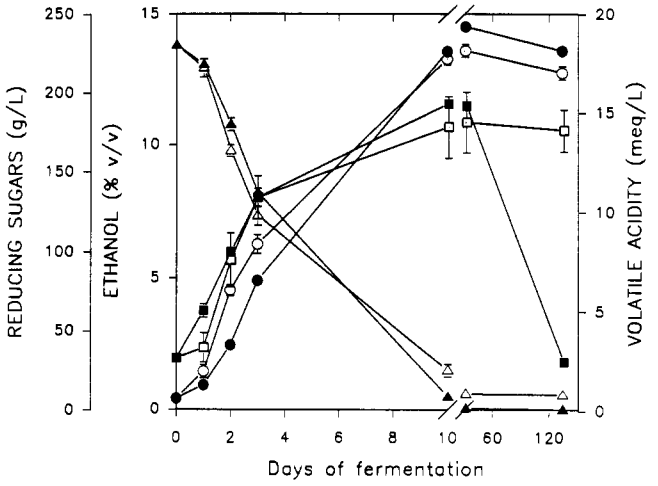

Figure 2. Reducing sugars (triangles), ethanol (circles), and volatile acidity (squares) during wine fermentation and flor veil formation by *S. cerevisiae* var. *cerevisiae* (hollow symbols) and *S. cerevisiae* var. *capensis* (solid symbols).

RESULTS

Yeast Growth and Chemical Parameters during Wine Fermentation. The exponential growth phase in *S. cerevisiae* var. *cerevisiae* lasted over 48 h, whereas that in *S. cerevisiae* var. *capensis* was significantly lower (data not shown). The maximum number of cells reached during the fermentation period was higher ($(125 \pm 5) \times 10^6$ cells/mL) in the fermentative strain, var. *cerevisiae*, than in var. *capensis* ($(91 \pm 5) \times 10^6$ cells/mL). In both strains, the maximum number of viable cells was reached after 48 h of fermentation (data not shown). After the sugar content was exhausted, cells of var. *capensis* remained alive and capable of growing and they even spontaneously built up a flor veil at the surface of the wine. In contrast, cells of var. *cerevisiae* were uncapable to keep growing, and nonviable cells were found at the end of the process (Figure 1). These results can explain the levels of the studied chemical compounds which are shown in Figure 2. After 31 days of fermentation, *S. cerevisiae* var. *capensis* consumed all of the reducing sugars and produced a higher alcoholic degree and slightly more volatile acidity than var. *cerevisiae*. However, cells of the *capensis* variety consumed most of the acetic acid content during the flor veil formation. Consequently, the corresponding wines

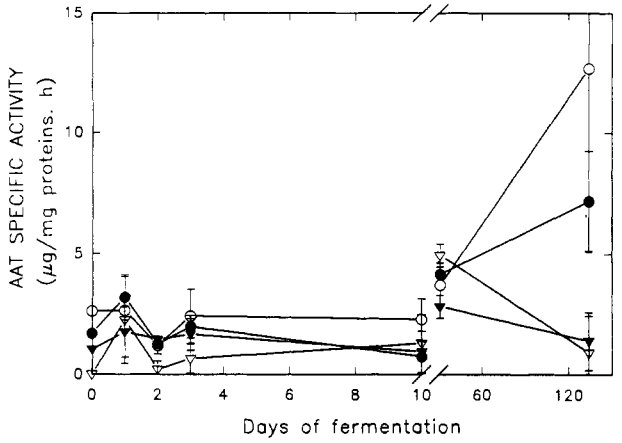

Figure 3. Alcohol acetyltransferase (ATT) activities of *S. cerevisiae* var. *cerevisiae* (circles) and *S. cerevisiae* var. *capensis* (triangles) during wine fermentation and flor veil formation. Ethanol/acetyl-CoA or isoamyl alcohol/acetyl-CoA were, respectively, used as substrates corresponding to the acetate esters formed (ethyl acetate ○ and ▽, isoamyl acetate ● and ▼).

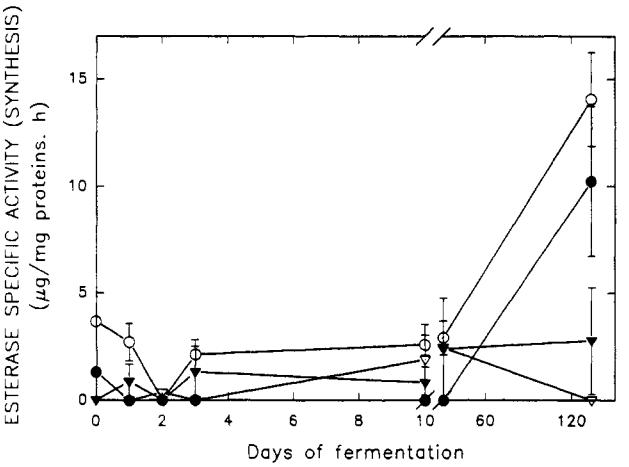

Figure 4. Synthesis of ethyl acetate (hollow symbols) and isoamyl acetate (solid symbols) by *S. cerevisiae* var. *cerevisiae* (circles) and *S. cerevisiae* var. *capensis* (triangles) during wine fermentation and flor veil formation. Ethanol/acetic acid or isoamyl alcohol/acetic acid were, respectively, used as substrates.

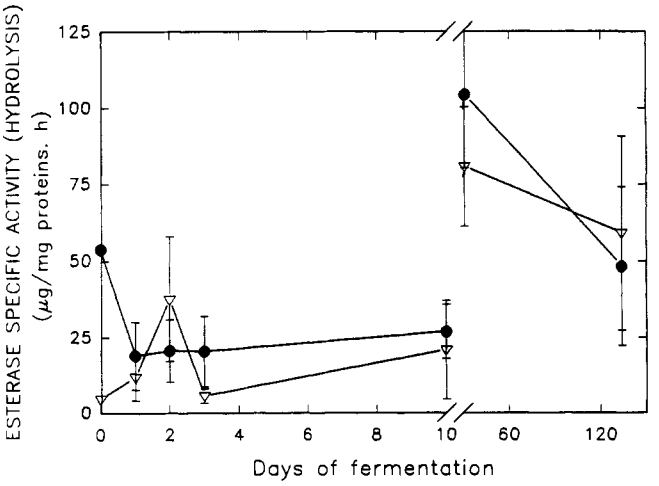

Figure 5. Hydrolysis of isoamyl acetate by *S. cerevisiae* var. *cerevisiae* (●) and *S. cerevisiae* var. *capensis* (▽) during wine fermentation and flor veil formation.

were lower in volatile acidity than the wines produced by the *cerevisiae* variety.

***In Vitro* Alcohol Acetyltransferase and Esterase Specific Activities during Fermentations.** AAT and esterase activities were not detected in whole yeast cells. In cell-free extracts, however, AAT and esterase activities were found, being, respectively, 1.5 and 1.25 times higher when assayed with Triton X-100 (data not shown). Then, all the enzymatic assays were done in the presence of Triton X-100, as reported under Materials and Methods.

Alcohol Acetyltransferase Activity. Values of AAT activities during the fermentation for the two varieties of *S. cerevisiae* assayed are given in Figure 3. AAT activity was detected during the entire fermentation process. It showed a maximum level at the midexponential phase of growth and decayed later on at the end of such a phase. This activity increased again during the stationary phase in both varieties.

After 134 days, AAT activity was lower in the veil cells (var. *capensis*, 43% of cellular viability) than in the non-film forming yeast cells (var. *cerevisiae*, 0% of cellular viability) (Figures 1 and 3).

Esterase Activities. We have studied the esterase activities in both senses: hydrolysis and synthesis of esters. Esterase activities measured as the synthesis of ethyl acetate and isoamyl acetate during fermentation are shown in Figure 4. The *cerevisiae* variety always showed higher levels of esterase activity related with the synthesis of ethyl acetate than the *capensis* variety. This activity was not detected in both varieties after 48 h. The esterase activity involved in the synthesis of isoamyl acetate was not detected during the first 31 days in the *cerevisiae* variety. At the stationary phase, esterase activities of both kinds of esters increased in both varieties. During the flor veil formation, a diminution of the esterase activity (synthesis of ethyl acetate) took place in var. *capensis*. However, the same activity for the synthesis of isoamyl acetate did not vary. Both kinds of esterase activities increased greatly in the *cerevisiae* variety after 134 days.

In both strains, the esterase activity measured as the hydrolysis of isoamyl acetate was always higher than the same activity measured as the synthesis of isoamyl acetate (Figure 5).

In the *capensis* variety, a maximum of esterase activity for the hydrolysis of isoamyl acetate was observed after 48 h of fermentation. The maximum level of this activity in both varieties was observed after 31 days, this activity being higher in var. *cerevisiae* than in var. *capensis*. It is worthwhile to mention that in both varieties, at the end of the process, a diminution of the hydrolysis-related esterase activity was observed, whereas the synthesis-related activity exhibited a significant increase (Figures 4 and 5).

Production and Cellular Accumulation of Esters and Alcohols. In this work, we studied the production of ethyl, isoamyl, hexyl, and phenethyl acetates and their alcohols. Figures 6–9 show the content of esters found in both cells and media during fermentations by the two varieties of *S. cerevisiae* tested. Ethyl acetate was the predominant compound followed by phenethyl, isoamyl, and hexyl acetates, respectively. The kinetics of the production of every acetate ester studied were similar in both varieties during the fermentation period (around 10 days). The differences observed between the *S. cerevisiae* varieties were stablished at the acetate esters content levels. So, the *cerevisiae* variety produced more ethyl, isoamyl, and hexyl acetates than the *capensis* variety, and the latter produced more phenethyl acetate than the former. During the first and second day of fermentation, the production of ethyl, isoamyl, and hexyl acetates was very high and afterwards decreased between the second and third day, where the production continued slowly until day 10. The

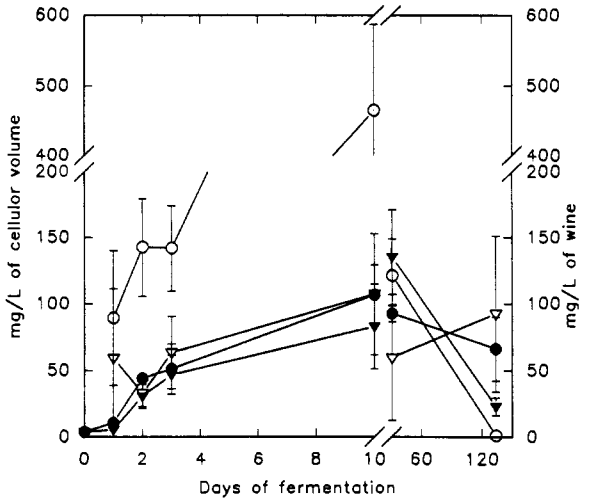

Figure 6. Concentration of ethyl acetate in the yeast cells (hollow symbols) and in the fermentation medium (solid symbols) by *S. cerevisiae* var. *cerevisiae* (circles) and *S. cerevisiae* var. *capensis* (triangles) during wine fermentation and film formation.

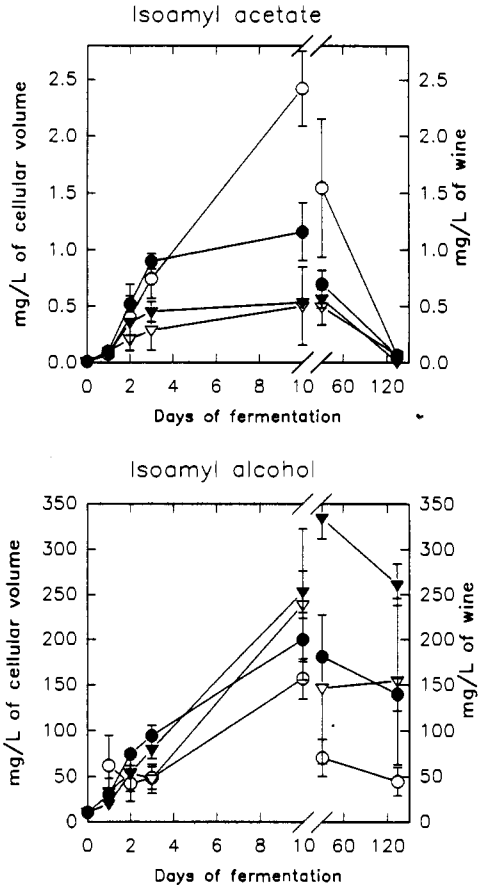

Figure 7. Concentration of isoamylic alcohols and their acetate esters in the yeast cells (hollow symbols) and in the fermentation medium (solid symbols) by *S. cerevisiae* var. *cerevisiae* (circles) and *S. cerevisiae* var. *capensis* (triangles) during wine fermentation and flor veil formation.

cerevisiae variety accumulated the greatest amount of all of the esters in the cells with the exception of phenethyl acetate.

The amounts of ethyl, isoamyl, and hexyl acetates in the wines after 10 days of fermentation were decreasing until day 134, except for hexyl acetate when fermented by the *cerevisiae* variety. The content of the same esters in the cells also decreased, except for ethyl acetate which increased in the *capensis* variety.

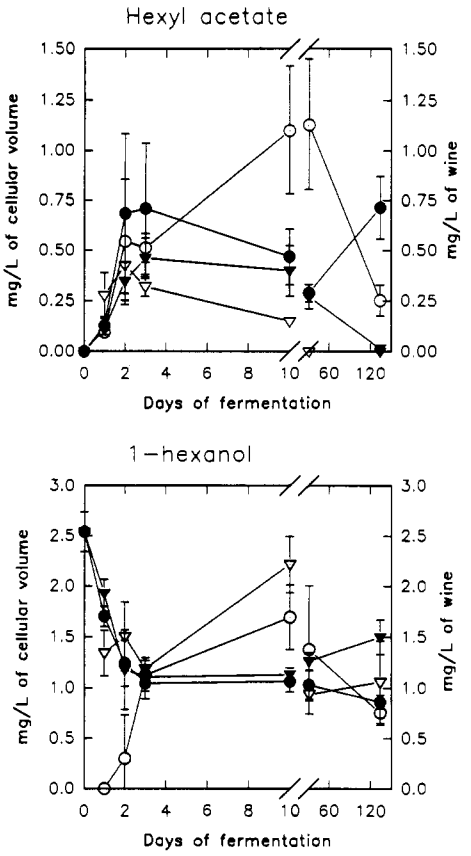

Figure 8. Concentration of 1-hexanol and its acetate ester in the yeast cells (hollow symbols) and in the fermentation medium (solid symbols) by *S. cerevisiae* var. *cerevisiae* (circles) and *S. cerevisiae* var. *capensis* (triangles) during wine fermentation and flor veil formation.

The kinetics of the phenethyl acetate appearance were different in all the cases assayed. Production occurred only after the third day of fermentation and was higher in var. *capensis*, which had a higher cellular concentration of this acetate ester. After 31 days, the amount of phenethyl acetate in the cells and in the medium decreased, except in the cells of the *capensis* variety in which it increased.

DISCUSSION

The first and the greatest number of current studies about AAT and esterase activities and ester production have been made on brewers' yeasts (Nordström, 1961, 1964, 1966; Yoshioka and Hashimoto, 1981, 1982a,b, 1983, 1984a,b; Peddie, 1990; Nakatani *et al.*, 1991). They reported that more ester production and increased enzyme activities were produced during the stationary phase of growth of the yeasts. Thurston *et al.* (1981, 1982), in studying the relationship between lipid metabolism and ester synthesis, reported a large increase in the specific rate of ester production at the latter half of fermentation, which is concurrent with the cessation of lipid synthesis. In wine yeasts, we found an increased production of ethyl acetate, isoamyl acetate, and hexyl acetate between 24 and 48 h of wine fermentation. This was related to the maximum AAT activity observed after 24 h of fermentation (midexponential phase of growth). This activity decreased after 48 h (at the end of the exponential phase), leading to a decreased ester production after 72 h of fermentation. The production of esters smoothly increased up to day 10, as a consequence of a new increase in the activities of both alcohol acetyltransferase and the synthesis-related activity

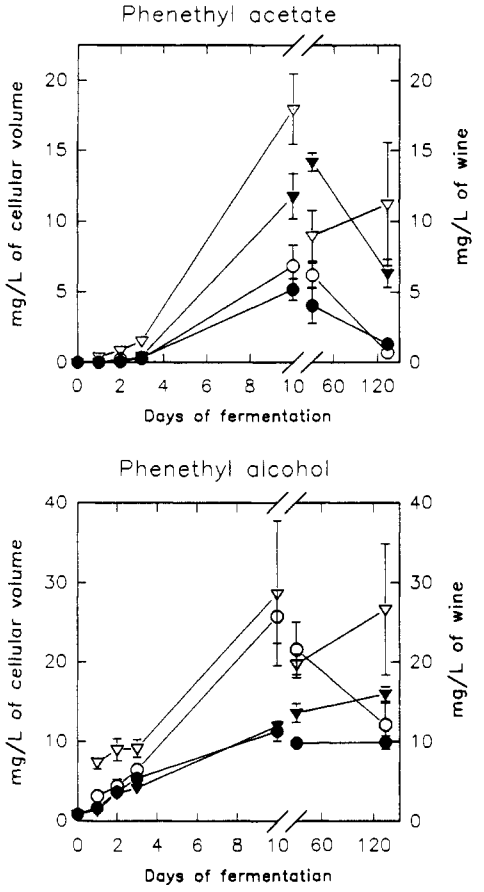

Figure 9. Concentration of phenethyl alcohol and its acetate ester in the yeast cells (hollow symbols) and in the fermentation medium (solid symbols) by *S. cerevisiae* var. *cerevisiae* (circles) and *S. cerevisiae* var. *capensis* (triangles) during wine fermentation and flor veil formation.

of esterases. After this, the production of esters decreased as the hydrolysis-related activity of esterases strongly increased. However, production of hexyl acetate by var. *cerevisiae* increased between days 31 and 134, which suggests that either another esterase with a synthesis-related activity might be present in this variety or the enzyme affinity for the corresponding alcohol is greater in the *cerevisiae* variety than in the *capensis* variety. The kinetics of phenethyl acetate production and its alcohol were different in the remaining esters studied in both varieties of *S. cerevisiae*, which increased their production greatly after 72 h of fermentation (beginning of the stationary phase). This is probably due to an abundant transport of the amino acid phenylalanine (grape musts are rich in this amino acid), and thus, the yeast transforms part of this to phenethyl alcohol and later to its acetate. We have observed different kinetics of the production of acetate esters. These differences could be explained if we take into consideration the different sources of their alcoholic substrates or, alternatively, could be due to different affinities of the enzymes by these substrates. So, ethanol is the main and final product of fermentation; isoamylic alcohols are intermediate products of amino acid biosynthesis (Rous *et al.*, 1983); 1-hexanol came from the must from where yeast take it up; phenethyl alcohol has its origin from phenylalanine, an amino acid found in the must. During the exponential phase, yeasts use this amino acid for the synthesis of protein, but later on, it behaves as a substrate for the production of phenethyl acetate.

The *cerevisiae* variety accumulated into the cells large amounts of ethyl, isoamyl, and hexyl acetates. We suggest that this could possibly be due to a deficient excretion of these esters to the medium. This deficiency might explain the lower viability found in the *cerevisiae* variety, since these compounds are toxic. The *capensis* variety, however, excreted more efficiently these acetates, thus explaining its higher viability. Between days 31 and 134, an accumulation of ethyl and phenethyl acetates by var. *capensis* was observed. This is probably due to the uptake of these esters from the medium for their utilization in cellular metabolism. Flor veil yeasts change from an anaerobic metabolism to an aerobic metabolism during the flor veil formation, and at this moment, reducing sugars are no longer present in the wine. Cells incorporate these esters and convert them to acetyl-CoA, intermediates of the TCA cycle, amino acids, lipids, nucleic acids, and sugars through the gluconeogenesis pathway. This is in accordance with the accumulation of alcohols observed in the var. *capensis* between days 31 and 134. We did not observe this accumulation in the var. *cerevisiae* as they were not viable at this period.

Yoshioka and Hashimoto (1981) reported that the formation of ethyl acetate depends on both AAT and the synthesis-related activity of esterases, whereas the formation of isoamyl acetate depends exclusively on the action of AAT. Our results with var. *cerevisiae* are in agreement with this hypothesis, but we also observed a strong activity of the synthesis-related activity of esterases in the synthesis of isoamyl acetate in the nonviable cells. This can be due to an activation of this activity when yeast cells lose their viability.

As previously demostrated, the AAT activity is inhibited *in vitro* by unsaturated fatty acids and ergosterol (Yoshioka and Hashimoto, 1983) and the activity of a crude membrane fraction is inversely related to the unsaturated fatty acid content of the cell membrane (Yoshioka and Hashimoto, 1982b, 1984a). Recently, Malcorps *et al.* (1991) explained the inhibition of ester synthesis by unsaturated fatty acids as a repression of the synthesis, or the processing of the enzyme, being the regulator presumably linked to the yeast lipid metabolism. Our results are in agreement with their findings. As the viability of var. *cerevisiae* cells after 134 days was nule, we assume that the ergosterol and unsaturated fatty acid contents were low (Larue *et al.*, 1980; Mauricio *et al.*, 1990, 1991). This can explain why *in vitro* the AAT and the synthesis-related esterase activities were found to be high after these 134 days. By contrast, the flor veil cells of var. *capensis* had low enzymatic activities in this period. Since yeast cells of this variety showed a 43% viability, these cells likely had high lipid contents at this time.

We have found a close parallelism in the appearance of the AAT and the synthesis-related esterase activities through all the processes, which suggests that both enzymes are coregulated in a similar way. The fact that the synthesis-related esterase activity increased over the stationary phase, whereas the hydrolysis-related esterase activity decrease during the same period, strongly suggests that both are different enzymes. Recently, Malcorps and Dufour (1992) have demonstrated the presence of at least three ester synthases which differ in their catalytic properties.

These enzymes might have the physiological function of removing toxic products from the cell, such as acetic acid (Pampulha and Loureiro-Diaz, 1990) and alcohols (Leao and van Uden, 1985). These compounds are then neutralized during the latter stage of alcoholic fermentation through ester formation. In addition, these esters might serve as a storage pool that the veil yeasts could use

during aerobic metabolism when the reducing-sugars content is over.

Yoshioka and Hashimoto (1981) believe that the AAT enzyme is bound to the plasma membrane. By contrast, Malcorps and Dufour (1987) suggest that the plasma membrane has low AAT activity and that this activity resides in vesicles of different shapes called "vacuomes". Recently, the enzymes which catalyze the synthesis of ethyl acetate and isoamyl acetate in the yeast *S. cerevisiae* were found to be in a fraction containing lipoproteins released during cell disruption (Malcorps and Dufour, 1992).

Further investigations will be addressed to the cellular location, purification, and quantification of ATT and esterase activities in the wine following yeast cell autolysis, when these enzymes are liberated into the medium.

We found a positive correlation between the appearance of the AAT and synthesis-related esterase activities and the formation of ethyl, isoamyl, and hexyl acetates produced during the first 72 h of fermentation. These compounds decreased after 10 days as a consequence of an increase in the hydrolysis-related esterase activity. In contrast, no correlation between the appearance of the studied activities and the formation of phenethyl acetate was observed, since no substrate was available during the first 72 h of fermentation.

We also reported the induction of the AAT and the synthesis-related sterase activities in two phases of yeast growth the first after 24 h and the second during the stationary phase, when the synthesis of fatty acids and the sterol synthesis are finished, in agreement with Peddie (1990).

ACKNOWLEDGMENT

This work was supported by grants from Junta de Andalucía and from C.I.C. y T. (ref. ALI 92-1065-CO2) of the Spanish Government. We gratefully acknowledge Dr. Antonio R. Franco for his help in writing the paper.

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Received for review March 23, 1993. Revised manuscript received July 22, 1993. Accepted August 11, 1993.*

* Abstract published in *Advance ACS Abstracts*, October 1, 1993.