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Investigation of Volatiles Evolution during the Alcoholic Fermentation of Grape Must Using Free and Immobilized Cells with the Help of Solid Phase Microextraction (SPME) Headspace Sampling

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A biocatalyst was prepared by immobilization of *Saccharomyces cerevisiae* strain AXAZ-1 on delignified cellulosic material (DCM). Repeated batch fermentations were conducted using these biocatalysts and free cells, separately, at temperatures of 20, 15, and 10 °C. Solid phase microextraction (SPME) was used in monitoring the formation of volatile alcohols, acetate esters, and ethyl esters of fatty acids. The kinetics of volatile production were similar for free and immobilized cells. In all cases immobilized cells showed a better rate of volatile production, which was directly connected to sugar consumption. The main difference observed was in propanol production, which increased with temperature decrease for the immobilized cells, whereas it remained constant for the free ones. In the case of immobilized cells significant amounts of esters were also produced. It is well-known that esters contribute to the fruity aroma of wine. It was also established that SPME is a very sensitive, accurate, and reliable technique and can be used without any reservation in the characterization of volatile constituents of wine.

KEYWORDS: Immobilization; volatiles; ethyl esters; acetate esters; higher alcohols; SPME; wine; kinetics; fermentation; formation; temperature

INTRODUCTION

Higher alcohols and esters belong to a group of volatile constituents that form the major part of wine fermentation aroma compounds (*1*−*3*). Ethyl esters of fatty acids and acetates are often described as exhibiting fruity and floral odors with the exception of ethyl acetate. In contrast, higher alcohols have never been considered as factors of wine quality. They possess odors described as fusel-like, harsh, pungent, and choking. 2-Phenylethanol is the only higher alcohol described with pleasant terms such as old rose, sweetish, and perfumed (*4*).

Higher alcohols and esters are byproducts of yeast fermentation. Their amounts in wine depend on many factors, such as yeast strain, grape must, and fermentation conditions (*5*−*7*).

Immobilized cell systems not only increase productivity and lower the cost of bioprocesses (*8*−*10*) but also influence yeast metabolism. Consequently, the quantities of alcohols and esters produced are higher and the resulting aroma is much better (*11*−*13*).

Monitoring these volatiles during fermentation is very important in understanding their synthesis from yeasts and the factors affecting their production. The newly developed method of solid phase microextraction (SPME) has been recently used for the monitoring of volatiles during fermentation (*14*, *15*). Among its advantages are its simplicity and versatility, and it provides linear results over a wide concentration of analytes (*16*, *17*).

In the present study, SPME was used to monitor higher alcohols and esters during wine fermentation by free and immobilized cells on delignified cellulosic material (DCM). The determination of the effect of immobilization and temperature on the formation of these important aroma compounds and the investigation of the existence of different pathways of biosynthesis were the aims of this study.

MATERIALS AND METHODS

Yeast Strain. The psychrophile and alcohol-resistant yeast strain AXAZ-1 of *Saccharomyces cerevisiae* was used. It was isolated from the Greek agricultural area (*18*). It was grown aerobically, in a medium consisting of glucose 2%, yeast extract 0.4%, $(NH_4)_2SO_4$ 0.1%, KH_2PO_4 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.5% at 25 °C.

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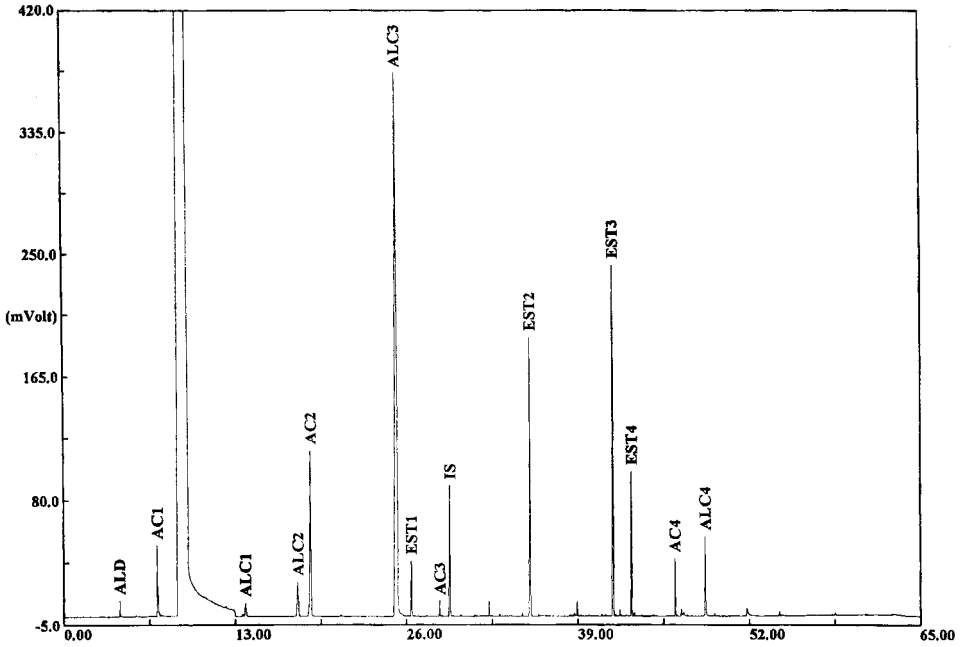

Figure 1. Typical gas chromatogram of the volatile compounds in fermenting grape must sampled by headspace SPME: ALD, acetaldehyde; AC1, ethyl acetate; ALC1, 1-propanol; ALC2, 2-methyl-1-propanol; AC2, 3- and 2-methylbutyl acetate; ALC3, amyl alcohols; EST1, ethyl hexanoate; AC3, hexyl acetate; IS, internal standard; EST2, ethyl octanoate; EST3, ethyl decanoate; EST4, ethyl 9-decenoate; AC4, 2-phenylethyl acetate; ALC4, 2-phenylethanol.

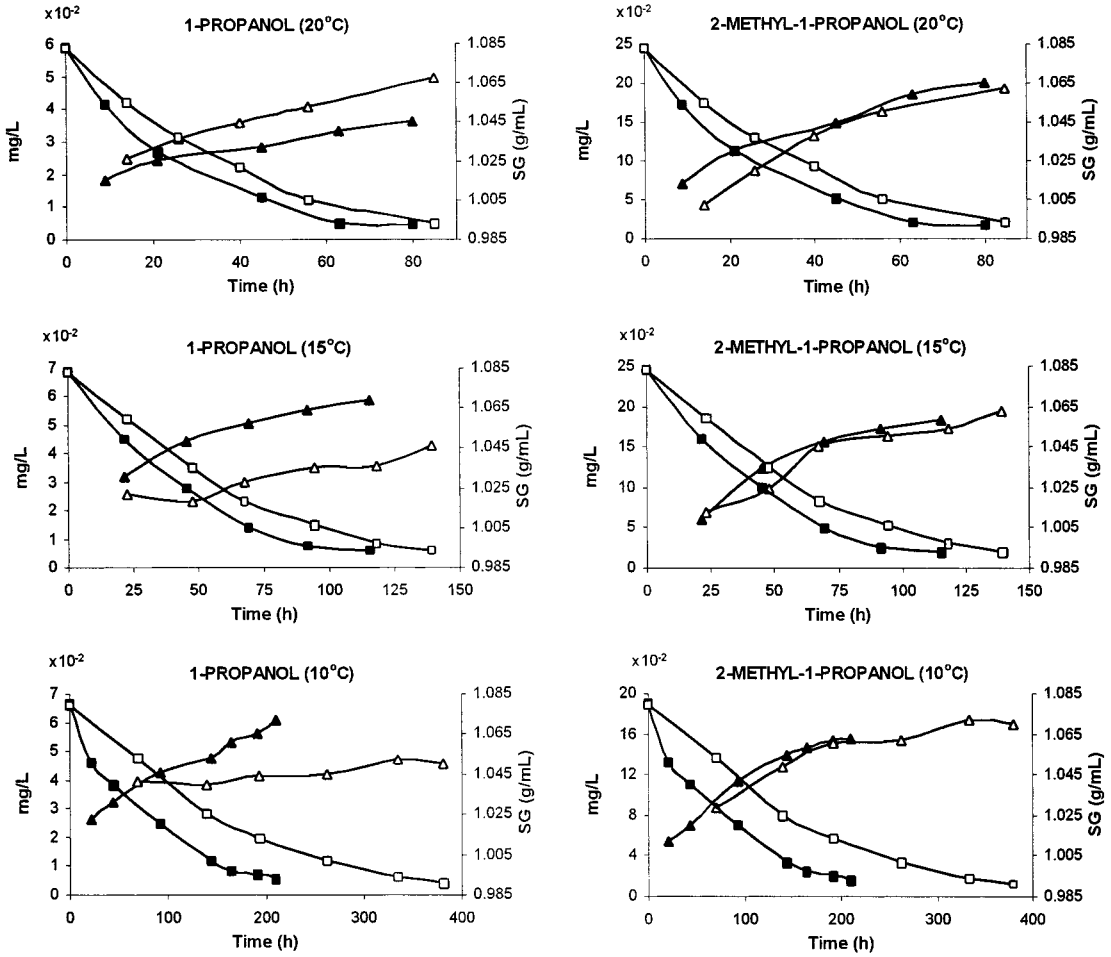

Figure 2. Production of 1-propanol and 2-methyl-1-propanol during fermentation at temperatures of 20, 15, and 10 °C: (▲, △) alcohol content produced during fermentation by immobilized and free cells, respectively; (■, □) progression of fermentation by immobilized and free cells, respectively, as monitored by SG.

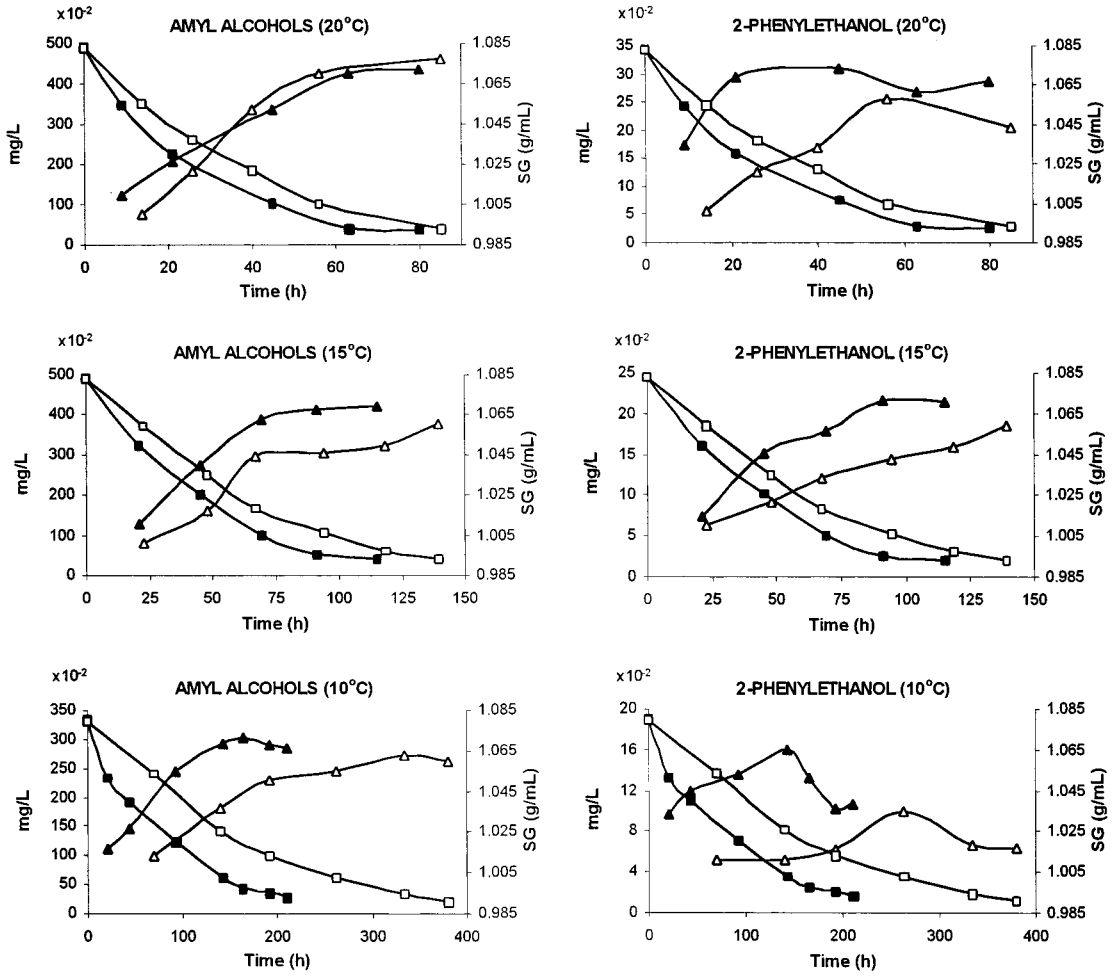

Figure 3. Production of amyl alcohols and 2-phenylethanol during fermentation at temperatures of 20, 15, and 10 °C: (▲, △) alcohol content produced during fermentation by immobilized and free cells, respectively: (■, □) progression of fermentation by immobilized and free cells, respectively, as monitored by SG.

Table 1. Test of Method Reproducibility

compound	mean concn[a] (mg/L × 10^{-2})	SD	% RSD
acetaldehyde	1.30	0.08	6.2
ethyl acetate	21.55	1.05	4.9
1-propanol	5.40	0.19	3.5
2-methyl-1-propanol	19.20	0.56	2.9
3- and 2-methylbutyl acetate	81.60	2.50	3.1
amyl alcohols	413.70	8.30	2.0
ethyl hexanoate	16.45	1.16	7.1
hexyl acetate	3.05	0.31	10.2
ethyl octanoate	90.35	3.57	4.0
ethyl decanoate	49.25	2.24	4.5
ethyl 9-decenoate	50.60	2.58	5.1
2-phenylethyl acetate	9.30	0.76	8.2
2-phenylethanol	21.45	0.64	3.0

[a] Mean concentration of six replicate analyses of the same sample.

Must. Grape must was prepared from the cultivar Moshofilero. It was sterilized at 130 °C for 15 min. The initial °Be density was 11 [specific gravity (SG) = 1.083 g/mL] and total acidity 5.9 expressed as grams of tartaric acid per liter. The must was used without any nutrient addition.

Immobilization. Delignified cellulosic material was used as support for the immobilization of yeast cells. It was prepared from sawdust after lignin's removal with a sodium hydroxide solution (*19*).

In a 1 L culture medium containing 12% glucose, 20 g of wet weight cells of the *S. cerevisiae* strain AXAZ-1 was spread. The pH was adjusted to 4.8 by addition of a dilute solution of sulfuric acid. The resulting broth was mixed with 200 g of wet DCM into a 2 L glass cylinder and left to ferment for 6−8 h, at 25 °C. Cotton plugs were fitted to prevent contamination. After that time, the supernatant liquid was decanted and the solid was washed twice with 400 mL of culture medium containing glucose and then with 400 mL of must. The resulting biocatalyst was directly used for wine-making by repeated batch fermentations.

Fermentations. Three repeated fermentation batches of 1 L of grape must were performed at temperatures of 20, 15, and 10 °C. Two hundred grams of DCM-supported biocatalyst (corresponding to 9.8 g of wet weight cells) was used. All fermentations were carried out without any agitation. The process of fermentation was monitored by measuring the specific gravity. When the fermentation was completed ($d = 0.992$ g/mL), the supernatant liquid was decanted and the support was washed successively twice with 400 mL of must each time. After that, the immobilized biocatalyst was used for the next fermentation batch.

Similar runs were carried out simultaneously with the same initial concentration (10 g/L) of free cells. Yeast cells immobilized on DCM weighed 4.9 g of wet weight cells/100 g of DCM (*19*).

Duplicate samples of fermenting must (5 mL) were taken during fermentation, filtered through 0.22 μm sterile filters (Gelman), and kept refrigerated.

After the end of each fermentation batch, the produced wines were centrifuged at 1310*g* for 10 min and stored at low temperature.

SPME. Two SPME fibers of Supelco [polyacrylate, 85 μm (PA-85), and poly(dimethylsiloxane), 100 μm (PDMS-100)] were evaluated for their selectivity in absorbing volatile alcohols and esters from the headspace of the samples. Preliminary trials showed that PA-85 coated fiber gave better response for the simultaneous analysis of alcohols and esters, so PA fibers were used for all subsequent analyses. The

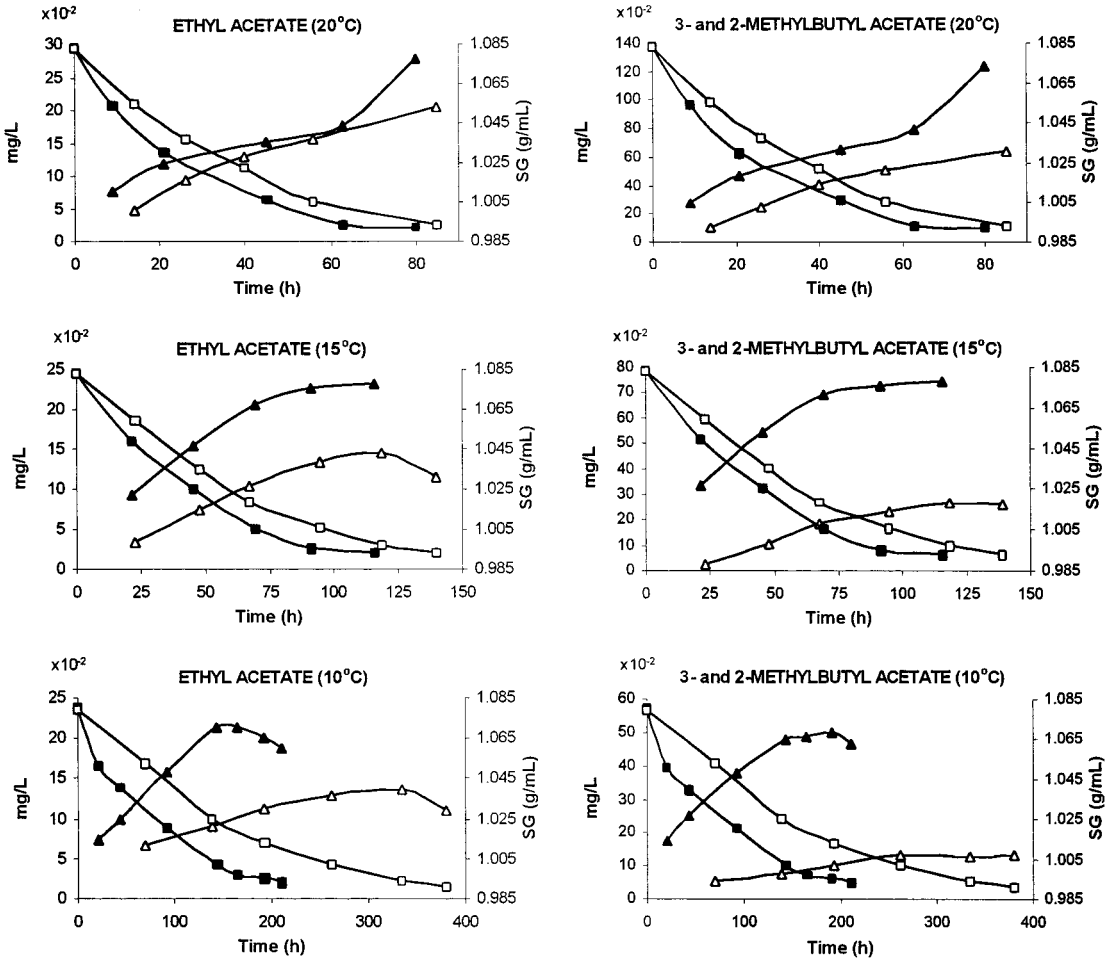

Figure 4. Production of ethyl acetate and 3- and 2-methylbutyl acetate during fermentation at temperatures of 20, 15, and 10 °C: (▲, △) ester content produced during fermentation by immobilized and free cells, respectively; (■, □) progression of fermentation by immobilized and free cells, respectively, as monitored by SG.

conditions of headspace SPME sampling used were as follows: 5 mL of liquid sample, 1 g of NaCl (20%), and 40 μL of internal standard (methyl heptanoate, 0.3 mg/L) were transferred into a 10 mL screw-capped glass vial with a rubber septum. The contents were magnetically stirred for 5 min at 25 °C. Then the fiber was exposed to the headspace for another 5 min, under the same conditions. The length of the fiber in the headspace was kept constant. Desorption of volatiles took place in the injector of the GC in splitless mode, at 240 °C for 5 min. Before each analysis, the fiber was exposed to the injection port for 10 min to remove any volatile contaminants.

Gas Chromatography. The volatile compounds [acetaldehyde, ethyl acetate, 1-propanol, 2-methyl-1-propanol, 3- and 2-methylbutyl acetate, amyl alcohols (3- and 2-methyl-1-butanol), ethyl hexanoate, hexyl acetate, methyl heptanoate (internal standard), ethyl octanoate, ethyl decanoate, ethyl 9-decenoate, 2-phenylethyl acetate, and 2-phenylethanol] were determined in a gas chromatograph of Fisons Instruments (GC 8000 series, model 8060), equipped with a split−splitless injector, FID detector, and Chromcard software (CE Instruments). The oven temperature was programmed from 30 °C for 5 min, and then it was raised to 60 °C at a rate of 2.0 °C/min. After a period of 2 min at 60 °C, the temperature was raised to 240 °C at a rate of 5 °C/min. It was held at this temperature for 5 min. The injector and detector temperatures were 240 and 250 °C, respectively. A Chrompack WCOT fused silica column was used (CP-Wax 52CB, 60 m × 0.32 mm, DF = 0.25 μm). The carrier gas used was helium at a flow rate of 2.32 mL/min. Splitless injections were carried out by using a 1 mm glass liner. A typical gas chromatogram is presented in **Figure 1**.

For the GC-MS analyses a mass spectrometer Fisons MD-800 was used. It was operated in the electron impact mode with the electron energy set at 70 eV and a mass range of *m*/*z* 29−400. Source and interface temperatures were 200 and 250 °C, respectively. The column parameters were similar to those adapted in the GC analysis. Identification of compounds was effected by comparing the retention times with those of authentic compounds and by spectral data obtained from Wiley and Nist libraries.

The reproducibility of the method (headspace SPME sampling and GC analysis) was tested by six replicate analyses of the same sample (**Table 1**). Quantitative data were expressed as milligrams per liter [(area of compound/area of internal standard) × concentration of internal standard].

RESULTS AND DISCUSSION

Higher Alcohols. The significance of this study can be attributed to the fact that one could determine the time of fermentation when the best proportion of volatiles is achieved and the finest wine aroma is produced. Subsequently, when the optimum conditions are attained, the fermentation can be stopped. This can be applied mainly in the making of semisweet and sweet wines.

The higher alcohols produced by yeasts are the aliphatic alcohols 1-propanol, 2-methyl-1-propanol, and amyl alcohols (3- and 2-methyl-1-butanol) and the aromatic 2-phenylethanol. All alcohols except 1-propanol were mainly produced during the first stages of fermentation. 2-Phenylethanol was formed, in most cases, early in the fermentation and then remained constant or slightly reduced. 1-Propanol was formed throughout alcoholic fermentation. An exception to this was the temperature of 10 °C for free cells. These findings are consistent with the results reported by Mauricio et al. (*20*) and Stashenko et al.

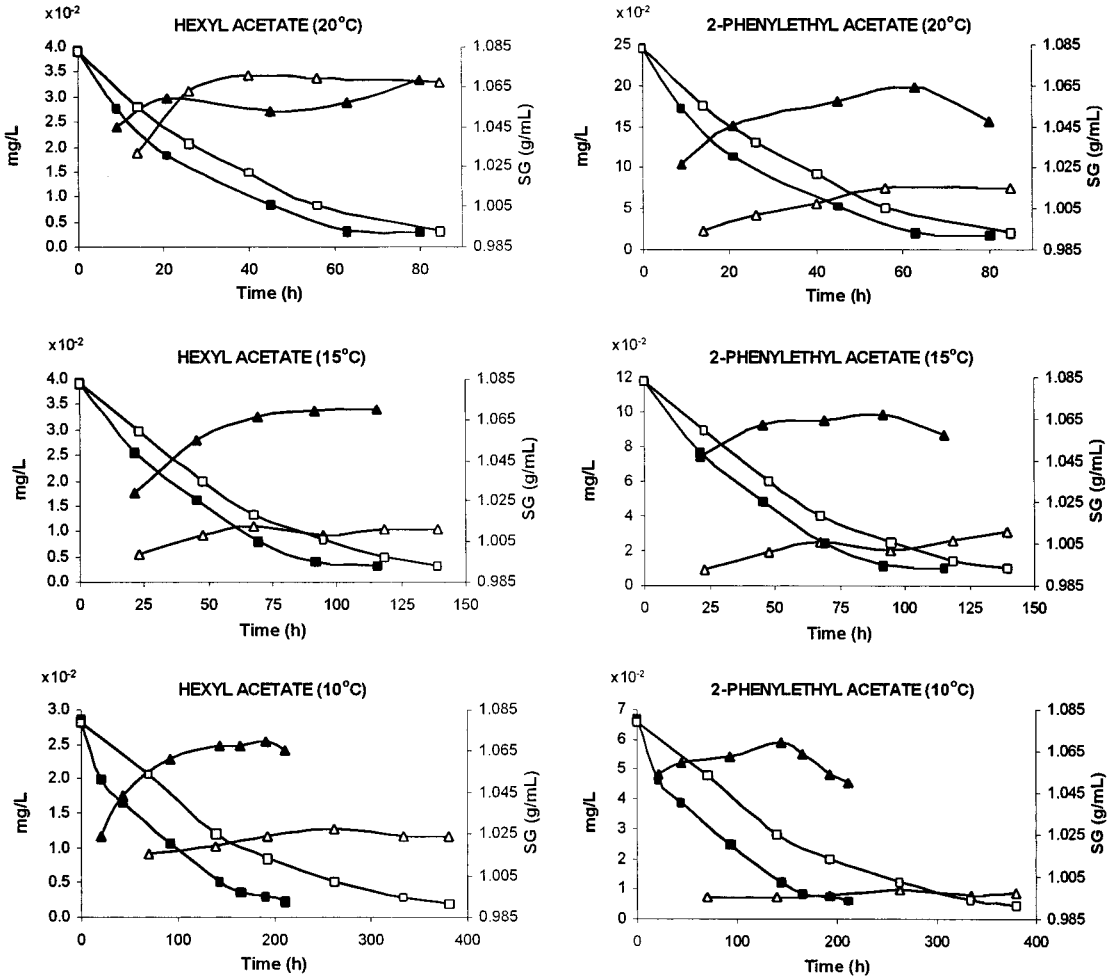

Figure 5. Production of hexyl acetate and 2-phenylethyl acetate during fermentation at temperatures of 20, 15, and 10 °C: (▲, △) ester content produced during fermentation by immobilized and free cells, respectively; (■, □) progression of fermentation by immobilized and free cells, respectively, as monitored by SG.

(*21*). The similar patterns of higher alcohol formation are shown in **Figures 2** and **3**.

However, immobilized cells produced higher alcohols (especially 2-phenylethanol) at a rate higher than that of free cells, with the exception of 1-propanol and amyl alcohols at 20 °C. Another important point was that immobilized cells produced more 1-propanol as the temperature decreased, whereas free cells produced similar amounts for the three temperatures examined. However, the relative percentages of 1-propanol on total volatiles increased as the temperature decreased for both immobilized and free cells. They were similar at every temperature.

2-Methyl-1-propanol contents seemed to decrease slightly with temperature decrease, especially from 15 to 10 °C. Also, free cells gave higher relative percentages than immobilized cells.

The amounts of amyl alcohols produced by immobilized and free cells decreased with temperature decrease and free cells gave higher relative percentages on total volatiles at every temperature studied. These results are in agreement with previous studies reported by Bardi et al. (*22*). Furthermore, the final concentrations of amyl alcohols on the vapor produced seemed to be approximately equal for free and immobilized cells. This differs slightly from previous results, where immobilized cells produced lesser amounts of amyl alcohols (*22*). This difference can be attributed to differentiation of the analytical procedure, because in the previous paper amyl alcohols were determined by direct injection of wine samples, whereas in this paper the analysis was exclusively based on the SPME method.

The contents of 2-phenylethanol also decreased with temperature decrease. Immobilized cells on DCM produced greater quantities of this desirable alcohol in every case.

Higher alcohols are synthesized by yeasts in two ways: the first is via Ehrlich's catabolism mechanism, which involves initial transamination between an amino acid and an α-ketoacid with subsequent decarboxylation and reduction. Alternatively, higher alcohols may be synthesized, in the absence of exogenous amino acids, from sugars utilizing part of the enzymatic pathways needed for the formation of amino acids (*5*, *23*).

Amyl alcohols, 2-methyl-1-propanol, and 2-phenylethanol, can be synthesized from leucine (and isoleucine), valine, and phenylalanine, respectively, via their ketoacids, α-ketoisocaproate (α-keto-*β*-methyl valerate), α-ketoisovalerate, and phenylpyruvate, the production of which depends on cellular growth and probably the presence of oxygen in the medium. This is likely the explanation for the better formation rates of higher alcohols observed in the case of immobilized cells. It is generally known that immobilized cells show greater metabolic activities for a number of reasons (*9*). Also, the repression of the metabolic activity of yeast due to the decrease of temperature explains the reduced amounts of higher alcohols that were observed by both free and immobilized cells.

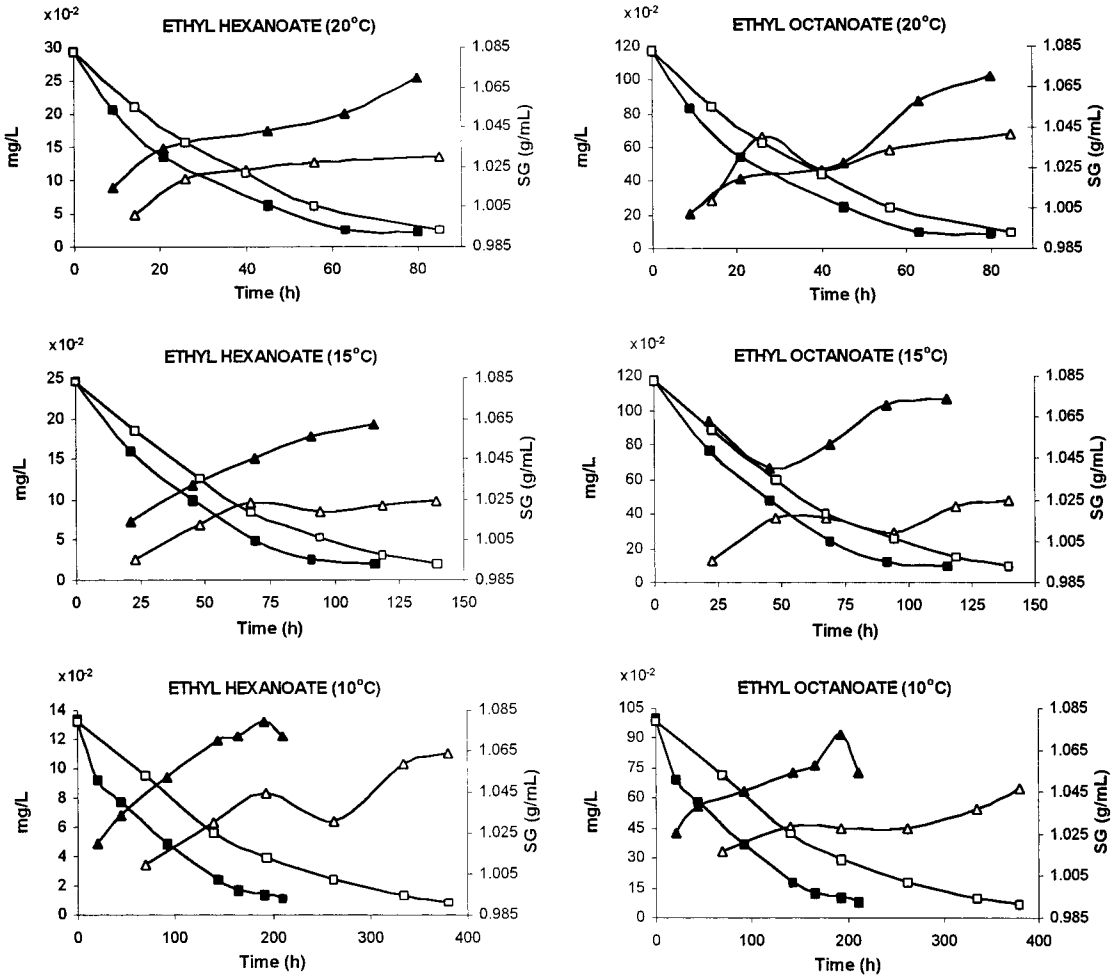

Figure 6. Production of ethyl hexanoate and ethyl octanoate during fermentation at temperatures of 20, 15, and 10 °C: (▲, △) ester content produced during fermentation by immobilized and free cells, respectively; (■, □) progression of fermentation by immobilized and free cells, respectively, as monitored by SG.

However, 1-propanol is primarily formed from sugar catabolism, by condensation of pyruvate and acetyl CoA, because its Ehrlich mechanism precursor, α-aminobutyric acid, does not occur in must (*23*, *24*). This is probably the reason that 1-propanol was synthesized throughout the fermentative process. It was also produced in greater quantities, with temperature decrease, by immobilized cells because of their increased metabolic activity. Another possible explanation for the increase of 1-propanol content with decrease of temperature can be elucidated by considering that the increase of 1-propanol content is related with the corresponding increase in acetaldehyde content. High acetaldehyde content can lead to the formation of high quantities of 1-propanol (*20*). However, in free cells a different profile for 1-propanol was observed, although high amounts of acetaldehyde were produced as in the case of immobilized cells.

Acetates. Ethyl acetate and 3- and 2-methylbutyl acetates followed similar pathways of production throughout the fermentation, for both immobilized and free cells. Their levels increased steadily, reaching a maximum near the end of fermentation (SG = 1.000). Then they remained constant or slightly decreased (**Figure 4**). An exception of this was observed at the temperature of 20 °C, when the levels of these two esters rapidly increased near the end of fermentation (especially for DCM biocatalyst). Immobilized cells produced greater quantities of these two acetates than free cells at every fermentation temperature studied. The final concentrations of ethyl acetate, for free and immobilized cells, are in agreement with previous findings (*22*).

Hexyl acetate and 2-phenylethyl acetate showed a slightly different mode of formation, especially for immobilized cells. Their amount increased rapidly during the early stage of fermentation, reached a maximum near the midpoint, and then decreased or remained constant (**Figure 5**). These data are in agreement with Vianna and Ebeler (*14*), who showed that hexyl acetate was produced more rapidly at the early stages of fermentation and ethyl acetate and 3- and 2-methylbutyl acetate after the midpoint and toward the end of fermentation.

The observed mode of acetate ester production resembles the formation of the corresponding higher alcohols, showing the dependency of fusel alcohol production on the formation of the corresponding ester. One can see the similarity in the curve of 3- and 2-methylbutyl acetate with amyl alcohols and of 2-phenylethyl acetate with 2-phenylethanol (**Figures 3** and **4**; **Figures 3** and **5**).

Ethyl Esters of Fatty Acids. The pathway of ethyl hexanoate production resembled mostly that of the ethyl acetate and 3- and 2-methylbutyl acetate. The other fatty acid esters showed in most cases a two-peak profile, which has been also observed by Vianna and Ebeler (*14*). Observed differences were dependent on temperature. In some cases such as ethyl decanoate at 15 and 10 °C, their maximum was observed in the early stages of fermentation (first peak in the diagram, **Figure 7**). Another peak was observed near the end of fermentation. In other cases,

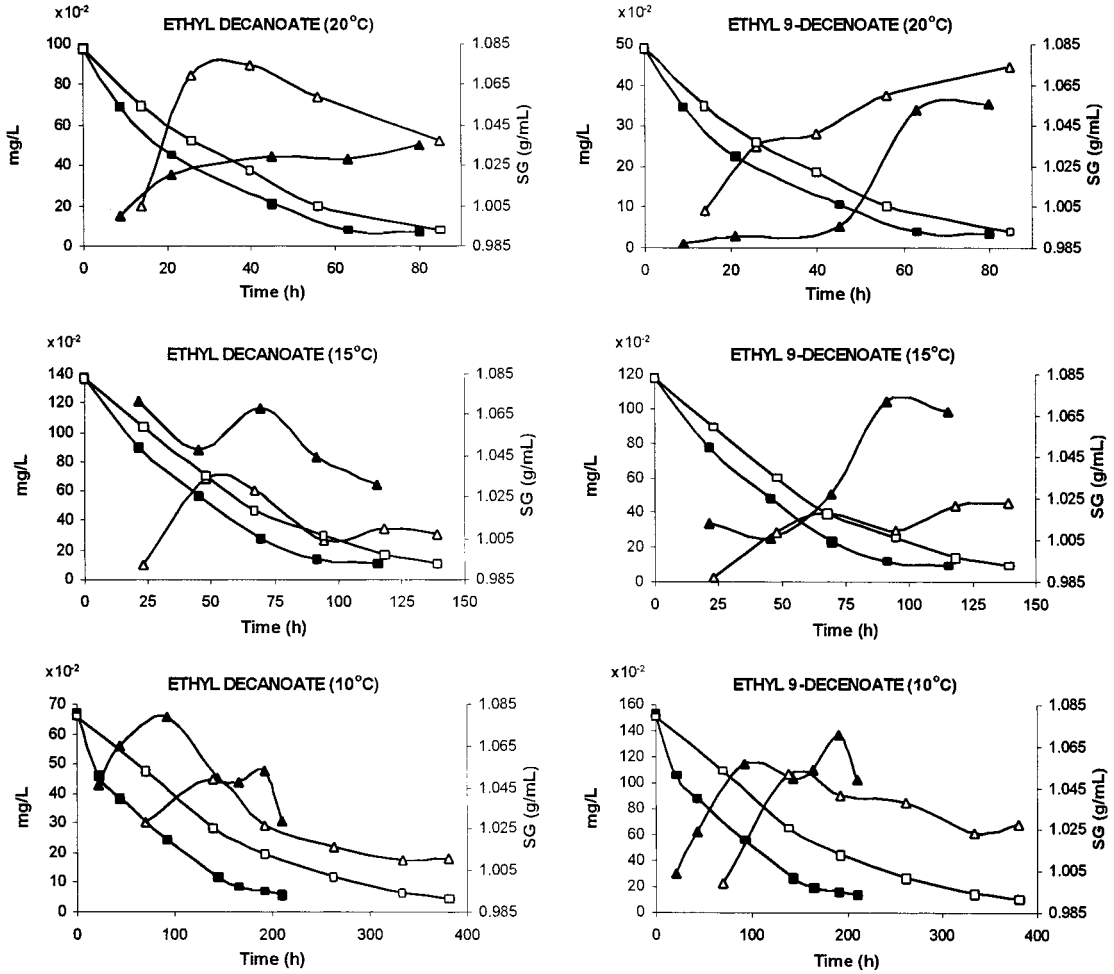

Figure 7. Production of ethyl decanoate and ethyl 9-decenoate during fermentation at temperatures of 20, 15, and 10 °C: (▲, △) ester content produced during fermentation by immobilized and free cells, respectively; (■, □) progression of fermentation by immobilized and free cells, respectively, as monitored by SG.

such as ethyl 9-decenoate at 15 and 10 °C (**Figure 7**) and ethyl octanoate (**Figure 6**), a two-peak pattern was also observed, although the first peak normally was smaller than the second one.

A noticeable point could be the clear tendency of the amount of ethyl 9-decenoate to increase with temperature decrease, for both immobilized and free cells. With regard to the other ethyl esters, the relative percentages of ethyl octanoate and ethyl decanoate remained nearly constant at every fermentation temperature in the case of DCM biocatalyst. Free cells gave the higher percentages of ethyl hexanoate and ethyl octanoate at 10 °C, whereas the percentage of ethyl decanoate was gradually reduced with the drop of temperature. In the majority of cases, immobilized cells produced greater quantities of ethyl esters of fatty acids than free cells at almost all temperatures.

In comparison with acetate ester production, the following important differences were observed. In most cases, the formation of ethyl esters reached a maximum earlier in fermentation than did that of acetate esters and a two-peak profile was observed during their formation. This has been previously reported by other workers (*14*). The data indicated that ethyl ester production follows a pattern of consistent increase with temperature decrease. The opposite phenomenon was observed for acetate esters.

Acetaldehyde. Acetaldehyde content increased remarkably during the first stage of fermentation, reaching a maximum. It decreased toward the end of the fermentation process. Before the end of fermentation, the acetaldehyde level reached a minimum, then increased slightly, and afterward remained approximately constant. This process was observed in both immobilized and free cells (**Figure 8**). Immobilized cells produced greater amounts of acetaldehyde than free cells, except at the temperature of 20 °C. Acetaldehyde contents increased with temperature decrease.

The major carbonyl compound (90% of total aldehydes in wines) produced during degradation of sugars by yeasts is acetaldehyde. It is formed by decarboxylation of the corresponding 2-ketoacid, 2-oxopropanoic acid, produced as an intermediate in the metabolism of amino acids. Its concentration in wine is dependent on the metabolism of yeasts in must and, particularly, on the pyruvate decarboxylase activity (*4*). Thus, the higher contents of acetaldehyde produced at low fermentation temperatures might be attributed to the increased activity of that particular enzyme.

The scheme of acetaldehyde formation has been described before by other authors (*1*, *23*). In alcoholic sugar fermentation, yeasts do not generate a net supply of NADH. However, yeasts need reducing power for growth and reproduction during the early stages of fermentation. In the decline phase, NADH and NADPH are probably accumulated. The associated redox disruption would suppress sugar fermentation by diminishing the supply of the requisite NAD^+. The changing needs of yeasts for reducing power during fermentation probably explain why acetaldehyde is initially released into the fermenting liquid and

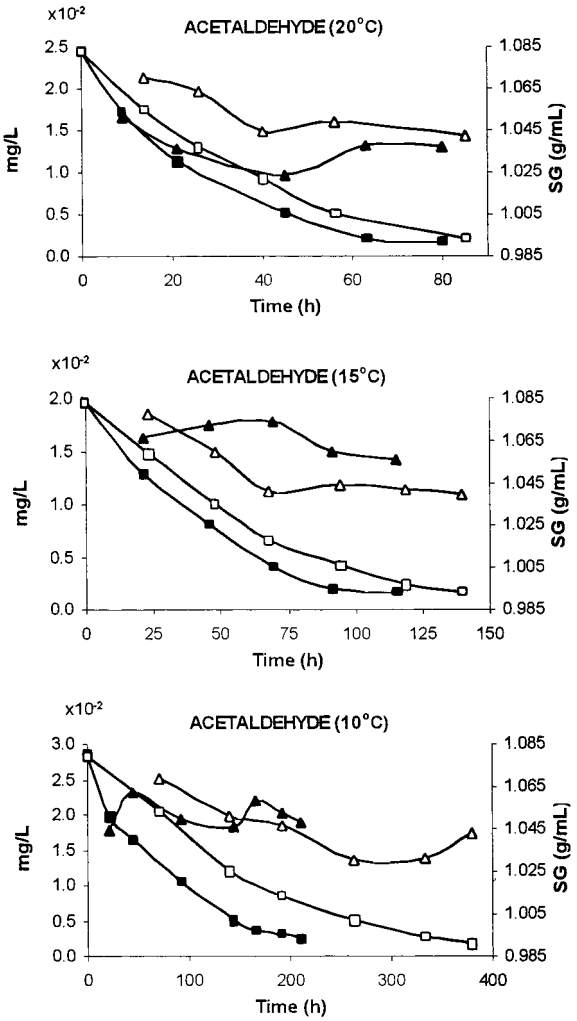

Figure 8. Production of acetaldehyde during fermentation at temperatures of 20, 15, and 10 °C: (▲, □) acetaldehyde content produced during fermentation by immobilized and free cells, respectively; (■, ○) progression of fermentation by immobilized and free cells, respectively, as monitored by SG.

subsequently reincorporated. This helps to balance the redox potential and allows fermentation to proceed (*25*).

Conclusions. The patterns of volatiles evolution were nearly similar for both immobilized and free cells. The main difference observed was the higher fermentation rates of immobilized cells, which resulted in faster evolution of volatiles. The production of higher alcohols is dependent on the formation of acetate esters. The formation patterns of acetate esters and ethyl esters were different.

Immobilized cells on DCM show an important increase in ester concentrations in comparison with free cells. The higher concentration of esters for DCM biocatalyst is obtained at the midpoint of fermentation time. This is very significant, as producers of sweet and semisweet wines can stop the fermentation at the point of the best synthesis of volatiles, producing thus wines with better aroma and taste.

ABBREVIATIONS USED

SPME, solid phase microextraction; GC-MS, gas chromatography−mass spectrometry; PA, polyacrylate; PDMS, poly(dimethylsiloxane); DCM, delignified cellulosic material; NAD^+, NADH nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; SD, standard deviation; RSD, relative standard deviation; SG, specific gravity.

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