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Volatile Compounds of Wines Produced by Cells Immobilized 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 the immobilized biocatalyst as well as the free yeast cells at 25, 20, 15, and 10 °C. The major volatile byproducts were determined by GC, whereas the minor volatile constituents were extracted in dichloromethane and analyzed by HRGC-MS. The qualitative profiles of the wines produced were similar in every case. Immobilized cells gave wines with higher contents of ethyl and acetate esters that increased with temperature decreases from 25 to 15 °C. The amount of volatile alcohols was more pronounced in wines produced by free cells and decreased dramatically at low fermentation temperatures (10 °C).

KEYWORDS: Immobilization; yeast; wine; volatiles; esters; acetates; higher alcohols; acids; temperature; fermentation; GC-MS

INTRODUCTION

The use of cell immobilization in alcoholic fermentation is an attractive research area because of its technical and economical advantages over that using free cell systems (*1*). However, for application in the wine industry, research is needed to find an immobilization support that meets the prerequisite of food grade purity and can be used to obtain a final product of improved aroma and taste. Particles of various synthetic or natural materials, organic and inorganic, have been used as supports in cell immobilization for wine fermentations (*2*−*5*). Recently, immobilized yeast cells on grape skins were used for conducting repeated batch fermentations of grape must (*6*). Immobilization on these supports modified cell metabolism, thus resulting in increased specific rates of substrate consumption and product formation, as well as ethanol productivity.

Wine aroma is the result of a complex combination of components that give each wine its distinctive character. It has been shown that the main fraction of the aroma compounds is produced during fermentation. These compounds are acetates and ethyl esters, higher alcohols, fatty acids, ketones, and aldehydes (*7*, *8*).

Esters have long been considered to be important contributors to wine aroma because they are major volatile compounds with fruity odors. Higher alcohols have never been considered as quality factors. Because their odors are rather unpleasant, they contribute more to the intensity of the odor of the wine than to its quality (*9*).

One of the most important and well-studied factors affecting wine aroma is temperature. Manufacturers recognize that wines produced at low temperatures have more fruity aroma because of the increased synthesis and/or reduced hydrolysis of esters (*10*). Several authors have reported the combined effect of temperature and immobilization on the formation of major volatiles during wine fermentation (*5*, *11*, *12*). However, publications concerning the analysis of these volatile components in wines produced by immobilized cells are scarce in the literature (*13*).

The aim of the present work was to investigate the differences in aroma compounds produced in wine by free and immobilized yeast cells on a support consisting of grape skins as well as the effect of fermentation temperature on 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 in static flasks under semiaerobic conditions at 25 °C for 24 h and separated by centrifugation at 1310*g* for 10 min, and the cells were harvested.

Fermentation Medium. Concentrated grape must (∼35 °Be) obtained from the winery Achaia Clauss (Patras, Greece) was used for alcoholic fermentations after appropriate dilution with deionized water to a sugar content of 235 g/L and without further addition of nutrients. The pH was adjusted to 3.4 using tartaric acid in order to obtain conditions similar to those of natural must. The medium was sterilized at 121 °C for 20 min.

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Table 1. Major Volatile Byproducts (Milligrams per Liter) in Wines Produced by Repeated Batch Fermentations Using Free Cells (FC) and Immobilized Cells (IC) on Grape Skins at Temperatures of 25, 20, 15, and 10 °C[a]

major volatile	IC 25	IC 20	IC 15	IC 10	FC 25	FC 20	FC 15	FC 10	*F* ratio Z	*F* ratio T	*F* ratio Z × T
acetaldehyde	72.4Aa	64.4Aa	60.6Aa	69.9Aa	96.1Aa	92.2Aa	82.2Aa	160.9Bb	**54.63**	**12.26**	**9.07**
ethyl acetate	97.4Aa	179.5Ab	202.8Ab	175.6Ab	65.4Ba	67.5Ba	42.6Bab	31.4Bb	**796.65**	**25.05**	**51.49**
1-propanol	67.9Aa	70.3Aa	78.1Aa	70.0Aa	54.4Ba	55.9Ba	47.3Ba	33.2Bb	**176.38**	**9.07**	**10.55**
2-methyl-1-propanol	19.9Aa	23.8Aab	30.6Ab	28.2Aab	25.4Aa	34.5Aab	40.7Ab	34.1Aab	**20.46**	**9.14**	0.58
amyl alcohols	138.3Aa	165.2Aa	164.6Aa	135.3Aa	182.0Ba	221.3Ba	196.8Aa	108.6Ab	**22.47**	**31.55**	**10.89**

[a] Data are the mean values from three repeated batches at each temperature. Z, fermentation type (immobilized or free cells); T, temperature; Z × T, interaction. Significant *F* ratios are in bold ($p < 0.05$). Statistical comparisons were made by ANOVA between fermentations using immobilized and free yeast under the same fermentation conditions (temperature) except for the biocatalyst; letters A and B following values indicate significant differences. Statistical comparisons were also made within each level of fermentation type (examining temperature under the same biocatalyst); letters a and b following values indicate significant differences. The level of significance was $p < 0.05$. There was no significant difference between samples with the same letter.

Immobilization. Skins of fresh grape berries (Tafeltrauben Grapes-Raisins, class I, S.A. San Miguel, Argentina) obtained from a local supermarket were removed by exhaustive pressing and used as a support for immobilization. Twenty grams of wet weight of *S. cerevisiae* cells, prepared as described above, was suspended in 1 L of semisynthetic medium containing 120 g of glucose/L at pH 4.8. Four hundred grams of wet grape skins (~88 g of dry weight) was sterilized at 121 °C for 20 min and added to the broth to ferment in static flasks at 25 °C for 6−8 h, under semiaerobic conditions. The supernatant was decanted, and the support was washed twice with 400 mL of must. The prepared biocatalyst was used directly in fermentations of grape must. To determine the degree of immobilized cells, samples of 10 g of wet grape skins (~1.2 g of dry weight) were taken during fermentation and homogenized for 4 min in a Stomacher with 90 mL of one-fourth strength Ringer solution. After appropriate decimal dilutions of the homogenates, the yeast cells were enumerated using a Neubauer improved hemacytometer (*6*). Approximately 4.5×10^8 ($\pm 1.0 \times 10^8$) cells were immobilized per gram of wet grape skins (corresponding to 2.96×10^{-3} g of dry weight of cells/g of wet grape skins).

Fermentation Experiments. Repeated batch fermentations were carried out using 800 mL of grape must and the immobilized 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 triplicate. 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. Fermentations were monitored by measuring specific gravity (SG) and stopped when it reached a value of 0.992−3 g/mL. At the end of each fermentation batch, the wines produced were centrifuged at 1310*g* for 10 min, and the supernatant was stored at low temperature until further use.

Volatile Analysis. The major volatile constituents [acetaldehyde, ethyl acetate, 1-propanol, 2-methyl-1-propanol, amyl alcohols (3-methyl- and 2-methyl-1-butanol)] were determined by direct injection of wine samples in a Fisons Instruments gas chromatograph (GC 8000 series, model 8060), equipped with a split−splitless injector (split ratio = 1/20) and an FID detector. A Chrompack WCOT fused silica column was used (CP-Wax 52CB, 60 m, 0.32 mm i.d., 0.25 μm film thickness). Helium was used as a carrier gas, at a flow rate of 2.05 mL/min. One microliter of wine was injected. The oven temperature was programmed as follows: 40 °C for 5 min and then raised to 120 °C at a rate of 5.0 °C/min, followed by an increase to 230 °C at a rate of 25 °C/min, at which it was held for 5 min. The injector temperature was maintained at 200 °C, whereas the detector temperature was at 240 °C.

4-Methyl-1-pentanol was used as an internal standard. Stock solutions of the standards were prepared in an alcoholic solution containing 12% (v/v) ethanol and 4 g of tartaric acid/L. The pH was adjusted to 3.2 with 0.1 N NaOH to simulate the wine matrix. Mixtures of the amyl alcohols and internal standard were dissolved in pure ethanol. The wine samples were transferred into a volumetric flask of 10 mL, and 50 μL of internal standard of known concentration was added. All samples were analyzed in triplicate.

The minor volatile constituents were isolated by liquid extraction (*13*). Dichloromethane was used as a solvent and methyl hexanoate as the internal standard. Analyses of the dichloromethane extracts were performed on a Fisons Instruments GC-MS (GC 8000 series, model 8060, MS MD800) with the injector in the splitless mode for 30 s. The column was the same as that used in the analysis mentioned above.

One microliter of sample extract was injected. Oven temperature was programmed as follows: 35 °C for 2 min and then raised to 50 °C at a rate of 4.0 °C/min, at which it stayed for 5 min, after which the temperature was raised to 230 °C at a rate of 4 °C/min and held for 10 min. The injector was set at 230 °C, the source at 200 °C, and the interface at 250 °C. The mass spectrometer was operated in the electron impact mode with the electron energy set at 70 eV and the mass range at *m*/*z* 29−400. Identification of compounds was obtained by comparing the retention times with those of authentic compounds and the spectral data obtained from the Wiley and NIST libraries. The volatile compounds were quantified by dividing the peak areas of the compounds of interest by the peak area of the internal standard (IS) and multiplying this ratio by the initial concentration of the IS (expressed as micrograms per liter). Each determination was carried out in duplicate.

Statistical Analysis. Analysis of variance (ANOVA) was carried out on volatile compounds' data. The General Linear Model was used to assess the significance of fermentation type (free or immobilized cells), temperature, and interaction of these factors on the amounts of wine volatiles. The means were compared by Tukey's honestly significant difference (HSD) procedure (Statgraphics Plus).

RESULTS AND DISCUSSION

Major Volatiles. Acetaldehyde is one of the most important carbonyl compounds formed during vinification and constitutes >90% of the total aldehyde content of wine aroma (*9*). Immobilized cells produced less acetaldehyde than free cells at the temperatures investigated (**Table 1**). However, a statistically significant difference was observed only at 10 °C. The concentration of acetaldehyde was similar at temperatures ranging between 25 and 10 °C and between 25 and 15 °C, for IC and FC, respectively. Further decrease in temperature resulted in a significant increase in acetaldehyde concentration. Generally, the concentration of acetaldehyde was near the levels determined in commercial white wines with the exception of that obtained at 10 °C for FC (*14*). Similar results have been reported by other workers in fermentations using either free or immobilized cells (*5*, *13*, *15*). The differences in the amounts of acetaldehyde produced may reflect the effect of temperature and immobilization on the activity of alcohol dehydrogenase, responsible for the reduction of aldehydes to alcohols.

The amount of ethyl acetate produced by IC was significantly higher than that produced by FC (**Table 1**). These quantities contribute to the fruity character of wine because they are higher

Table 2. Volatile Compounds in Wines Produced by Free Cells and Immobilized Cells on Grape Skins

t_R (min)	compound	reliability of identification[a]	t_R (min)	compound	reliability of identification[a]
	Esters			Alcohols	
5.159	ethyl acetate	a	9.310	propanol	a
7.026	ethyl propanoate	a	11.760	2-methyl-1-propanol	a
7.209	ethyl isobutanoate	b	14.443	butanol	a
7.426	propyl acetate	a	17.744	amyl alcohols	a
8.560	isobutyl acetate	a	19.610	1-pentanol	b
9.393	ethyl butanoate	a	21.927	4-penten-1-ol	a
9.810	methyl ethanethioate	b	22.477	4-methyl-1-pentanol	a
11.026	butyl acetate	a	23.010	3-methyl-1-pentanol	a
13.543	3-methyl- and 2-methyl acetate	a	23.577	3-pentanol	a
16.027	pentyl acetate	b	24.094	hexanol	a
16.727	methyl hexanoate (IS)	a	24.927	3-ethoxy-1-propanol	b
18.327	4-penten-1-yl acetate	b	31.378	α-linalool	a
18.743	ethyl hexanoate	a	31.711	1-octanol	a
20.660	ethyl pyruvate	b	35.211	2-furanmethanol	a
23.760	ethyl lactate	b	36.278	α-terpineol	a
25.394	2,3-butanediyl diacetate	b	36.928	3-(methythio)-1-propanol	b
27.377	ethyl octanoate	a	42.745	2-phenylethanol	a
30.361	ethyl 3-hydroxybutanoate	b			
31.111	2-furanmethyl acetate	b		Acids	
34.512	ethyl decanoate	a	28.144	acetic acid	a
35.645	diethyl succinate	b	31.261	propanoic acid	b
36.111	ethyl 9-decenoate	b	32.244	2-methylpropanoic acid	b
39.962	2-phenylethyl acetate	a	34.211	butanoic acid	b
			35.528	3-methylbutanoic acid	b
	Carbonyls		40.812	hexanoic acid	b
5.326	2-propanone	a	46.662	octanoic acid	b
21.110	3-hydroxy-2-butanone	a	51.979	decanoic acid	b
29.961	1-(2-furanyl)ethanone	b	53.429	10-undecenoic acid	b
30.594	benzaldehyde	b			
30.744	dihydro-2-methyl-3(2*H*)-thiophenone	b		Others	
34.861	3-methoxy-4,5-dimethyl-2(5*H*)-furanone	b	28.578	linalool oxide	b
37.228	5-ethoxydihydro-2(3*H*)-furanone	b			
39.728	dihydro-4-methyl-2(3*H*)-furanone	b			
41.212	2,5-dimethyl-4-hydroxy-3(2*H*)-furanone	b			

[a] a, identification by comparison of retention times and mass spectral data with those of authentic compounds; b, tentative identification.

than the odor threshold and lower than the level considered to have a negative impact on wine aroma (*9*). Similar results have been reported by Bardi (*11*) and Bakoyianis (*12*). A dramatic increase in ethyl acetate content for IC was observed at temperatures from 25 to 20 °C. This amount remained relatively constant until 10 °C. A further decrease in temperature to 5 °C resulted in a significant decrease of ethyl acetate concentration (results not shown). These results are in agreement with those of other investigators (*15*−*17*). The concentration of ethyl acetate produced by FC seems to decrease with a decrease in temperature (**Table 1**).

The synthesis of esters by *S. cerevisiae* occurs via an intracellular process catalyzed by the enzyme alcohol acetyltransferase (AAT), the activity of which is strongly repressed under aerobic conditions or the addition of unsaturated fatty acids (*18*). The higher amounts of ethyl acetate produced by IC may have been the result of a higher activity of AAT, due to lower oxygen levels in the immobilization matrix, as a result of limited diffusion (*1*).

Fusel alcohols compose the largest group of aroma compounds in alcoholic beverages (*8*, *19*). In the present study, IC produced more 1-propanol than FC, at the fermentation temperatures used (**Table 1**). However, the concentrations of 1-propanol were near the mean value found in commercial wines. These concentrations were lower than the odor threshold reported by Etievant (*9*). Similar amounts of 1-propanol were produced at 25−10 °C for IC and at 25−15 °C for FC. Further decreases in temperature resulted in significant decreases in 1-propanol concentration.

The interaction of temperature with fermentation type (IC or FC) was not statistically significant for 2-methyl-1-propanol (**Table 1**). However, the main effects were significant when it was synthesized in smaller quantities by IC than by FC. Decreases in fermentation temperature from 25 to 15 °C resulted in an increased concentration of 2-methyl-1-propanol. Generally, the levels of 2-methyl-1-propanol were low.

FC produced large amounts of amyl alcohols. Significant differences between FC and IC were observed at 25 and 20 °C (**Table 1**). The concentrations of amyl alcohols were similar at 25−10 °C for IC (nonsignificant differences). Further decreases in temperature resulted in significant decreases in amyl alcohols concentrations (results not shown). Similar results were obtained for FC.

Finally, the total amounts of major fusel alcohols decreased significantly at low fermentation temperatures (10−5 °C) for both immobilized and free cells. The values of the major fusel alcohols were always below 300 mg/L. This fact was considered to contribute positively to the complexity of wine aroma (*7*). Decreases in the amounts of higher alcohols produced with decreases in temperature have been reported by other workers (*9*, *15*, *20*). Similar results have also been observed in fermentations using cells immobilized on kissiris, γ-alumina, delignified cellulosic material, and apple pieces (*3*, *11*, *12*).

Minor Volatiles—Qualitative Composition. Detailed investigation of the minor volatile components produced in wines by free or immobilized cells was effected by GC-MS.

The volatile compounds identified in all wines are presented in **Table 2**. The major components were ethyl and acetate esters

Table 3. Esters (Micrograms per Liter) Determined by GC-MS in Wines Produced by Free Cells (FC) and Immobilized Cells (IC) on Grape Skins at Temperatures of 25, 20, 15, and 10 °C[a]

									F ratio		
compound	IC 25	IC 20	IC 15	IC 10	FC 25	FC 20	FC 15	FC 10	Z	T	Z × T
ethyl esters											
ethyl propanoate	5.9Aa	7.9Aab	9.8Ab	7.0Aa	2.8Ba	3.9Ba	3.5Ba	4.0Ba	**106.04**	**5.99**	**4.16**
ethyl isobutanoate	1.2Aa	1.2Aa	1.1Aa	0.6Ab	0.6Ba	0.7Ba	0.5Ba	0.5Aa	**54.72**	**6.36**	2.67
ethyl butanoate	23.7Aa	29.2Aab	31.6Aab	36.3Ab	14.0Aa	17.8Aa	15.6Ba	16.8Ba	**76.64**	**3.64**	1.83
ethyl hexanoate	38.1Aa	48.6Aa	85.2Ab	95.4Ab	30.2Aa	38.2Aa	38.8Ba	16.8Ba	**52.68**	**6.64**	**11.02**
ethyl pyruvate	12.7a	4.5b	3.1b	1.6b	3.4a	2.5b	1.1c	tr			
ethyl lactate	239Aa	205Aa	155Aa	43.2Ab	53.7Ba	59.0Ba	25.1Ba	15.8Aa	**64.59**	**11.30**	**4.67**
ethyl octanoate	18.5Aa	20.6Aa	26.5Aab	34.5Ab	28.4Aa	28.8Aa	18.1Ab	11.9Bb	**5.38**	0.53	**28.86**
ethyl 3-hydroxybutanoate	0.7Aa	0.7Aa	0.9Aab	1.1Ab	0.3Aa	0.5Ba	0.3Aa	0.4Aa	**106.55**	**5.06**	7.32
ethyl decanoate	0.5Aa	0.4Aa	0.4Aa	0.7Aa	1.5Ba	1.2Aab	0.8Aab	0.4Ab	**16.57**	2.62	5.69
diethyl succinate	3.3Aa	3.5Aa	2.4Aa	1.3Aa	1.2Aab	1.5Aab	1.2Aa	4.6Bb	1.37	1.37	**8.28**
ethyl 9-decenoate	0.6Aa	0.6Aa	0.6Aa	1.3Aa	1.0Aa	1.3Aa	2.0Ba	2.0Aa	**21.24**	**3.98**	1.43
sum of ethyl esters	344	322	317	223	137	155	107	73.2			
acetate esters											
propyl acetate	9.0Aa	20.2Ab	22.8Ab	16.2Aab	3.4Aa	3.7Ba	2.4Ba	0.8Ba	**92.09**	**3.97**	**4.44**
isobutyl acetate	9.8Aa	20.8Aab	26.3Ab	17.3Aab	8.3Aa	8.7Aa	5.5Ba	1.3Ba	**46.67**	**3.91**	**5.10**
butyl acetate	2.6a	5.7b	5.2b	3.6b	0.7a	0.7b	0.4c	tr			
isoamyl acetate	2191Aa	5798Ab	7717Ac	5538Ab	860Aa	1550Ba	560Ba	35.6Ba	**470.35**	**30.48**	**35.37**
4-pentenyl acetate	3.1ac	5.6ab	7.4b	6.1b	1.9a	1.4ab	0.6b	tr			
2-phenylethyl acetate	689Aa	1030Ab	1043Ab	673Aa	512Aa	484Ba	151Bb	19.6Bb	**267.58**	**22.81**	**19.12**
sum of acetate esters	2905	6880	8822	6254	1386	2049	720	57.3			
total ester content	3249	7202	9139	6477	1523	2204	827	131			

[a] Data are the mean values from three repeated batches at each temperature. Z, fermentation type (immobilized or free cells); T, temperature; Z × T, interaction. Significant *F* ratios are in bold ($p < 0.05$). Statistical comparisons were made by ANOVA between fermentations using immobilized and free yeast under the same fermentation conditions (temperature) except for the biocatalyst; letters A and B following values indicate significant differences. Statistical comparisons were also made within each level of fermentation type (examining temperature under the same biocatalyst); letters a–c indicate significant differences. The level of significance was $p < 0.05$. There was no significant difference between samples with the same letter.

and higher alcohols. Low molecular weight fatty acids are the only acids that may have an impact on the wine aroma because they have low odor thresholds. They are present in rather high concentrations in wines and show enough volatility at room temperature (*7*, *9*). The majority of carbonyl compounds identified are furanones, and they are possibly formed during the condensation of grape must or during the process of sterilization.

The wines produced by either free or immobilized cells on grape skins contained the same number and type of volatile compounds. Most of them were formed during alcoholic fermentation. No differences were observed in the qualitative profile of the wines aroma. This is in accordance with a previous study using other supports (*13*). However, significant differences were observed in the quantitative profile.

Minor Volatiles—Quantitative Composition. *Ethyl and Acetate Esters.* In most cases, the concentrations of esters in wines produced by IC were significantly higher than those in wines produced by FC except for ethyl decanoate, ethyl 9-decenoate, and diethyl succinate. The effect of temperature as well as the interaction of temperature with fermentation type was in most cases statistically significant (**Table 3**).

IC produced more acetate esters than FC, which increased with a decrease in temperature from 25 to 15 °C. In wines produced by FC, the concentrations of acetate esters seem to decrease with a decrease in temperature, although the differences observed were not significant. The optimum condition for acetate ester production was observed to be at 15 °C, at which 12 times more acetate esters were obtained by IC than by FC. Similar findings were observed for ethyl acetate, as stated earlier.

The concentration of the ethyl esters of propanoic, butanoic, hexanoic, and octanoic acids in relation to fermentation temperature showed similar production patterns. For IC, their concentrations increased significantly with temperature decreases from 25 to 10 °C. A further decrease in temperature (5 °C) resulted in a low ester production (results not shown). For FC, the amounts of these esters were approximately similar at the various temperatures (no significant differences). An exception was ethyl octanoate, the amount of which decreased. In most cases, the wines produced by IC contained more esters.

In the case of IC, the effect of temperature on the concentrations of ethyl isobutanoate and ethyl lactate was different when their concentrations decreased with decrease in temperature. However, statistically significant differences were observed between 25 and 10 °C. The amounts of these esters in wines produced by FC were similar.

The concentration of ethyl 3-hydroxybutanoate increased with a decrease in temperature for IC, whereas for FC it remained almost constant, and the opposite was observed for ethyl decanoate. The interaction of temperature with fermentation type was not significant in the formation of ethyl 9-decenoate. However, the main effects were significant when FC produced greater amounts than IC. Decreases in temperature resulted in wines with higher concentrations of ethyl 9-decenoate, whereas the opposite was observed for diethyl succinate.

Generally, the concentrations of ethyl and acetate esters were higher in wines produced by IC on grape skins for the fermentation temperatures studied. The total ethyl esters' content decreased with decreases in temperature. In particular for IC, this decrease was more intense at low temperatures (<15 °C), at which the content of acetate esters increased with a decrease in temperature, reaching a maximum at 15 °C.

The effect of temperature on the formation of esters has been well studied (*9*, *16*). Low fermentation temperatures (15 °C)

Table 4. Alcohols (Micrograms per Liter) Determined by GC-MS in Wines Produced by Free Cells (FC) and Immobilized Cells (IC) on Grape Skins at Temperatures of 25, 20, 15, and 10 °C[a]

									F ratio		
compound	IC 25	IC 20	IC15	IC 10	FC 25	FC 20	FC15	FC 10	Z	T	Z × T
butanol	12.4Aa	13.8Aa	10.6Aab	7.9Ab	6.7Ba	5.6Ba	3.7Ba	2.1Ba	**128.33**	**13.60**	0.91
4-pentenol	4.0Aa	3.8Aa	4.2Aa	4.0Aa	3.8Aa	2.6Aa	2.3Aa	2.0Aa	**18.23**	1.61	1.72
4-methyl-1-pentanol	0.5Aa	0.6Aa	0.5Aab	0.4Ab	0.4Aab	0.5Aa	0.3Bab	0.2Ab	**44.00**	**18.87**	0.89
3-methyl-1-pentanol	0.9Aa	1.3Ab	1.7Ac	1.7Ac	0.5Ba	0.7Ba	0.6Ba	0.4Ba	**369.78**	**22.51**	**24.26**
3-pentanol	3.6Aa	20.5Aab	15.4Abc	8.4Ac	14.9Ba	30.5Ab	27.9Bb	3.1Aa	0.05	**29.27**	**17.11**
hexanol	0.6Aa	0.6Aa	0.5Aa	0.5Aa	0.6Aa	0.6Aa	0.5Aa	0.6Aa	0.09	**3.50**	2.39
3-ethoxy-1-propanol	1.4Aa	1.2Aa	1.5Aa	1.7Aa	2.8Ba	2.2Aab	1.5Abc	1.0Ac	**8.74**	**4.73**	**9.46**
α-linalool	1.2Aa	1.2Aa	0.9Aa	0.8Aa	0.8Aa	0.7Aa	0.6Aa	0.5Aa	**22.19**	**5.07**	0.39
octanol	0.5Aa	0.4Aa	0.4Aa	0.5Aa	0.4Aa	0.4Aa	0.4Aa	0.2Aa	3.24	0.67	**4.29**
2-furanmethanol	13.6Aa	11.6Aa	12.8Aa	12.8Aa	13.3Aa	8.0Aab	7.6Bb	6.9Bb	**23.97**	**5.01**	2.58
α-terpineol	4.7Aa	4.4Aab	4.0Aab	3.6Ab	3.8Aa	2.8Bab	2.5Bb	2.5Bb	**72.53**	**11.59**	1.18
3-(methylthio)-1-propanol	143Aa	140Aa	186Aab	207Ab	45.2Ba	89.7Aa	89.1Ba	88.1Ba	**92.39**	**6.25**	2.26
2-phenylethanol	4161Aa	4239Aa	4056Aa	3275Aa	5284Aa	5369Aa	4923Aa	2680Ab	**11.92**	**21.01**	**4.88**
total alcohols	4347	4438	4294	3524	5377	5513	5060	2788			

[a] Data are the mean values from three repeated batches at each temperature. Z, fermentation type (immobilized or free cells); T, temperature; Z × T, interaction. Significant *F* ratios are in bold ($p < 0.05$). Statistical comparisons were made by ANOVA between fermentations using immobilized and free yeast under the same fermentation conditions (temperature) except for the biocatalyst; letters A and B following values indicate significant differences. Statistical comparisons were also made within each level of fermentation type (examining temperature under the same biocatalyst); letters a–c following values indicate significant differences. The level of significance was $p < 0.05$. There was no significant difference between samples with the same letter.

Table 5. Acids (Micrograms per Liter) Determined by GC-MS in Wines Produced by Free Cells (FC) and Immobilized Cells (IC) on Grape Skins at Temperatures of 25, 20, 15, and 10 °C[a]

									F ratio		
compound	IC 25	IC 20	IC 15	IC 10	FC 25	FC 20	FC15	FC 10	Z	T	Z × T
acetic	134Aa	59.3Aa	23.2Aa	36.0Aa	745Ba	219Ab	245Ab	258Ab	**41.47**	**9.80**	**4.64**
propanoic	3.0Aa	2.0Aa	1.8Aa	2.0Aa	3.0Aa	1.7Aa	1.9Aa	2.0Aa	0.04	**8.77**	0.41
2-methylpropanoic	13.4Aa	10.4Aa	8.4Aa	8.9Aa	16.4Aa	9.0Aa	9.2Aa	12.0Aa	1.84	**7.48**	1.08
butanoic	16.6Aa	15.9Aa	18.0Aab	21.9Ab	14.2Aa	10.1Aa	10.0Ba	11.9Ba	**66.17**	**4.34**	**3.97**
3-methylbutanoic	13.0Aa	10.5Aab	8.6Ab	8.3Ab	10.5Aa	8.2Aa	8.3Aa	8.5Aa	**5.71**	**8.74**	1.73
hexanoic	156Aa	229Aa	400Ab	617Ac	233Aa	181Aa	195Ba	125Ba	**57.93**	**13.84**	**29.68**
octanoic	1458Aa	1357Aa	1961Aab	2649Ab	1948Aa	1536Aab	1552Aab	666Bb	**8.54**	0.85	**13.20**
decanoic	33.4Aa	25.3Aa	41.0Aa	39.7Aa	261Ba	143Aab	152Aab	23.9Ab	**14.86**	2.61	2.88
total fatty acids	1828	1710	2462	3383	3231	2108	2173	1107			

[a] Data are the mean values from three repeated batches at each temperature. Z, fermentation type (immobilized or free cells); T, temperature; Z × T, interaction. Significant *F* ratios are in bold ($p < 0.05$). Statistical comparisons were made by ANOVA between fermentations using immobilized and free yeast under the same fermentation conditions (temperature) except for the biocatalyst; letters A and B following values indicate significant differences. Statistical comparisons were also made within each level of fermentation type (examining temperature under the same biocatalyst); letters a–c following values indicate significant differences. The level of significance was $p < 0.05$. There was no significant difference between samples with the same letter.

favor the production of esters by yeasts. This was attributed to changes in the metabolic activities of yeasts. The low production of esters at higher temperatures may be the result of increased hydrolysis and not necessarily a low rate of formation (*10*). The differences observed between IC and FC could be caused by a mass transfer phenomenon or reflect different enzymic activities. In addition, because oxygen represses the synthesis of esters (*19*), a favorable microenvironment for ester synthesis in the vicinity of the biocatalyst might have been created due to limited oxygen diffusion.

Alcohols. The amounts of butanol, 4-methyl-1-pentanol, α-linalool, α-terpineol, and 2-phenylethanol were significantly affected by the fermentation type and temperature. The interaction of these main effects was not significant for any of the compounds studied except for 2-phenylethanol. The concentrations of these alcohols were higher in wines produced by IC (except 2-phenylethanol) and decreased with decreases in temperature (**Table 4**).

The terpenoid alcohols, α-linalool and α-terpineol, originate from grapes, in which they either exist as free compounds or are bound with glycosides. It is known that *S. cerevisiae* is capable of modifying the terpenic profile of wine either by transforming the existing terpenes or by hydrolysis of the glycoconjugates (*21*). Therefore, the higher amounts of α-linalool and α-terpineol can be attributed to the changes of the metabolic activity of *S. cerevisae* because of immobilization and contribution in terpenoids by the grape skins.

The amount of 4-pentenol was greater in wines produced by IC. Fermentation temperature did not have a significant effect on this alcohol. The amount of 3-methyl-1-pentanol increased with a decrease in temperature in wines produced by IC, whereas in those produced by FC its content remained almost constant. 3-Pentanol was significantly affected by temperature but not by fermentation type.

The contents of hexanol and octanol were not affected by these main factors, because these alcohols originate from grapes (*9*). 2-Furanmethanol decreased significantly at temperatures from 25 to 20 °C and then remained constant. It was found at higher levels in wines produced by IC. The effect of temperature was significant in the formation of 3-(methylthio)-1-propanol, which increased with temperature decreases. Wines produced by IC contained greater amounts of this alcohol. It originates

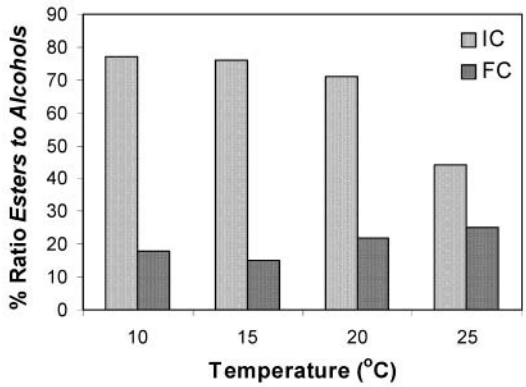

Figure 1. Percentage of total esters (including ethyl acetate) to total alcohols (including major alcohols) in wines produced by free and immobilized cells on grape skins at various temperatures.

from the metabolism of methionine in yeasts (*22*), and its contribution to wine aroma is considered to be negligible (*9*).

Generally, the total content of alcohols decreased with decreases in temperature for wines produced by either FC or IC (**Table 4**). These results are in agreement with findings of other workers (*15*, *23*, *24*). Although the IC produced wines with a higher content of the minor alcohols, FC gave wines with greater amounts of the major alcohols. Thus, the total content of alcohols was always larger in wines produced by FC.

Acids. The concentrations of acetic, propanoic, and 2-methylpropanoic acids decreased significantly with temperature decreases from 25 to 20 °C, after which they remained constant (**Table 5**). The effect of fermentation type on the production of these two acids was not significant. FC produced wines containing more acetic acid, whereas butanoic and hexanoic acids were present at higher levels in wines produced by IC and increased with temperature decreases. The inverse relationship was observed for 3-methylbutanoic acid. The interaction of temperature with fermentation type had a profound effect on the concentration of octanoic acid. It increased with temperature decreases for fermentations performed with IC, whereas the opposite was observed for those with FC. Decanoic acid was affected significantly by fermentation type. FC wines contained greater quantities of this acid.

The amounts of volatile fatty acids were larger in wines produced by FC at higher temperatures (25−20 °C). Low fermentation temperatures (15−10 °C) had the opposite effect in fermentations performed using IC (**Table 5**). Thus, decreases in temperature had a positive influence, on fatty acid content, as stated by Etievant (*9*), but only for fermentations with IC.

Conclusively, wines produced by IC were characterized by better ratios of *esters to alcohols*, at every temperature studied, in comparison with wines produced by FC. These ratios increased with temperature decreases for IC, whereas they decreased for FC. The highest ratios of esters to alcohols were observed at 10 and 25 °C for IC and FC, respectively (**Figure 1**).

These results show that wines produced by immobilized cells on grape skins had a potentially better fruity aroma. The combination of this better aroma with increased fermentation rates (*6*) show that grape skins can be used as a good immobilization support for wine fermentations.

ABBREVIATIONS USED

GC-MS, gas chromatography−mass spectrometry; SG, specific gravity; IC, immobilized cells; FC, free cells; AATase, alcohol acetyltransferase; EHase, ester hydrolase; NAD^+, NADH nicotinamide adenine dinucleotide; t_R retention time.

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