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Evolution of Volatile Byproducts during Wine Fermentations Using Immobilized Cells on Grape Skins

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A biocatalyst was prepared by immobilization of *Saccharomyces cerevisiae* cells on grape skins. Repeated batch fermentations were conducted using this biocatalyst as well as free cells, at 25, 20, 15, and 10 °C. Solid phase microextraction (SPME) was used in monitoring the evolution of volatile byproducts. The effect of immobilization and temperature on evolution patterns of volatiles was obvious. The major part of esters was formed after consumption of 40−50% of the sugars. Similar processes were observed for amyl alcohols and 2-phenylethanol, whereas 1-propanol and 2-methyl-1-propanol were formed during the whole alcoholic fermentation period at an almost constant formation rate. Acetaldehyde and acetoin were synthesized in the early stages of fermentation. Afterward, their amount decreased. In most cases, immobilized cells exhibited higher formation rates of volatiles than free cells. The final concentration of esters was higher in wines produced by immobilized biocatalyst. Their amount increased with temperature decrease. The opposite was observed for higher alcohols.

KEYWORDS: Immobilization; yeast; wine; volatiles formation; esters; acetates; higher alcohols; temperature; SPME

INTRODUCTION

Immobilized cell systems offer many prospects for enology, such as improved performance of alcoholic and malolactic fermentation, adaptation to continuous processes, and simplified systems for removing and reusing microbial cells in batch processes (*1*). Particles of various synthetic or natural materials, organic and inorganic, have been used as supports for cell immobilization in wine fermentations (*2*−*5*). Recently, immobilized yeast cells on grape skins were used for conducting repeated batch fermentations of grape must (*6*). Immobilization on the supports modified cell metabolism, resulting thus in increased specific rates of substrate consumption and product formation, as well as ethanol productivities.

The modifications on yeast metabolism are accompanied by a change in the concentrations of volatile byproducts (*5*, *7*−*9*). Ethyl and acetate esters, higher alcohols, fatty acids, ketones, and aldehydes constitute the most important substances that influence wine aroma (*10*−*13*). Monitoring these volatiles during wine fermentation is very important in understanding their synthesis and the factors that affect their production. Research papers on this subject are very scarce and focus mostly on free cell fermentations (*14*−*18*).

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The aim of the present work was the investigation of the evolution patterns of some important volatiles during wine fermentation using immobilized cells on grape skins. The effect of temperature was also examined as it is one of the most influential factors in the formation of wine aroma.

MATERIALS AND METHODS

Yeast and Growth. The psychrophilic, alcohol-resistant yeast strain AXAZ-1 (yeast collection of Food Biotechnology Group, University of Patras, Greece) of *Saccharomyces cerevisiae* was grown on a semisynthetic medium containing 20 g of glucose/L, 4 g of yeast extract/L, 1 g of $(NH_4)_2SO_4$/L, 1 g of KH_2PO_4/L, and 5 g of $MgSO_4 \cdot 7H_2O$/L, previously sterilized at 121 °C for 20 min. The strain was cultivated on static flasks under semiaerobic conditions at 25 °C for 24 h and separated by centrifugation at 3000 rpm for 10 min.

Fermentation Medium. Concentrated grape must was used for alcoholic fermentations after appropriate dilution with deionized water (sugar content = 235 g/L) and without any addition of nutrients. The pH was adjusted at 3.4 using tartaric acid to obtain conditions similar to those of natural must. It was sterilized at 121 °C for 20 min.

Immobilization. The skins of fresh grape berries were removed by exhaustive pressing and used as support for immobilization. Twenty grams of wet weight cells of the *S. cerevisiae*, prepared as described above, was spread in 1 L of the semisynthetic medium (containing 120 g of glucose/L, pH 4.8). Four hundred grams of wet grape skins (∼88 g dry weight), sterilized at 121 °C for 20 min, was added, and the broth was left to ferment in static flasks at 25 °C for 6−8 h, under semiaerobic conditions. The supernatant liquid was decanted, and the support was washed twice with 400 mL of must. The prepared

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biocatalyst was used directly for fermentations of grape must. Determination of immobilized cells was carried out according to the method of Mallouchos et al. (*6*). It was found that 4.5×10^8 cells (corresponding to 2.96×10^{-3} g of dry weight cells) were immobilized per gram of wet grape skins.

Fermentation Experiments. Repeated batch fermentations of 800 mL of grape must were carried out using the aforementioned biocatalyst (400 g of wet grape skins corresponding to 1.18 g of dry weight cells) in spherical flasks without agitation. The biocatalyst was kept submerged by means of plastic netting. Experiments were performed at temperatures of 25, 20, 15, and 10 °C. Each experiment was carried out in duplicate. Free cell fermentations were used as controls. The number of cells per milliliter during the fermentation with free cells was the same as that of the immobilized ones. Fermentation was monitored by measuring specific gravity (SG) and stopped when SG reached 0.992−3 g/mL. Liquid samples were taken during fermentation and kept refrigerated for further analyses.

Sugar Analysis. Glucose and fructose were determined in a Jasco PU-980 HPLC using an RI detector (Jasco RI-930) and a LiChrospher 100NH$_2$ (5 μm) column (Merck LiChroCART 250-4). The eluent was acetonitrile/water (80:20, v/v) at a flow rate of 1.0 mL/min. Column and detector temperatures were set at 35 °C. The fermentable sugars were expressed as the sum of glucose and fructose.

Solid Phase Microextraction (SPME). The fiber used for the absorption of volatiles was a Carbowax−divinylbenzene 65 μm (Supelco, Bellefonte, PA). The best conditions of headspace SPME sampling were determined after preliminary trials examining the effect of incubation temperature and absorption time on volatiles' peak area and better resolution. The following conditions were used: 500 μL of liquid sample and 50 μL of internal standard (4-methyl-1-pentanol, 5.5 mg/L in final solution) were transferred into a 1.2 mL screw-capped glass vial with a Teflon−rubber septum. The contents were stirred for 10 min at 35 °C. Then, a constant length of the fiber was exposed to the headspace for another 5 min, under the same conditions.

Gas Chromatography (GC). The volatile compounds 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, Milan, Italy). Desorption of volatiles took place in the injection chamber in the splitless mode, at 240 °C for 4 min. Prior to the GC analysis, the fiber was exposed to the injection port for 10 min to remove any volatile residues. The oven temperature was programmed from 30 °C for 5 min and then raised to 60, 200, and 250 °C at rates of 2.0, 5.0, and 25.0 °C/min, respectively. It was held at 250 °C for 10 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 2.05 mL/min.

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 mass range m/z 29−400. Source and interface temperatures were set at 200 and 250 °C, respectively. The column parameters were similar to those adapted in the GC analysis. Identification of the volatile compounds was effected by comparing the retention times with those of authentic compounds, by mass spectra of these authentic compounds generated in the laboratory, and by mass spectra obtained from Wiley and NIST libraries.

The best reproducibility of the SPME analyses was that of 3- and 2-methylbutyl acetates (% RSD of 2.7) and the worst that of 2-phenylethanol (% RSD of 14.7). Semiquantitative data were expressed in milligrams per liter [(area of compound/area of internal standard) × concentration of internal standard].

RESULTS AND DISCUSSION

According to a previous study (*6*), faster glucose and fructose uptake rates and ethanol productivities characterized the metabolism of *S. cerevisiae* when it was attached to grape skins. Thus, fermentations using this biocatalyst are advantageous over those of free cells. The advantages of grape skins' use (natural, food-grade purity, cheap, and abundant) and the ease of immobilization technique (fast, cheap, and free of chemicals) make possible the application of this process to wine-making. The next step was to examine the formation of volatile byproducts and detect any differences that would play a significant role in aroma and would affect the quality of the final product.

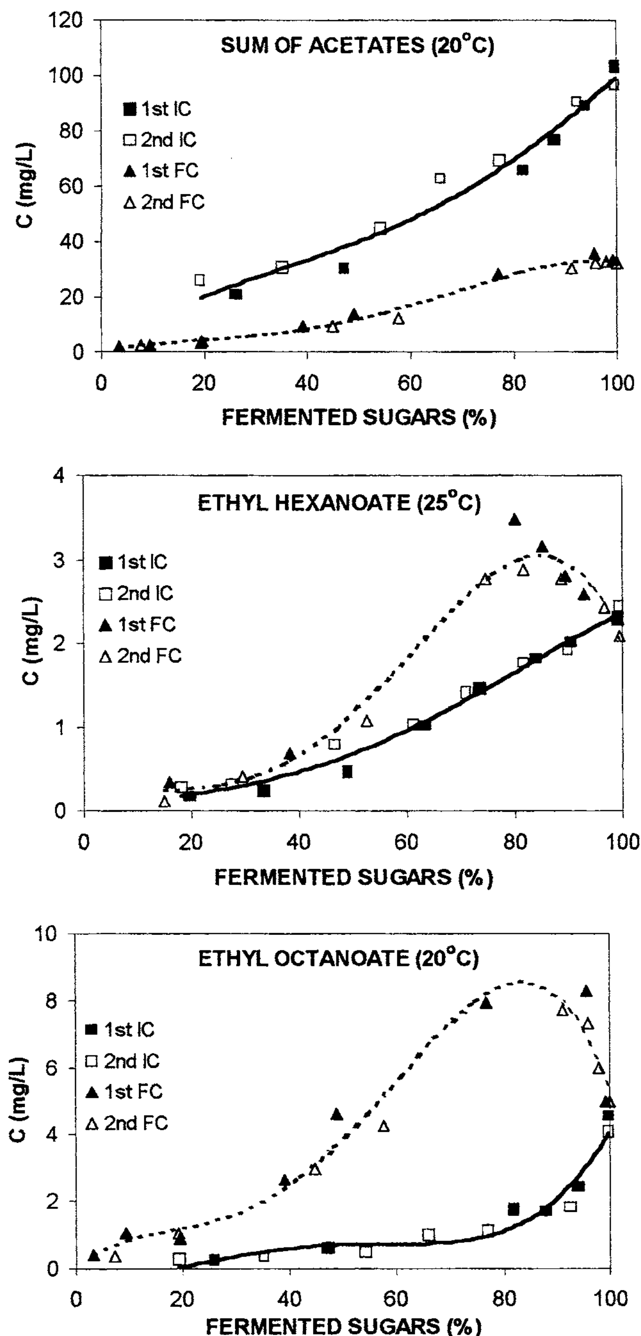

Figure 1. Production of esters during repeated batch fermentations of grape must using immobilized (IC) and free cells (FC): (squares) IC; (triangles) FC; (solid symbols) first repeated batch; (open symbols) second repeated batch. Lines represent polynomial fittings through the points of the two batches in each case.

Evolution of Esters. Fusel alcohol acetates and fatty acid ethyl esters are the volatiles providing the major aroma impact of freshly fermented wines. They have fruity odors and often occur at concentrations much higher than their respective odor thresholds. It has been demonstrated that there is a positive correlation of the wine quality with the concentration of esters.

Table 1. Relative Concentrations[a] (Milligrams per Liter) of Acetate Esters

	ethyl acetate				3- and 2-methylbutyl acetate				2-phenylethyl acetate			
	25 °C	20 °C	15 °C	10 °C	25 °C	20 °C	15 °C	10 °C	25 °C	20 °C	15 °C	10 °C
20% of fermented sugars												
FC	0.26	0.82	0.67	0.41	0.14	3.27	2.09	0.44	0.07	0.15	0.08	0.06
IC	2.46	4.55	4.91	5.16	6.52	18.01	19.50	20.12	0.14	0.45	0.50	0.36
40% of fermented sugars												
FC	1.00	1.87	1.03	0.72	2.30	5.96	4.07	0.50	0.13	0.37	0.10	0.06
IC	3.65	6.38	6.67	8.24	10.53	24.54	27.90	30.44	0.32	0.70	0.78	0.58
60% of fermented sugars												
FC	2.97	3.41	1.71	1.19	9.76	12.80	7.28	0.74	0.46	0.65	0.21	0.08
IC	5.79	9.76	9.22	12.86	17.11	36.92	37.97	44.46	0.42	1.03	1.12	0.94
80% of fermented sugars												
FC	5.44	5.03	3.15	1.88	16.80	21.95	12.26	1.14	0.98	1.03	0.42	0.10
IC	8.10	14.26	14.00	18.78	23.80	54.48	58.05	65.67	0.74	1.75	1.89	1.37
100% of fermented sugars												
FC	6.76	5.94	5.80	2.83	18.28	25.12	19.55	1.24	1.20	1.70	0.58	0.07
IC	8.28	19.45	20.12	23.10	31.92	76.52	93.61	88.00	1.55	3.18	3.41	2.21

[a] Values have been calculated from the polynomial fittings through the points of two repeated batches in each temperature.

Table 2. Relative Concentrations[a] (Milligrams per Liter) of Ethyl Esters

	ethyl butanoate				ethyl hexanoate				ethyl octanoate			
	25 °C	20 °C	15 °C	10 °C	25 °C	20 °C	15 °C	10 °C	25 °C	20 °C	15 °C	10 °C
20% of fermented sugars												
FC	0.17	0.11	0.09	0.07	0.26	0.53	0.43	0.26	1.10	1.20	1.15	1.53
IC	0.17	0.17	0.23	0.27	0.22	0.39	0.62	1.20	0.07	0.06	0.59	2.40
40% of fermented sugars												
FC	0.21	0.15	0.11	0.11	0.66	1.59	1.03	0.47	1.55	2.48	2.00	3.00
IC	0.24	0.24	0.32	0.39	0.43	0.89	1.35	2.37	0.20	0.60	1.37	5.35
60% of fermented sugars												
FC	0.27	0.20	0.16	0.18	1.81	2.73	1.60	0.66	3.95	5.56	4.78	3.13
IC	0.34	0.30	0.39	0.54	0.99	1.61	2.42	4.09	0.24	0.73	2.38	6.23
80% of fermented sugars												
FC	0.39	0.32	0.33	0.30	2.97	3.42	2.65	0.82	6.64	8.44	6.92	2.06
IC	0.46	0.44	0.55	0.82	1.65	2.69	3.98	6.38	0.43	1.13	3.12	7.03
100% of fermented sugars												
FC	0.51	0.55	0.71	0.49	1.99	3.11	3.91	1.00	3.70	5.32	0.53	2.10
IC	0.60	0.75	0.94	1.29	2.36	4.26	6.18	8.78	1.01	4.13	9.23	14.73

[a] Values have been calculated from the polynomial fittings through the points of two repeated batches in each temperature.

The only exception is ethyl acetate, which is considered to be undesirable if present at concentrations >150−200 mg/L (*12*, *13*, *23*, *25*).

Acetate esters were produced in small amounts at the initial stages of fermentation. Two-thirds of the total amount was synthesized after the consumption of 40−50% of sugars. The formation of acetate esters by immobilized cells (IC) was continuous up to the end of fermentation. Stabilization in the synthesis of acetates by free cells (FC) was observed toward the end of fermentation (**Figure 1**). The formation rates for IC were greater than those for FC. The difference between them increased with temperature decrease. In all cases, immobilized biocatalyst favored the production of larger quantities of acetates and most notably of 3- and 2-methylbutyl acetates. These amounts increased with temperature decrease. The maximum concentration was observed at 15 °C (**Table 1**).

The evolution patterns of ethyl butanoate and ethyl hexanoate were similar to those of acetates. The major part of their synthesis took place after the midpoint of fermentation. However, a slight differentiation was observed in the formation of ethyl hexanoate by FC at 25 and 20 °C. The formation rates were greater, but near the end of fermentation (80% of consumed sugars) its concentration decreased, reaching levels lower than those of IC (**Figure 1**). At every temperature, IC produced more ethyl butanoate and ethyl hexanoate than FC. Temperature decrease resulted in the production of increased amounts of the aforementioned esters (**Table 2**).

Ethyl octanoate followed a different evolution pattern (**Figure 1**). FC produced this ester after the consumption of 40% of sugars. Its concentration reached a maximum after 80% of sugars were fermented. After that point, it decreased dramatically. The optimum synthesis of ethyl octanoate by IC took place after the depletion of 80% of the sugars. After this point, its concentration increased steadily. At 25−20 °C, FC produced greater amounts of this ester. The opposite was observed at 15−10 °C. IC gave higher concentrations of ethyl octanoate with temperature decrease (**Table 2**).

In *S. cerevisiae* and other yeasts, the synthesis of esters is effected via an intracellular process catalyzed by an alcohol acyltransferase. With regard to acetate esters, these are believed to be synthesized from alcohols and acetyl-CoA by the alcohol acetyltransferase AAT (*19*). The AAT-encoding gene is not transcribed in the presence of oxygen or unsaturated fatty acids, and it is expressed only during the later stages of the fermentation. At the beginning of fermentation, ester synthesis is very slow due to the high metabolic demands of yeasts for acetyl-CoA. During this phase of the fermentation curve, oxygen and acetyl-CoA are rapidly consumed to support the production of unsaturated fatty acids and sterols. After this, an equilibrium is established between acetyl-CoA consumption for fatty acid and

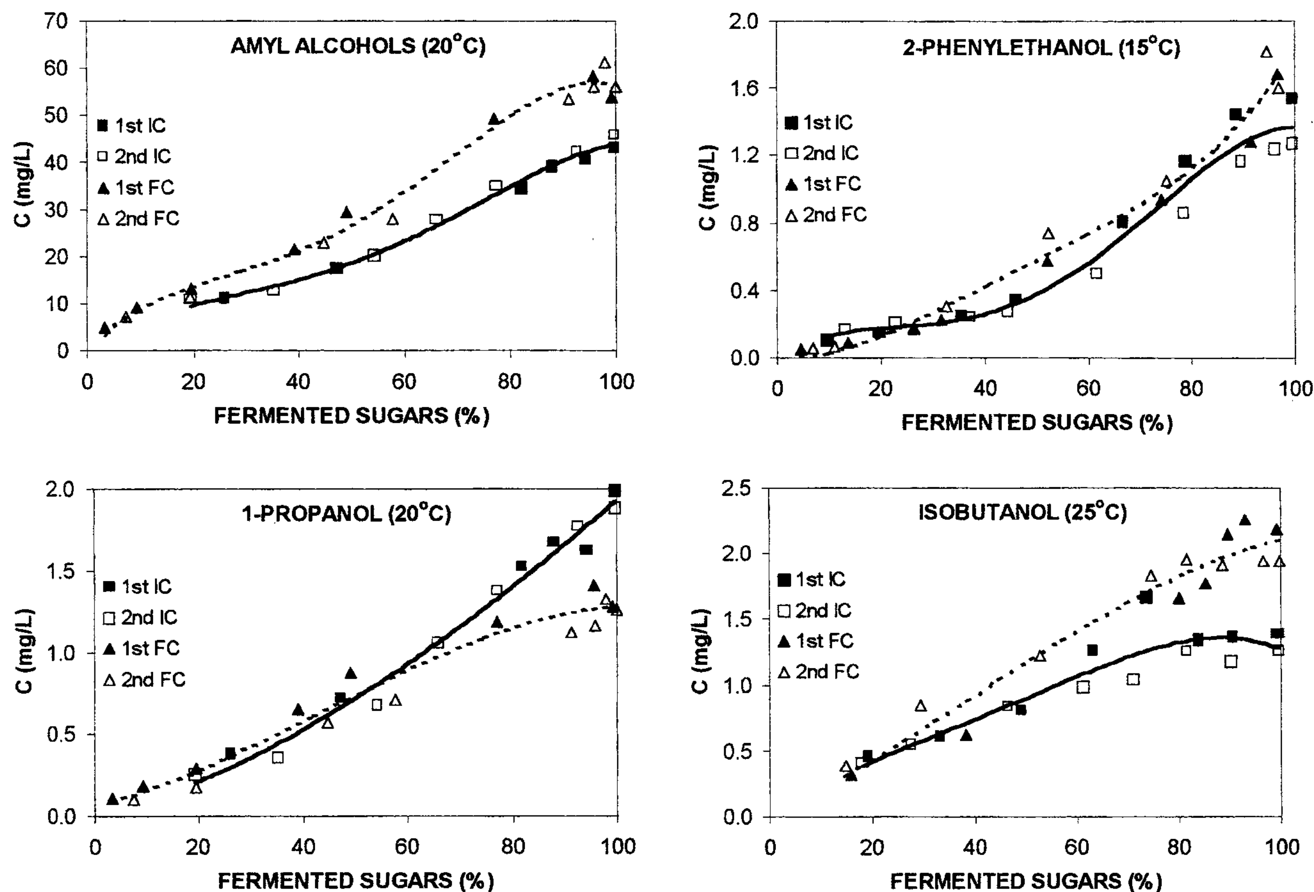

Figure 2. Production of higher alcohols during repeated batch fermentations of grape must using immobilized (IC) and free cells (FC): (squares) IC; (triangles) FC; (solid symbols) first repeated batch; (open symbols) second repeated batch. Lines represent polynomial fittings through the points of the two batches in each case.

sterol synthesis and that for ester production. This happens near the midpoint of fermentation (*20*). The evolution patterns of acetate esters were in agreement with the previous findings. However, yeasts are also able to hydrolyze esters via intracellular ester-hydrolases EHase (*19*). Thus, the production of esters is believed to be dependent on the balance of ester synthesis by AATase and ester hydrolysis by EHase. Usually, near the end of fermentation there is a decrease in ester concentration due to the greater action of hydrolase (*21*, *22*). This phenomenon was also observed in the fermentations using FC. On the contrary, similar action was not evident for IC, with the exception of ethyl acetate at 25 °C. The different evolution patterns of IC and FC can be attributed to the following: (i) the immobilization of cells changed the activity of the enzymes AATase and EHase (even at the genetic level), (ii) a favorable microenvironment was established for the action of acetyltransferase due to limited diffusion of oxygen at the IC, and (iii) greater amounts of acetyl-CoA were available for ester synthesis due to limited growth of IC on grape skins (*6*).

Evolution of Alcohols. Propanol, isobutanol, amyl alcohols, and 2-phenylethanol are the most abundant higher alcohols found in the aroma of wine. Their odors are rather unpleasant, and they are regarded as a negative quality factor. 2-Phenylethanol is the only higher alcohol that has been described in pleasant terms such as old rose (*13*, *25*).

The synthesis of amyl alcohols started from the beginning of fermentation. The formation rate was slightly increased after the fermentation of 40% of the sugars (**Figure 2**). As a result of this, greater amounts of amyl alcohols were formed after the midpoint of fermentation. The same profile was observed for all temperatures, with either FC or IC. However, the temperature effect on the amyl alcohol production rate was different for IC and FC. Thus, the final quantities of amyl alcohols in the produced wines were different (**Table 3**).

2-Phenylethanol was formed mainly after the consumption of 50−60% of the sugars (**Figure 2**). This was more evident for IC. However, at 20 and 15 °C, the production of 2-phenylethanol by FC followed an almost linear trend with sugar consumption. The decrease of temperature resulted in a lowering of the amount of 2-phenylethanol in the final product. FC produced larger amounts of this alcohol (**Table 3**). The above results are in agreement with those of Fraile et al. (*16*).

Propanol and isobutanol followed a different formation pattern (**Figure 2**). Their synthesis was initiated at the beginning of fermentation and continued until the depletion of sugars, at an almost constant rate. The same was observed at every temperature using either IC or FC. In some cases, a slight increase or decrease of production rate appeared mainly near the last stages of fermentation. FC gave higher final concentrations of isobutanol than IC, whereas the opposite was observed for propanol. The amount of propanol produced was found to be nearly constant at every temperature. Isobutanol's content increased with temperature decrease (**Table 4**).

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 from sugars utilizing part of the enzymatic pathways needed for the formation of amino acids (*10*). In a typical wine fermentation, the nitrogen-

Table 3. Relative Concentrations[a] (Milligrams per Liter) of Amyl Alcohols and 2-Phenylethanol

	amyl alcohols				2-phenylethanol			
	25 °C	20 °C	15 °C	10 °C	25 °C	20 °C	15 °C	10 °C
20% of fermented sugars								
FC	5.16	13.37	9.30	8.53	0.11	0.34	0.12	0.23
IC	8.85	9.72	9.50	9.05	0.12	0.36	0.18	0.17
40% of fermented sugars								
FC	13.14	21.05	14.89	13.59	0.32	0.89	0.42	0.34
IC	13.50	15.15	14.88	13.63	0.35	0.42	0.26	0.25
60% of fermented sugars								
FC	26.07	33.64	20.99	19.39	0.93	1.63	0.74	0.57
IC	21.34	23.39	22.85	20.62	0.55	0.81	0.57	0.47
80% of fermented sugars								
FC	40.13	49.68	32.74	25.77	1.82	2.36	1.12	0.84
IC	29.34	34.96	33.85	31.26	0.84	1.51	1.07	0.84
100% of fermented sugars								
FC	45.80	56.45	57.51	29.09	2.52	2.70	1.84	0.80
IC	34.02	44.04	43.65	41.49	1.59	1.83	1.37	1.14

[a] Values have been calculated from the polynomial fittings through the points of two repeated batches in each temperature.

Table 4. Relative Concentrations[a] (Milligrams per Liter) of Propanol and Isobutanol

	propanol				isobutanol			
	25 °C	20 °C	15 °C	10 °C	25 °C	20 °C	15 °C	10 °C
20% of fermented sugars								
FC	0.10	0.28	0.15	0.20	0.43	0.92	0.83	0.94
IC	0.13	0.22	0.25	0.25	0.42	0.50	0.64	0.65
40% of fermented sugars								
FC	0.33	0.58	0.26	0.38	0.91	1.46	1.14	1.46
IC	0.37	0.53	0.53	0.52	0.74	0.90	1.00	0.96
60% of fermented sugars								
FC	0.64	0.89	0.41	0.56	1.40	1.97	1.54	1.96
IC	0.70	0.93	0.92	0.90	1.07	1.29	1.45	1.40
80% of fermented sugars								
FC	0.92	1.14	0.65	0.73	1.82	2.39	2.21	2.37
IC	1.07	1.41	1.37	1.35	1.32	1.65	1.86	1.97
100% of fermented sugars								
FC	1.05	1.28	1.03	0.85	2.10	2.66	3.35	2.67
IC	1.43	1.93	1.76	1.85	1.27	1.96	2.09	2.28

[a] Values have been calculated from the polynomial fittings through the points of two repeated batches in each temperature.

Table 5. Relative Concentrations[a] (Milligrams per Liter) of Acetoin, Acetaldehyde, and Acetic Acid

	acetoin				acetaldehyde				acetic acid			
	25 °C	20 °C	15 °C	10 °C	25 °C	20 °C	15 °C	10 °C	25 °C	20 °C	15 °C	10 °C
20% of fermented sugars												
FC	0.88	1.70	0.93	1.70	1.07	0.91	0.41	0.57	1.15	0.92	0.93	1.53
IC	0.30	0.40	0.52	0.29	0.60	0.91	0.52	0.81	0.35	0.12	0.18	0.21
40% of fermented sugars												
FC	1.74	2.55	1.31	2.04	0.79	0.73	0.48	0.82	1.63	1.74	1.91	2.46
IC	1.23	0.96	0.65	0.42	0.83	0.89	0.36	0.69	0.61	0.41	0.27	0.47
60% of fermented sugars												
FC	1.65	2.03	1.03	1.62	0.90	0.67	0.42	1.06	2.99	2.89	3.10	3.69
IC	1.79	1.18	0.54	0.36	0.66	0.88	0.30	0.51	1.22	0.83	0.46	0.74
80% of fermented sugar												
FC	0.70	0.63	0.37	0.86	0.52	0.57	0.34	0.95	5.61	4.15	4.35	4.88
IC	1.43	0.85	0.33	0.17	0.43	0.71	0.25	0.60	2.24	1.27	0.66	0.90
100% of fermented sugars												
FC	0.05	0.13	0.18	0.07	0.37	0.32	0.30	0.56	8.53	4.71	5.18	4.38
IC	0.13	0.06	0.10	0.05	0.17	0.16	0.15	0.27	3.32	1.61	0.56	0.85

[a] Values have been calculated from the polynomial fittings through the points of two repeated batches in each temperature.

containing compounds of grape must are incorporated in the yeast cell at the early stages and fill the biosynthetic pools. After this, nitrogen compounds will be taken up and degraded in a specific order of preference, which is dependent on many factors (environmental, physiological, and strain-specific) (*23*). It is difficult to explain the observed differences in the evolution patterns of higher alcohols because of the complicated metabolism. However, it would be interesting to investigate both the uptake of nitrogen compounds and the consumption of sugars and relate it to the formation of higher alcohols.

Evolution of Acetoin, Acetaldehyde, and Acetic Acid. Acetoin is a byproduct of alcoholic fermentation. Under normal vinification conditions, the amounts of acetoin produced do not exceed its threshold value. However, acetoin is important as it

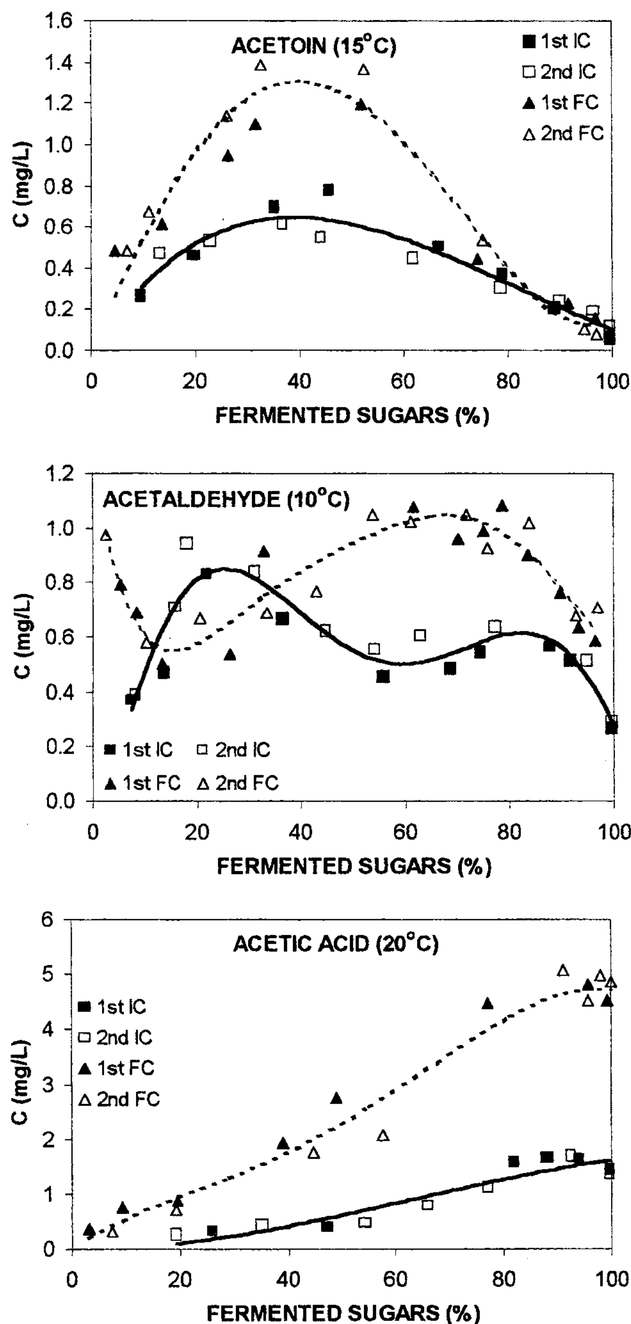

Figure 3. Production of acetoin, acetaldehyde, and acetic acid during repeated batch fermentations of grape must using immobilized (IC) and free cells (FC): (squares) IC; (triangles) FC; (solid symbols) first repeated batch; (open symbols) second repeated batch. Lines represent polynomial fittings through the points of the two batches in each case.

is the key compound in the biosynthesis of diacetyl, which creates an off-flavor in alcoholic beverages, even in very small amounts (*24*).

In this work, acetoin production was observed in the early stages of fermentation and reached a maximum near the midpoint (40−60% of fermented sugars). Afterward, its levels declined continuously until the end of fermentation (**Figure 3**). The maximum value in the evolution patterns of acetoin produced by IC decreased with temperature decrease. At 25 and 20 °C, the maximum of acetoin concentration for IC was observed when 60% of the sugars had been consumed. At lower temperature, this maximum value was observed when 40% of the sugars had been fermented (**Table 5**).

In wine yeasts, acetoin is formed only in the presence of fermentable carbohydrates or pyruvic acid. The produced acetoin is reduced to 2,3-butanediol with simultaneous oxidation of NADH to NAD^+. The ratio of NAD^+/NADH determines the extent of acetoin formation or degradation (*24*). In the early stages of fermentation, the need of yeasts for NADH is increased. Thus, significant amounts of acetoin are accumulated. Afterward, the redox balance changes, resulting in the simultaneous production of acetoin and NAD^+. The different maximum concentrations of acetoin between IC and FC reflect the different needs of these biocatalysts for reducing power.

The major aldehyde found in the produced wines is acetaldehyde. At low levels, it gives a pleasant fruity aroma, but at higher concentrations it gives a pungent irritating odor, which is considered to be undesirable for table wines (*13*, *25*). Acetaldehyde's content increased dramatically during the first stages of fermentation, reaching a maximum when 20% of the sugars were fermented or even earlier (**Figure 3**). Afterward, the amount of acetaldehyde decreased toward the end of fermentation. In some cases, fluctuations were observed. A similar evolution pattern has been described by other workers (*25*). The explanation is similar to that given for acetoin. It seems that yeasts initially release certain compounds, such as acetaldehyde and acetoin, to the medium and later they reincorporate them. In this way, the redox potential is balanced and the fermentation proceeds without any problem.

FC produced slightly greater amounts of acetaldehyde than IC. These amounts were similar at 25, 20, and 15 °C, whereas they increased with further decrease of temperature to 10 °C (**Table 5**). The amount of acetaldehyde is dependent on the activity of alcohol dehydrogenase, which reduces the aldehyde to alcohol. This activity seems to be greater in IC and depends on temperature.

Acetic acid is the most important volatile acid produced during alcoholic fermentation. It gives the note of vinegar when it is present in high amounts (*13*). IC were less favorable to acetic acid production at every temperature (**Table 5**). Acetic acid was synthesized during alcoholic fermentations in a similar manner by both biocatalysts. Its concentration increased constantly until the end of fermentation (**Figure 3**). The decrease of temperature affected the production of acetic acid, lowering its concentration in the final product.

From the finding of this investigation it is obvious that the evolution patterns of the volatiles studied were similar for both immobilized and free cells. Significant differences were observed only in the formation rates, especially those of fruity esters. In most cases, no hydrolytic phenomena of esters were observed for IC. Immobilized cells, in comparison with the free ones, produced wines containing larger amounts of esters. The difference in the amounts of esters increased with temperature decrease. This, in relation with the reduction of the amount of higher alcohols with temperature decrease, favors low-temperature wine-making using immobilized cells, leading thus to the production of wines of improved quality. From an economic point of view, immobilized yeast cells on grape skins improved the rate and efficiency of transformation (*6*), leading thus to reduced costs. It should be mentioned that grape skins are a cheap and relatively resistant material that is not affected by the fermentation environment. Their easy handling makes simple the design of the bioreactor. The use of grape skins as support for cell immobilization offers a good alternative to the wine-making industry, which considers and treats them as a waste byproduct.

Finally, monitoring the evolution of volatile byproducts in the wine production by the SPME technique is very useful because the industrialist can determine when, how, and which factors influence their production. This makes easy the manipulations before or during fermentation to produce wines of better quality.

ABBREVIATIONS USED

SPME, solid phase microextraction; GC-MS, gas chromatography−mass spectrometry; RSD, relative standard deviation; SG, specific gravity; IC, immobilized cells; FC, free cells; AATase, alcohol acetyltransferase; EHase, ester hydrolase; NAD^+, NADH nicotinamide adenine dinucleotide.

LITERATURE CITED

(1) Divies, C.; Cachon, R.; Cavin, J. F.; Prevost, H. Theme 4: Immobilized cell technology in wine production. *Crit. Rev. Biotechnol.* **1994**, *14*, 135−153.
(2) Bardi, E.; Koutinas, A. A. Immobilization of yeast on delignified cellulosic material for room temperature and low-temperature winemaking. *J. Agric. Food Chem.* **1994**, *42*, 221−226.
(3) Kourkoutas, Y.; Komaitis, M.; Koutinas, A. A.; Kanellaki, M. Wine production using yeast immobilized on apple pieces at low and room temperatures. *J. Agric. Food Chem.* **2001**, *49*, 1417−1425.
(4) Bakoyianis, V.; Kanellaki, M.; Koutinas, A. A.; Agelopoulos, K. Comparative study of kissiris, γ-alumina and Ca-alginate as supports of cells for batch and continuous wine making at low temperatures. *J. Agric. Food Chem.* **1997**, *45*, 4884−4888.
(5) Yajima, M.; Yokotsuka, K. Volatile compound formation in white wines fermented using immobilized and free yeast. *Am. J. Enol. Vitic.* **2001**, *52*, 210−218.
(6) Mallouchos, A.; Reppa, P.; Aggelis, G.; Kanellaki, M.; Koutinas, A. A.; Komaitis, M. Grape skins as a natural support for yeast immobilization. *Biotechnol. Lett.* **2002**, *24*, 1331−1335.
(7) Ferraro, L.; Fatichenti, F.; Ciani, M. Pilot scale vinification process using immobilized *Candida stellata* cells and *Saccharomyces cerevisiae*. *Process Biochem. (Oxford)* **2000**, *35*, 1125−1129.
(8) Bardi, E.; Koutinas, A. A.; Psarianos, C.; Kanellaki, M. Volatile by-products formed in low-temperature wine making using immobilized yeasts. *Process Biochem. (Oxford)* **1997**, *32*, 579−584.
(9) Mallouchos, A.; Komaitis, M.; Koutinas, A.; Kanellaki, M. Wine fermentations by immobilized and free cells at different temperatures. Effect of immobilization and temperature on volatile by-products. *Food Chem.* in press.
(10) Nykanen, L.; Suomalainen, H. Formation of aroma compounds by yeast. In *Aroma of Beer, Wine and Distilled Alcoholic Beverages*; Reidel Publishing: Dordrecht, The Netherlands, 1983; pp 3−15.
(11) Schreier, P. Flavor composition of wines: A review. *CRC Crit. Rev. Food Sci. Nutr.* **1979**, *12*, 59−11.
(12) Rapp, A.; Mandery, H. Wine aroma. *Experientia* **1986**, *42*, 873−884.
(13) Etievant, X. P. Wine. In *Volatile Compounds in Foods and Beverages*; Maarse, H., Ed.; Dekker: New York, 1991; pp 483−533.
(14) Vianna, E.; Ebeler, E. S. Monitoring ester formation in grape juice fermentations using solid-phase microextraction coupled with gas chromatography−mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 589−595.
(15) Vas, G.; Blechschmidt, I.; Kovacs, T.; Vekey, K. Examination of aroma production kinetics of different commercial wine yeast in fermenting Muscat Ottonel wines with the help of SPME headspace sampling and fast GC analysis. *Acta Aliment*. **1999**, *28*, 133−140.
(16) Fraile, P.; Garrido, J.; Ancin, C. Influence of a *Saccharomyces cerevisiae* selected yeast strain in the volatile composition of rose wines. Evolution during fermentation. *J. Agric. Food Chem.* **2000**, *48*, 1789−1798.
(17) Fraile, P.; Garrido, J.; Ancin, C. Effect of *S. cerevisiae* selected killer strains on the volatile composition of rose wines. *Eur. Food Res. Technol.* **2001**, *213*, 126−131.
(18) Mallouchos, A.; Komaitis, M.; Koutinas, A.; Kanellaki, M. 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. *J. Agric. Food Chem.* **2002**, *50*, 3840−3848.
(19) Rojas, V.; Gil, V. J.; Manzanares, P.; Gavara, R.; Piñaga, F.; Flors, A. Measurement of alcohol acetyltransferase and ester hydrolase activities in yeast extracts. *Enzyme Microb. Technol.* **2002**, *30*, 224−230.
(20) Lilly, M.; Lambrechts, M. G.; Pretorious, I. S. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Appl. Environ. Microbiol.* **2000**, *66*, 744−753.
(21) Inoue, Y.; Trevanichi, S.; Fukuda, K.; Izawa, S.; Wakai, Y.; Kimura, A. Roles of esterase and alcohol acetyltransferase on production of isoamyl acetate in *Hansenula mrakii*. *J. Agric. Food Chem.* **1997**, *45*, 644−649.
(22) Fukuda, K.; Yamamoto, N.; Kiyokawa, Y.; Yanagiuchi, T.; Wakai, Y.; Kitamoto, K.; Inoue, Y.; Kimura, A. Balance of activities of alcohol acetyltransferase and esterase in *Saccharomyces cerevisiae* is important for production of isoamyl acetate. *Appl. Environ. Microbiol.* **1998**, *64*, 4076−4078.
(23) Boulton, B. R.; Singleton, L. V.; Bisson, F. L.; Kunkee, E. R. Yeast and biochemistry of ethanol fermentation. In *Principles and Practices of Winemaking*; Chapman and Hall: New York, 1996; pp 102−192.
(24) Romano, P.; Suzzi, G. Origin and production of acetoin during wine yeast fermentation. *Appl. Environ. Microbiol.* **1996**, *62*, 309−315.
(25) Jackson, S. R. Fermentation. In *Wine Science. Principles and Applications*; Academic Press: San Diego, CA, 1994; pp 220−272.

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