JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Corn Starch Gel for Yeast Cell Entrapment. A View for Catalysis of Wine Fermentation

PANAGIOTIS KANDYLIS, AMALIA GOULA, AND ATHANASIOS A. KOUTINAS*

Food Biotechnology Group, Department of Chemistry, University of Patras, GR-26500 Patras, Greece

A new biocatalyst was prepared by immobilization of *Saccharomyces cerevisiae* AXAZ-1 yeast cells in the matrix of corn starch gel. This biocatalyst was used for repeated batch fermentations of glucose and grape must at various sugar concentrations (110−280 g/L) and low-temperature winemaking (5 °C). The biocatalyst retained its operational stability for a long period, and it was proved to be capable of producing dry and semisweet wines. The produced wines were analyzed for volatile byproducts by GC and GC-MS, and the results showed an increase in the number and amount of esters by immobilized cells. In addition, an increase in the percentages of esters and a decrease in those of alcohols with the drop of fermentation temperature were reported. The activation energy (E_a) was lower (∼36%) and the reaction rate constant (k) was higher (∼78% at 30 °C and ∼265% at 15 °C) in the case of immobilized cells compared to free cells, especially at low temperatures. These results show that corn starch gel may act as a promoter for the enzymes that are involved in the process or as a catalyst of the alcoholic fermentation and can explain the capability of immobilized cells for extremely low-temperature winemaking. Therefore, these results open a new way for research to find new catalysts in biotechnological processes.

KEYWORDS: Immobilization; wine; volatiles; GC-MS; corn starch gel; activation energy

INTRODUCTION

In recent years several immobilized cell systems have been proposed for use in bioconversions such as alcoholic fermentation. This can be attributed to their numerous advantages, such as improved productivity, compared to free cell systems. Among the techniques that have been proposed for cell immobilization, the most important are mechanical containment behind a barrier, entrapment within a porous matrix, cell flocculation (aggregation), and immobilization on solid carrier surfaces (*1*).

Various materials (synthetic, natural, organic, and inorganic) have been used as supports of cell immobilization (*2*−*7*). In recent years several supports of food grade purity, such as delignified cellulosic materials (*8*), gluten pellets (*9*), brewer's spent grains (*10*), dried figs (*11*), dried raisin berries (*12*), fruit pieces (*13*), and potatoes (*14*), have been proposed as ideal for yeast immobilization for winemaking and brewing. The immobilization of alcohol resistant and cryotolerant yeasts on these supports led to low-temperature winemaking and brewing, resulting in wines and beers with improved taste and aroma. Although several immobilization supports have been proposed for alcoholic fermentation, only a few find application at the industrial level, and therefore the search for new material is of great interest.

In previous studies (*8, 15*) the use of delignified cellulosic material as support for yeast immobilization proved to be very effective for low-temperature winemaking and brewing. These studies, based on a theoretical approach of the Arrhenius equation, showed that the low-temperature fermentations obtained by the immobilized cells are responsible for the reduction of the activation energy, E_a, by the presence of this support. In a recent study, using yeast cells immobilized on potatoes (*14*), the reduced activation energy and the higher reaction rate constant in the case of immobilized cells led to the conclusion that potatoes may behave as a catalyst or a promoter of the enzymes involved in the process.

The aim of the present study was to evaluate the use of corn starch gel as a support for yeast immobilization. The new biocatalyst was used for dry and semisweet winemaking at 27 °C and also for low-temperature wine making (5 °C). The effect of initial sugar concentration and that of temperature on volatile formation and fermentation kinetics during winemaking were also examined. Finally, the possible catalytic effects of the immobilization support to the enzymes that are involved in the alcoholic fermentation were studied and the reaction rate constants and activation energies of free and immobilized systems calculated.

MATERIALS AND METHODS

Yeast Strains and Media. The alcohol resistant and cryotolerant *Saccharomyces cerevisiae* AXAZ-1, isolated from a Greek vineyard plantation (*16*), was used in the present study. It was grown on culture medium consisting of 4 g of yeast extract/L, 1 g of $(NH_4)_2SO_4$/L, 1 g of KH_2PO_4/L, 5 g of $MgSO_4 \cdot 7H_2O$/L, and 40 g of glucose monohydrate/L at 30 °C and harvested at 4000 rpm for 10 min. All media

* Author to whom correspondence should be addressed (telephone +30 2610 997104; fax +30 2610 997105; e-mail A.A.Koutinas@upatras.gr).

10.1021/jf802476w CCC: $40.75
Published on Web 11/26/2008

were sterilized at 130 °C and 1.5 atm for 15 min. Must of roditis grape variety was provided from the local wine industry "Achaia Clauss", with 11.3−12.1 initial °Be density (197.5−214.6 g/L), total acidity of 6−7 g of tartaric acid/L, and sulfur dioxide content of ∼60 mg/L. When necessary, concentrated must (∼ 35 °Be) from the same grape variety was used to obtain an initial density higher than 11.5 °Be.

Preparation of Support and Cell Immobilization. Corn starch was mixed with 100 mL of deionized water heated to 90 °C (from 25 °C it was raised to 65, 80, 86, and 90 °C at a rate of 5, 3, 1.5, and 0.5 °C/min, respectively, and it was held at 90 °C for 5 min) and then left to cool at room temperature. Different concentrations (2−10 g/100 mL) of corn starch were evaluated to produce the more stable gel for immobilization. The amount of starch needed for the optimum stability of the gels was 8 g of starch/100 mL. For the immobilization 10 mL of glucose medium containing 10 g of wet weight AXAZ-1 was mixed with the prepared gel (still a dilution; complete gel formation had not occurred) at ∼40 °C during its cooling. The whole was left at 15 °C overnight for complete gel formation and cell immobilization. The next day the immobilized biocatalyst was used in the form of small cakes (having different shapes with lengths ranging from 0.2 to 1 cm) for fermentations.

Enumeration of Immobilized Cells. Representative 1 g portions of duplicate corn starch gel samples taken after immobilization and at the end of fermentations were blended with 99 mL of sterilized ringer solution (1/4 strength). Then 1 mL of the homogenized sample was transferred to 9 mL of sterilized solution and mixed well, and the procedure was repeated several times. From each dilution 0.1 mL was spread on the surface of the appropriate agar. The enumeration of immobilized yeast cells was made on malt agar and on an agar with the following (w/v) content: glucose, 4%; yeast extract, 0.4%; $(NH_4)_2SO_4$, 0.1%; KH_2PO_4, 0.1%; and $MgSO_4 \cdot 7H_2O$, 0.5%, after incubation at 30 °C for at least 72 h (*14*). The number of immobilized cells on corn gel was approximately constant during fermentations (6.3×10^8 cells g^{-1} of wet corn gel corresponding to 4.14×10^{-3} g of dry weight cells g^{-1} of wet corn gel) with a small reduction with the drop of temperature.

Fermentation. The immobilized biocatalyst of 85 g was used for a series of repeated batch fermentation of 400 mL of glucose medium (6 and 8 °Be) and must (10, 12, 14, and 17 °Be) at 27 °C without any agitation. When the fermentation was completed, the liquid was collected for analyses and the support was washed twice with 200 mL of glucose medium or must and then used for the next fermentation batch. After the completion of these fermentations, separate series of repeated batch fermentations were carried out initially at 30 °C using glucose medium (11.5 °Be), and then must was used and the temperature was successively decreased 25, 20, 15, 10, 5, and 2 °C. When the fermentation was complete, the liquid was collected for analyses and the support was washed twice with 200 mL of glucose medium or must and then used for the next fermentation batch. For comparison, must fermentations using free cells were also carried out.

Analyses. Fermentation kinetics were performed by measuring the °Be density at various time intervals. Residual sugar was determined by high-performance liquid chromatography, using a Shimadzu chromatograph (Kyoto, Japan) with an SCR-101N stainless steel column, an LC-9A pump, a CTO-10A oven at 60 °C, and an RID-6A refractive index detector. Three times distilled water was used as mobile phase with a flow rate of 0.8 mL/min, and 1-butanol (Sigma-Aldrich, Poole, U.K.) was used as internal standard. Samples of 0.5 and 2.5 mL of a 1% (v/v) solution of 1-butanol were diluted to 50 mL, and 40 μL was injected directly into the column. Residual sugar concentrations were calculated using standard curves prepared by at least seven standard solutions by correlating the ratio of residual sugar peak areas/1-butanol peak areas to residual sugar concentrations.

Total acidity of the wines expressed as grams of tartaric acid per liter was determined by titration with 0.1 N NaOH. Volatile acidity expressed as grams of acetic acid per liter was determined by titration with 0.1 N NaOH of the distillates obtained after steam distillation of the wine samples (*17*). Wet weight free cell concentrations in the fermentation liquid were determined according to the method of a previous study (*18*). Wine productivity was calculated as grams of wine per liter of total volume produced per day, using the following equation:

$$(\text{g of wine} \times 24)/\text{total vol of fermentation} \times \text{fermentation time} \quad (1)$$

The grams of wine was calculated from the volume of wine by assuming that the density of wine is 1 g/mL, whereas the total volume of fermentation is the volume of grape must and the volume of immobilization support. Ethanol productivity was expressed as grams of ethanol produced per day per liter of liquid volume of bioreactor. Conversion was calculated with the following equation:

$$(\text{initial sugar concn} - \text{residual sugar concn})/\text{initial sugar concn} \times 100 \quad (2)$$

Determination of Ethanol and Volatile Byproduct. Ethanol and methanol were determined by gas chromatography using a Shimadzu GC-8A gas−liquid chromatograph (Kyoto, Japan) with a Porapac S column. Nitrogen was used as carrier gas at 40 mL/min. The column temperature was settled at 120−170 °C at a rate of 10 °C/min. The temperatures of the injector and FID detector were 210 and 220 °C, respectively. For the ethanol and methanol determination, a total volume of 2 μL for each sample was injected directly into the column. 1-Butanol was used as internal standard at a concentration of 0.5% (v/v). Ethanol and methanol concentrations were calculated using standard curves prepared by at least seven standard solutions by correlating the ratio of ethanol and methanol peak areas/1-butanol peak areas to ethanol and methanol concentrations.

Volatiles such as acetaldehyde, ethyl acetate, 1-propanol, isobutanol, and amyl alcohols were determined by gas chromatography using a Shimadzu GC-8A gas−liquid chromatograph with a stainless steel column packed with Escarto-5905 consisting of 5% squalene, 90% Cabowax-300, and 5% bis(2-ethylhexyl) sebacate. Nitrogen was used as carrier gas at 20 mL/min. The injection port and FID detector temperatures were 210 °C, and the column temperature was 70 °C. The internal standard was 1-butanol (0.5% v/v). Samples of 4 μL were injected directly into the column, and the concentrations of the above compounds were calculated using standard curves as described above.

Headspace SPME-GC-MS Analysis. The volatile constituents of the produced wines were determined by means of gas chromatography−mass spectroscopy as described in a previous study (*14*). More specifically, the volatiles were isolated by the headspace solid phase microextraction (SPME) method. The fiber used for the absorption of volatiles was a 2 cm fiber coated with 50/30 mm divinylbenzene/Carboxen on poly dimethylsiloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA). The conditions of headspace SPME sampling were as follows: 10 mL of liquid sample, 3 g of NaCl, and internal standard (4-methyl-2-pentanol) were transferred into a 20 mL headspace vial fitted with a Teflon-lined septum sealed with an aluminum crimp seal. The contents were magnetically stirred for 5 min at 60 °C, and then the fiber was exposed to the headspace for 45 min. The length of the fiber in the headspace was kept constant. Desorption of volatiles took place in the injector of the gas chromatograph in the splitless mode, at 240 °C for 3 min. Before each analysis, the fiber was exposed to the injection port for 5 min to remove any volatile contaminants. GC-MS analysis was performed on a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu MS QP5050 mass spectrometer. Helium was used as carrier gas (1.8 mL/min). Separation of compounds was performed on a capillary column (Supelco CO Wax-10 60 m, 0.32 mm i.d., 0.25 μm film thickness). Oven temperature was programmed at 35 °C for 6 min and then raised to 60, 200, and 250 °C at a rate of 2, 5, and 25 °C/min, respectively. It was held at 250 °C for 6 min. The injector and interface temperatures were set at 240 and 240 °C, respectively. The mass spectrometer was operated in the scan range m/z 45−400. Identification of the compounds was effected by comparing (i) the linear retention indices based on the even n-alkanes (C10−C24) with those of standard compounds and by the literature retention indices and (ii) MS data with those of standard compounds and by MS data obtained from Wiley and NIST libraries.

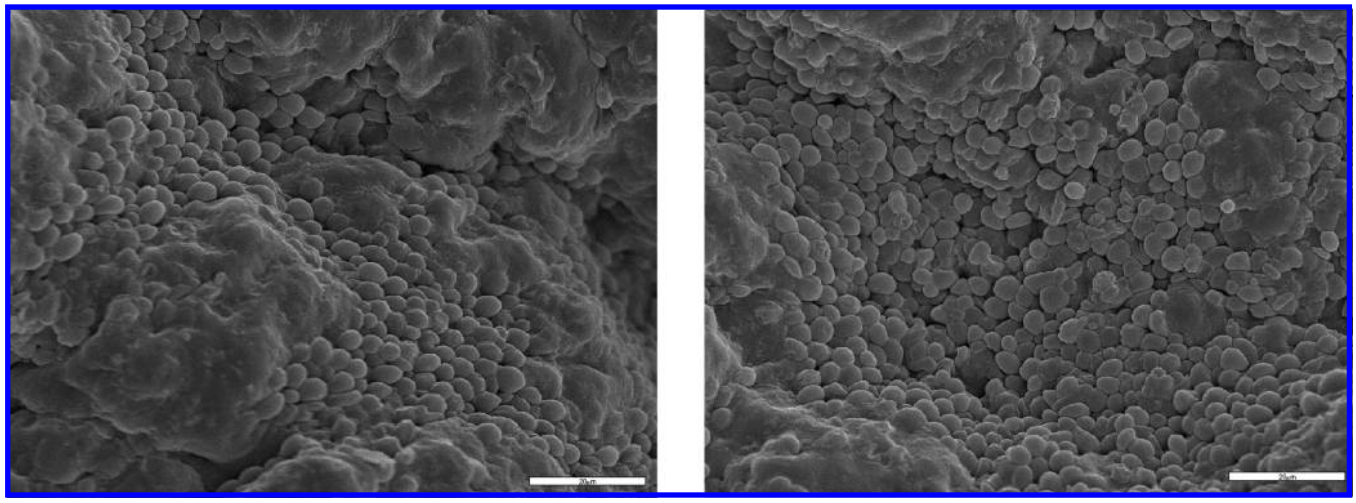

Figure 1. Electron micrographs of corn starch gel with the immobilized cells of *S. cerevisiae* AXAZ-1.

Table 1. Effect of Initial Sugar Concentration on Kinetic Parameters Observed during Repeated Batch Fermentations of Glucose Medium and Must at 27 °C Using Immobilized *Saccharomyces cerevisiae* AXAZ-1 Yeast Cells on Corn Starch Gel

batch	initial density (°Be)	initial sugar (g/L)	fermentation time (h)	residual sugar (g/L)	ethanol (% v/v)	conversion (%)	ethanol productivity (g/L/day)	wine productivity (g/L/day)
1−5	6[a]	111.0 ± 2.7	22.2 ± 1.5	Tr[c]	6.3 ± 0.5	100	54.2 ± 7.3	
6−8	8[a]	141.7 ± 3.1	24.0 ± 1.0	5.7 ± 2.3	7.9 ± 0.4	96.0 ± 1.5	62.7 ± 4.6	
9−11	10[b]	176.3 ± 4.7	36.0 ± 2.0	7.5 ± 1.3	10.0 ± 0.3	95.7 ± 0.9	52.3 ± 1.5	534.3 ± 30.0
12−14	12[b]	208.0 ± 2.6	55.3 ± 1.2	5.7 ± 1.4	11.9 ± 0.4	97.2 ± 0.7	41.0 ± 2.0	347.3 ± 7.5
15−17	14[b]	239.0 ± 3.6	311.3 ± 25.1	30.7 ± 13.3	13.6 ± 0.4	87.2 ± 5.4	8.0 ± 1.0	61.7 ± 5.0
18	17[b]	280	483	57.4	13.9	79.5	5	40

[a] Glucose medium. [b] Grape must. [c] Tr, traces.

Semiquantitative analysis was performed by dividing the peak area of a compound with the peak area of the internal standard and multiplying the result with the concentration of the internal standard (1.62 mg/L).

Calculation of Activation Energy E_a. Fermentations of 400 mL of 12% (w/v) glucose medium were carried out with immobilized and free cells at various temperatures (85 g of wet corn gel corresponding to 0.35 g of dry weight cells). The activation energies of the fermentation systems were calculated using an equation based on the Arrhenius equation according to a previous study (*19*) by a curve obtained by plotting $\ln(dP/d3t)$ versus $1/T$.

Electron Microscopy. Pieces of the immobilized biocatalysts prepared with corn starch gel having immobilized *S. cerevisiae* AXAZ-1 cells were washed with deionized water and dried overnight at 30 °C. The samples were coated with gold in a Balzers SCD 004 sputter coater for 3 min and examined in a JEOL model JSM-6300 scanning electron microscope.

Experimental Design and Statistical Analysis. All analyses were carried out in triplicate, and the mean values are presented (standard deviation for all values was about ±5% in most cases). In the experiments conducted, the effects of initial sugar concentration, immobilization, and temperature on fermentation parameters and formation of volatiles (ethanol, acetaldehyde, ethyl acetate, isobutanol, 1-propanol, and amyl alcohols) during glucose synthetic medium and must fermentations were studied. In addition, the effects of temperature and immobilization on activation energy E_a and rate coefficient k were also studied. The experiments were designed and analyzed statistically by ANOVA. Duncan's multiple-range test was used to determine significant differences among results [coefficients, ANOVA tables, and significance ($p < 0.05$) were computed using Statistica v.5.0 (StatSoft, Inc., Tulsa, OK)].

RESULTS AND DISCUSSION

This is the first time that corn starch gel has been evaluated as a support for yeast cell immobilization, and its feasibility for repeated batch fermentation of glucose and grape must at various sugar concentrations and temperatures is examined. In addition, the possible catalytic effect of corn starch gel on alcoholic fermentation was examined by calculating the activation energies of both free and immobilized cells and the corresponding reaction rate constants at various temperatures between 30 and 15 °C.

A solution of corn starch in water was prepared and heated to 90−95 °C. At this temperature, higher than its gelatinization temperature, corn starch granules undergo irreversible swelling, and as they cool, crystallization is favored and an elastic gel is formed (*20, 21*). During the cooling process and prior to gel formation, at ∼40 °C, the yeast cells are added and the whole is left at 15 °C overnight for complete gel formation. The immobilization was confirmed by electron microscopy (**Figure 1**), showing yeast cells attached on the surface of corn starch gel and also mixed and entrapped inside the gel. In addition, the immobilization was proved by using the new biocatalyst for repeated batch fermentations of glucose and grape must at various sugar concentrations and temperatures.

Repeated Batch Fermentations. Initially, the immobilized biocatalyst was used for repeated batch fermentations of glucose medium (6 and 8 °Be) and grape must (10−17°Be) at 27 °C, and the effects of initial sugar concentration were studied (**Table 1**).

Sugar concentration affected significantly fermentation time, residual sugar, conversion, ethanol content, and ethanol and wine productivities ($p < 0.01$ in all cases). The immobilized biocatalyst was used for 18 repeated batches and retained its operational stability for more than 2.5 months. All fermentations led to products with low residual sugar content (from traces to 8.8 g/L) and subsequently high conversions (from 94.5 to 100%); however, when grape must of 14 and 17 °Be density was used significantly ($p < 0.01$), higher contents of residual

Table 2. Effect of Initial Sugar Concentration on the Production of Volatiles and Acidities in Repeated Batch Fermentations of Glucose Medium and Must at 27 °C Using Immobilized *Saccharomyces cerevisiae* AXAZ-1 Yeast Cells on Corn Starch Gel

batch	initial density (°Be)	volatile acidity (g of acetic acid/L)	total acidity (g of tartaric acid/L)	acetaldehyde (mg/L)	ethyl acetate (mg/L)	1-propanol (mg/L)	isobutyl alcohol (mg/L)	amyl alcohols (mg/L)	methanol (mg/L)	total volatiles, methanol excluded (mg/L)
1−5	6[a]			9.6 ± 0.9	25.0 ± 7.1	1.0 ± 1.0	16.6 ± 1.3	102.8 ± 16.8	36.0 ± 7.8	155.2 ± 23.1
6−8	8[a]			25.7 ± 12.0	41.7 ± 6.7	8.7 ± 1.5	21.3 ± 7.0	118.3 ± 3.1	38.7 ± 12.0	215.7 ± 3.5
9−11	10[b]	0.3 ± 0.1	5.2 ± 0.2	39.0 ± 6.6	42.7 ± 4.0	5.0 ± 2.0	19.0 ± 2.0	107.7 ± 8.6	70.3 ± 11.7	213.3 ± 4.9
12−14	12[b]	0.6 ± 0.1	5.5 ± 0.1	49.0 ± 4.6	49.3 ± 4.9	6.0 ± 5.3	24.0 ± 1.0	136.7 ± 28.9	72.0 ± 12.5	265.0 ± 30.8
15−17	14[b]	0.7 ± 0.1	5.7 ± 0.2	64.7 ± 8.5	75.3 ± 16.1	8.7 ± 3.1	18.7 ± 1.5	124.0 ± 8.0	85.0 ± 17.4	280.3 ± 14.6
18	17[b]	0.6	5.9	69	81	Tr[c]	17	147	84	314

[a] Glucose medium. [b] Grape must. [c] Tr, traces.

Table 3. Kinetic Parameters of Low-Temperature Repeated Batch Fermentations of Grape Must with Immobilized *Saccharomyces cerevisiae* AXAZ-1 Yeast Cells on Corn Starch Gel

temp (°C)	fermentation medium	batch	initial sugar (g/L)	fermentation time (h)	residual sugar (g/L)	ethanol (% v/v)	conversion (%)	ethanol productivity (g/L/day)	wine productivity (g/L/day)
30	glucose	1	112	24	0.3	6	99.7	47.4	
		2	117	26	0.6	6.4	99.5	46.7	
		3	139	28	6.7	7.7	95.2	52.1	
		4	145	28	1.6	7.7	98.9	52.1	
30	must	5	205	42	2.3	11.2	98.9	50.6	457.1
25		6−10	211.8 ± 1.8	76.2 ± 3.5	8.8 ± 4.6	11.8 ± 0.3	95.8 ± 2.2	29.4 ± 1.7	252.4 ± 11.7
20		11−15	206.9 ± 4.8	93.6 ± 2.7	6.4 ± 3.0	11.5 ± 0.3	96.9 ± 1.4	23.3 ± 0.7	205.3 ± 5.9
15		16−20	202.2 ± 3.1	135.8 ± 4.2	6.9 ± 2.3	11.4 ± 0.3	96.6 ± 1.1	16.0 ± 0.7	141.5 ± 4.5
10		21−25	208.5 ± 6.1	404.8 ± 92.0	9.8 ± 2.2	11.6 ± 0.2	95.3 ± 1.0	5.7 ± 1.4	49.5 ± 11.7
5		26−30	207.5 ± 2.7	1341.4 ± 48.5	10.8 ± 3.3	11.5 ± 0.1	94.8 ± 1.6	1.6 ± 0.1	14.3 ± 0.5

sugar (up to 57.4 g/L) and lower conversions were observed. Ethanol and wine productivities ranged from 67.3 to 5 g/L/day and from 564.3 to 40 g/L/day, respectively. Total and volatile acidities of the wines produced were in the usual levels of commercial dry and semisweet wines (**Table 2**). Both were affected by the initial sugar concentration ($p < 0.01$), and a small increase was observed with the increase of initial sugar concentration. In general, the biocatalyst was capable of producing dry wines with 11.5−12%v/v ethanol content, whereas when grape musts of 14 and 17 °Be were used, semisweet wines with ∼14% v/v ethanol and 16.7−57.4 g/L residual sugars were produced.

With regard to the effect of the corn starch gel supported biocatalyst and the initial sugar concentration on the aroma, the formation of major volatile byproducts was examined, and the results are summarized in **Table 2**. Initial sugar concentration affected significantly the most volatile byproducts ($p < 0.01$ for ethyl acetate, acetaldehyde, and methanol and $p < 0.05$ for 1-propanol), but did not affect isobutanol and amyl alcohol concentrations ($p > 0.05$). More specifically the concentrations of isobutanol and amyl alcohols ranged from 14.3 to 28.3 mg/L and from 86 to 165.6 mg/L, respectively. 1-Propanol was found in low concentration from traces to 11.8 mg/L. Ethyl acetate and acetaldehyde were detected in usual levels that contribute positively to wine aroma (*22, 23*), and a small increase in their concentrations was observed at high initial sugar concentrations (14 and 17 °Be). The methanol content was significantly affected by the fermentation medium ($p < 0.01$), ranging from 26.7 mg/L, when glucose medium was used, to 102.4 mg/L, when grape must was used. These values were expected as methanol has its origin mainly from the enzymatic breakdown of pectins contained in grapes (*24*).

Winemaking by Corn Starch Gel Supported Biocatalyst. The first four fermentations were carried out at 30 °C using synthetic glucose medium with glucose concentration from 112 to 145 g/L for better adaptation of the biocatalyst. All other fermentations were carried out using grape must with initial sugar concentration ranging between 199 and 215 g/L, whereas temperature was successively decreased to 25, 20, 15, 10, and 5 °C with five repeated fermentation batches at each temperature. Fermentation temperature affected significantly fermentation time and ethanol and wine productivities ($p < 0.01$), but did not affect residual sugar, ethanol concentration, and conversion ($p > 0.05$). The immobilized biocatalyst retained its operational stability for a period longer than 12 months, even at low temperatures, and produced wines in 3 days at 25 °C and in 55 days at 5 °C (**Table 3**). The wines produced were of fine clarity and contained alcohol at concentrations similar to those of dry table wines. More specifically, the final free cell concentrations were low [from ∼2.5 g/L at 30 °C to ∼0.2 g/L at 5 °C (wet weight)], especially at low temperatures, showing that the fermentations were carried out by the immobilized cells. Ethanol and wine productivities were relatively high, whereas total acidity and volatile acidity were at the levels of commercial dry wines (**Table 4**). Fermentation temperature did not affect the volatile acidity ($p > 0.05$), but did affect significantly ($p < 0.01$) total acidity. More specifically, the values of total acidity at 30, 25, and 20 °C were significantly higher ($p < 0.05$) than those observed at lower temperatures (15, 10, and 5 °C).

Major Volatile Byproducts. The majority of the compounds that contribute to the aroma of wines are produced during alcoholic fermentation of grape must; very few are derived from grapes. The most abundant compounds in the wine aroma are acetaldehyde, ethyl acetate, 1-propanol, isobutanol, and amyl alcohols (*25*). The concentrations of these compounds in the wines produced are summarized in **Table 4**. As is obvious, the fermentation temperature affected significantly ($p < 0.01$) all of the major volatile byproducts. Acetaldehyde ranged in concentration from 64 to 23 mg/L. The higher concentration was observed at 25 and 20 °C, whereas reduction in fermentation temperature led to lower concentration. No significant difference ($p > 0.05$) was observed at 10 and 5 °C, where the concentration

Table 4. Volatiles and Acidity of Wines Produced by Low-Temperature Repeated Batch Fermentations of Grape Must Using Immobilized *Saccharomyces cerevisiae* AXAZ-1 Yeast Cells on Corn Starch Gel

temp (°C)	batch	volatile acidity (g of acetic acid/L)	total acidity (g of tartaric acid/L)	acetaldehyde (mg/L)	ethyl acetate (mg/L)	1-propanol (mg/L)	isobutyl alcohol (mg/L)	amyl alcohols (mg/L)	methanol (mg/L)	total volatiles, methanol excluded (mg/L)
30	1[a]			11	39	7	16	90	32	163
	2[a]			13	29	5	12	81	17	140
	3[a]			27	39	Tr[c]	18	113	33	197
	4[a]			31	35	5	15	99	38	185
30	5[b]	0.5	5.7	38	41	3	17	101	40	200
25	6−10[b]	0.4 ± 0.1	5.7 ± 0.1	58.8 ± 5.7	66.6 ± 12.3	9.6 ± 1.1	35.8 ± 4.0	158.5 ± 10.2	87.2 ± 7.5	329.6 ± 11.2
20	11−15[b]	0.5 ± 0.1	5.4 ± 0.1	53.6 ± 4.6	58.2 ± 4.6	4.4 ± 4.3	34.4 ± 2.1	174.8 ± 16.6	73.4 ± 11.4	325.4 ± 18.3
15	16−20[b]	0.4 ± 0.1	5.0 ± 0.1	45.8 ± 4.0	73.2 ± 3.7	2.6 ± 1.5	20.4 ± 1.8	124.4 ± 6.2	70.4 ± 15.7	266.4 ± 8.0
10	21−25[b]	0.4 ± 0.1	5.0 ± 0.1	27.0 ± 3.5	78.8 ± 2.2	1.4 ± 1.0	15.6 ± 2.2	111.8 ± 2.4	70.6 ± 7.4	234.2 ± 6.8
5	26−30[b]	0.3 ± 0.1	5.1 ± 0.1	25.2 ± 2.4	91.2 ± 2.9	1.8 ± 1.8	12.8 ± 1.3	91.0 ± 6.2	68.4 ± 3.2	222 ± 3.9

[a] Glucose medium. [b] Grape must. [c] Tr, traces.

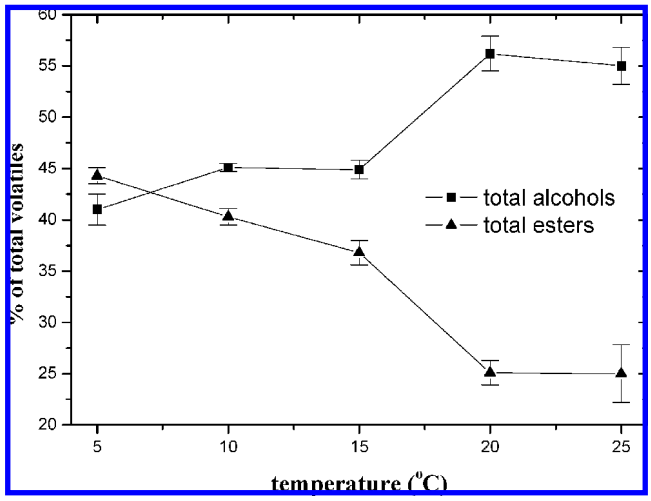

Figure 2. Effect of temperature on percentages of total esters and total alcohols on total volatiles of wines produced by immobilized cells.

of acetaldehyde ranged from low concentrations to 30 mg/L. Ethyl acetate concentration ranged from 41 mg/L at 30 °C to 94 mg/L at 5 °C, and a significant increase ($p < 0.01$) with the drop of fermentation temperature was observed. Amyl alcohols, isobutanol, and 1-propanol were detected at concentrations that contribute to the pleasant flavors of the wines produced (*22, 26*), and a significant drop ($p < 0.01$) in their concentrations with the drop of fermentation temperature was observed, as in many previous studies (*14, 27, 28*).

SPME-GC-MS Analysis of Wines. The results from the SPME-GC-MS analysis of the wines are presented in **Table 5**. In total, 80 compounds were detected, of which 42 in must and 48 and 49 in wines were produced at 15 °C by free and immobilized cells, respectively, whereas further reduction of temperature led to a small increase to 54 compounds in the case of immobilized cells. Semiquantitative analysis showed that immobilized cells produced significantly higher concentrations of esters and other compounds that provide improved characteristic flavor. The reduction of fermentation temperature from 25 to 15 °C led to higher concentrations of all compounds, whereas further reduction of temperature to 5 °C led to reduced concentrations (*14, 29*). However, an increase in the percentage of total esters and a decrease in the percentage of higher alcohols with the drop of temperature from 15 to 5 °C were also observed (**Figure 2**), which is in agreement with other recent studies and is considered to have a positive impact on wine aroma (*14, 29*).

Esters. A total of 29 esters were detected in wines of the present study. In both free and immobilized biocatalysts, an increase in the concentrations of esters was observed with the decrease of temperature to 15 °C, whereas further reduction of temperature led to a decrease. However, at all temperatures wines produced by immobilized cells presented higher numbers and concentrations of esters than those produced by free cells, especially at 15 °C. The main ester in our samples and wines in general was ethyl acetate. Other esters present in all wine samples were those of fusel alcohols and short-chain fatty acids, the so-called "fruit esters". In the case of immobilized cells their content was higher compared to free cells. Some examples are ethyl hexanoate, contributing fruity notes to wine aroma, ethyl octanoate, having a floral, fruity, musty impact, and ethyl dodecanoate, which is known for its smoky, earthy, dried fruit, spicy, and toasty aroma (*30*). Acetates other than ethyl acetate were detected in our samples; these compounds are responsible for a pleasant fruit-like aroma (*26*). Two esters detected in all wine samples but in higher concentrations in immobilized cells were 2-phenylethyl acetate, giving a banana−apple aroma (*22*), and ethyl-9-decenoate, which described as exhibiting a very pleasant odor (*27*).

Acids. Fatty acids, due to their low odor threshold values and rather high concentrations in wines, are considered to have flavor impact in wines (*27*). This impact is positive, contributing to the complexity of the aroma bouquet, at concentrations up to their threshold values, but negative at higher concentrations (*24*). A positive correlation between concentrations of hexanoic, octanoic, and decanoic acid and the quality of the produced wines has been reported (*27*). In the present study these acids are detected in all wines but in higher concentrations in the case of immobilized cells, and the same trend is reported for all volatile fatty acids.

Alcohols. Fusel alcohols are generally considered to have rather unpleasant odors; therefore, it is believed that they contribute more to the intensity of the odor of the wine that to its quality (*27*). 2-Phenylethanol, which is one of the few fusel alcohols described with a pleasant odor as old rose (*12, 27*), was detected in all wine samples, and a reduction in its content at low temperatures (10 and 5 °C) was observed, which is in accordance with other studies (*14, 29*).

Carbonyl Compounds. Benzaldehyde detected in must and wines has a bitter almond odor, whereas β-damascenone detected in must and in higher concentrations in wines with immobilized cells at all temperatures has a complex smell of flowers, tropical fruit, and stewed apple (*22, 31*).

Terpene Compounds. Terpenes, which are mainly derived from the grape, are principal components that contribute to the characteristic aroma of a wine (*32*). Linalool gives a flowery odor (*32*) and was detected only in grape must at levels little

Table 5. Volatile Compounds (Milligrams per Liter) Identified in Grape Must and in Wines Produced by Free and Immobilized *Saccharomyces cerevisiae* AXAZ-1 Yeast Cells on Corn Starch Gel Using SPME-GC-MS Technique

			free cells			immobilized cells on corn starch gel				
RI[d]	compound	grape must	25 °C	15 °C	5 °C	25 °C	20 °C	15 °C	10 °C	5 °C
	esters									
925	ethyl formate[b]		0.013		0.009					0.107
1040	ethyl butanoate[a]	0.012	3.246		0.175	0.022	0.226	0.213	0.201	0.342
1258	ethyl hexanoate[a]	0.139	0.797	0.606	0.612	0.558	1.173	2.856	1.823	1.629
1361	ethyl heptanoate[c]				0.012				0.040	0.062
1386	ethyl 2-hydroxypropanoate[c]		1.591	3.464	2.121	0.356	1.080	2.366	1.148	0.746
1401	methyl octanoate[a]					0.025	0.008	0.008	0.010	0.009
1451	ethyl octanoate[b]	0.181	2.495	4.505	1.658	4.077	5.110	8.257	4.462	3.803
1506	ethyl 7-octenoate[b]		0.117	0.022		0.015	0.022	0.035	1.204	0.360
1553	ethyl 3-hydroxybutanoate[c]								0.179	
1564	2-furanmethanol acetate[c]				1.123	0.040	0.042	0.383	0.150	0.057
1652	ethyl decanoate[b]		1.772	1.297	1.176	12.880	13.151	14.830	9.277	7.807
1676	3-methylbutyl octanoate[b]					0.152	0.378	0.435	0.440	0.405
1679	3,7-dimethyl-6-octen-1-ol acetate[c]								0.843	
1700	diethyl butanedioate[b]	0.206	0.900	2.382	1.981	0.558	1.117	1.048	0.976	0.924
1709	ethyl 9-decenoate[b]	0.128	1.312	2.166	3.623	2.435	3.558	9.382	10.163	8.755
1808	methyl salicylate[c]	0.009		0.018						0.033
1809	ethyl benzeneacetate[b]	0.003	0.133	0.038	0.148	0.057	0.143	0.089	0.141	0.152
1847	2-phenylethyl acetate[b]	1.425	0.893	2.585	1.817	3.556	3.727	5.302	3.015	2.517
1850	ethyl dodecanoate[b]	0.049	0.734	0.184	0.267	8.096	7.594	12.357	6.886	4.630
1889	3-methylbutyl pentadecanoate[c]					0.225		0.803	0.291	0.242
2094	ethyl tetradecanoate[c]	0.040	0.238	0.154	0.341		0.543	0.632	0.575	0.386
2185	dibutyl phthalate[c]			0.269						
2260	isopropyl palmitate[c]	0.087		0.370						
2271	ethyl hexadecanoate[c]		0.845	0.590	0.955	1.484	1.133	1.569	1.246	1.045
2279	3,7,11-trimethyl-2,6,10-dodecatrien-1-ol acetate[c]					0.405	0.658	0.607	0.505	0.485
2292	ethyl 9-hexadecenoate[b]		2.330	0.443	2.364	5.007	9.276	8.737	6.547	5.120
2365	diethyl phthalate[c]	0.055	8.231	10.121	5.002	6.039	8.238	13.090	16.816	13.262
2416	ethyl octadecanoate[c]		0.024	0.100	0.330		0.108	0.128	0.508	0.106
2435	ethyl 9-octadecenoate[c]		0.231		0.326		0.549	0.586	0.558	0.422
	sum of esters	**2.334**	**25.902**	**29.314**	**24.040**	**45.987**	**57.834**	**83.713**	**68.004**	**53.406**
	total no. of esters	**12**	**18**	**18**	**19**	**19**	**21**	**22**	**25**	**25**
	organic acids									
1534	acetic acid[c]		0.322	6.689	11.314				1.167	
1633	butanoic acid[b]				0.007					
1962	hexanoic acid[c]		0.726	0.850	0.626	0.166	0.567	0.550	0.493	0.400
2156	octanoic acid[c]	1.397	9.880	11.145	8.730	17.158	19.602	21.285	19.996	13.247
2213	nonanoic acid[c]		0.354	0.790			0.450	1.252		0.588
2336	decanoic acid[c]	0.508	1.723	2.113	0.580	11.165	9.765	6.344	1.658	6.328
2390	undecylenic acid[c]					1.090	1.203	1.501	1.611	1.309
2425	dodecanoic acid[c]		0.366							
	sum of acids	**1.905**	**13.471**	**21.587**	**21.257**	**29.579**	**31.587**	**30.932**	**24.925**	**21.872**
	total no. of acids	**2**	**6**	**5**	**5**	**4**	**5**	**5**	**5**	**5**
	alcohols									
1179	1-butanol[b]		0.038	0.114		0.379	0.039	0.079	0.065	0.030
1312	4-penten-1-ol[b]		0.086	0.323	0.150	0.420	0.137	0.138	0.537	0.042
1329	4-methyl-1-pentanol[b]					0.015		0.008		
1350	3-methyl-1-pentanol[b]	0.002	0.009	0.014		0.036	0.019	0.038	0.080	0.012
1370	1-hexanol[a]	0.094				0.103	0.090	0.061		0.016
1419	3-ethoxy-1-propanol[c]		0.035						0.090	0.032
1470	1-heptanol[b]	0.009	0.031	0.079	0.183	0.033	0.036	0.378	0.374	0.060
1520	2-ethyl-1-hexanol[c]	0.015	0.007	0.013		0.037				
1538	6-methyl-5-hepten-2-ol[c]	0.028								
1559	2,3-butanediol[b]	0.339	3.143	3.883	2.791	12.038	14.276	11.062	8.746	6.877
1570	1-octanol[a]	0.137	0.601		0.488	0.322	0.301	0.238	0.105	
1590	1,3-butanediol[b]	0.540	2.673	2.343	1.765	5.410	8.241	7.844	7.276	3.261
1680	2-furanmethanol[a]	0.212	0.367			0.371	0.445	0.623	0.670	0.339
1730	3-(methylthio)-1-propanol[b]	0.077	0.385	0.244	0.530	1.572	2.015	1.153	0.883	0.557
1783	decanol[c]			0.150	0.241	0.151	0.145	0.197	0.105	0.072
1843	anthenol[c]			1.105						
1916	benzyl alcohol[a]	0.032	0.045		0.049	0.022				0.103
1928	isophytol[c]	0.020	0.148	0.025						
1933	2-phenylethanol[a]	3.292	9.596	6.086	4.923	17.694	16.382	17.658	13.449	13.780
1998	dodecanol[a]	0.030	0.150	0.105	0.176		0.029	0.163		
2312	2,4-bis(1,1-dimethylethyl)phenol[c]	0.270	0.516	0.537	0.587	2.998	3.056	2.546	1.742	1.482
2359	hexadecanol[c]			0.395						

Table 5. Continued

			free cells			immobilized cells on corn starch gel				
RI[d]	compound	grape must	25 °C	15 °C	5 °C	25 °C	20 °C	15 °C	10 °C	5 °C
	sum of alcohols	**5.097**	**17.830**	**15.416**	**11.883**	**41.601**	**45.211**	**42.186**	**34.122**	**26.663**
	total no. of alcohols	**15**	**16**	**15**	**11**	**16**	**14**	**15**	**13**	**14**
	carbonyls									
958	3-methylbutanal[b]	0.034								
1195	5-methyl-3-heptanone[c]	0.004								
1358	6-methyl 5-hepten-2-one[c]	0.003								
1390	nonanal[b]								0.015	0.017
1445	5-methyl 2-furanone[c]	0.001								
1486	furfural[a]	0.930	0.352	0.135	0.042	0.054	0.049	0.056	0.048	0.035
1533	benzaldehyde[b]	0.022	0.293		0.934	0.141				
1578	5-methylfurfural[c]	0.180	0.498		0.732	0.505	0.689	0.709	0.350	0.214
1834	β-damascenone[b]	0.038		0.024		0.108	0.115	0.090	0.105	0.085
	sum of carbonyl compounds	**1.212**	**1.143**	**0.159**	**1.708**	**0.808**	**0.853**	**0.855**	**0.518**	**0.351**
	total no. of carbonyl compounds	**8**	**3**	**2**	**3**	**4**	**3**	**3**	**4**	**4**
	terpenes									
1189	limonene[a]	0.038		0.081						
1454	*cis*-linalool oxide[b]			0.022	0.034	0.089	0.087	0.091	0.062	0.050
1556	β-linalool[b]	0.053								
1715	terpineol[b]	0.287	0.267		0.645	0.297	0.397	0.379	0.423	0.205
1741	geranial[c]			0.056						
1790	β-citronellol[c]			0.101					0.480	0.545
2090	nerolidol[c]			0.058						0.165
2343	farnesol[c]		0.136	0.181	0.520	0.900	0.935	1.638	0.987	0.576
	sum of terpenes	**0.378**	**0.403**	**0.499**	**1.199**	**1.286**	**1.419**	**2.108**	**1.952**	**1.541**
	total no. of terpenes	**3**	**2**	**6**	**3**	**3**	**3**	**3**	**4**	**5**
	miscellaneous									
690	chloromethane[c]	0.008								
850	tetrahydrofuran[c]	0.005								
930	1,1-diethoxyethane[c]			0.429						
1063	3-fluoro-1-propene[c]		0.141	0.388	0.047	0.631	0.209	0.211	0.605	0.237
	sum of miscellaneous	**0.013**	**0.141**	**0.817**	**0.047**	**0.631**	**0.209**	**0.211**	**0.605**	**0.237**
	total no. of miscellaneous	**2**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**
	sum of compounds detected	**10.939**	**58.890**	**67.792**	**60.134**	**119.892**	**137.113**	**160.005**	**130.126**	**104.070**
	total no. of compounds detected	**42**	**46**	**48**	**42**	**47**	**47**	**49**	**52**	**54**

[a] MS data and retention index in agreement with those of authentic compound. [b] MS data and retention index in agreement with those in the literature. [c] MS data in agreement with those in NIST and Wiley libraries. [d] RI, retention index.

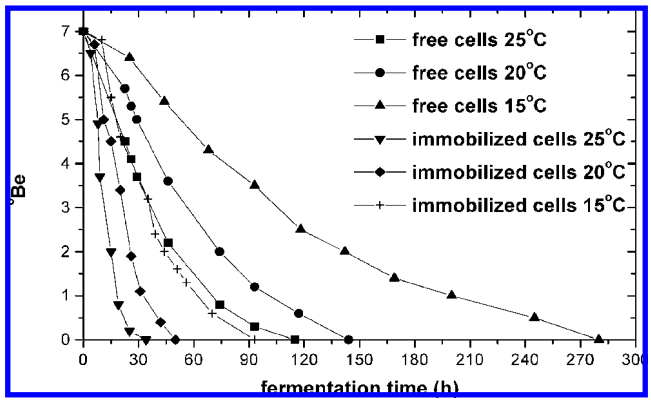

Figure 3. Fermentation kinetics observed with the use of 12% w/v glucose at 25, 20, and 15 °C by free and immobilized yeast cells on corn starch gel.

higher than its perception threshold [0.05 mg/L (*22*)]. α-Terpineol, farnesol, and *cis*-linalool oxide were detected in almost all wine samples produced by free and immobilized cells; however, due to their high perception thresholds, varying from 0.4 mg/L (for α-terpineol) to 1−5 mg/L (for *cis*-linalool oxide), they have very little olfactory impact on wines (*22*). Limonene, having a citrus-like odor note, was detected only in grape must and wine produced by free cells at 15 °C. β-Citronellol was detected at levels much higher than its perception threshold [0.018 mg/L (*22*)] in wines produced by immobilized cells at low temperatures (10 and 5 °C) and is considered to give a citrus, sweet, floral note (*32, 33*).

Catalytic Effect of Corn Starch Gel on Alcoholic Fermentation. The fermentation kinetics observed with 12% w/v glucose at several temperatures by free and immobilized on corn starch gel cells are presented in **Figure 3**. Using these results [calculating the ethanol productivity (dP/dt) at each temperature] and plotting $\ln(dP/dt)$ versus $1/T$, the curves of **Figure 4** for free and immobilized cells were graphed, which are described by the following equation based on the Arrhenius equation (*19*):

$$\ln(dP/dt) = \ln(AX) - E_a/RT \quad (3)$$

The activation energy E_a and the Arrhenius pre-exponential factors for free and immobilized cells were calculated by the

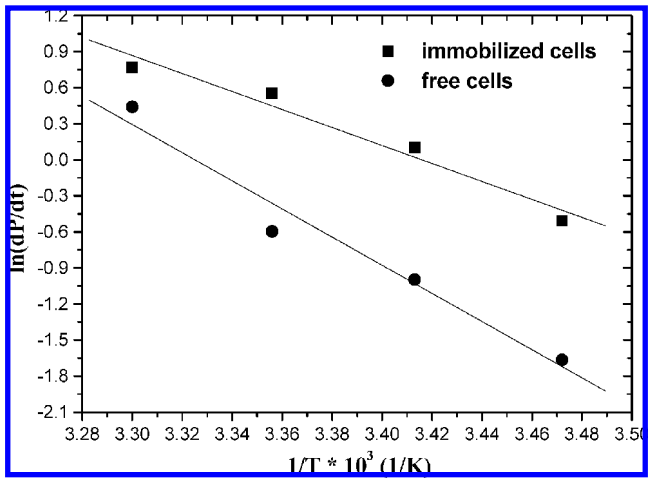

Figure 4. Arrhenius plot for the graphic evaluation of the activation energy and the pre-exponential factor of alcoholic fermentation performed with free and immobilized cells.

Table 6. Activation Energies and Reaction Rate Constants of the Fermentations Made Using Free and Immobilized *Saccharomyces cerevisiae* AXAZ-1 Yeast Cells on Corn Starch Gel

support[a]	activation energy (E_a, kJ/mol)	reaction rate constant (h^{-1})			
		k_{30}	k_{25}	k_{20}	k_{15}
FC	97.4	0.454	0.237	0.121	0.061
IC	62.2	0.808	0.533	0.348	0.223

[a] FC, free cells; IC, immobilized cells.

slope and intercept of these curves. The activation energy of the immobilized on corn starch gel cells was 36% smaller than that of free cells (62.2 and 97.4 kJ/mol, respectively), confirming the catalytic activity of the immobilization support as in the case of potato pieces in a previous study (*14*). Furthermore, using the Arrhenius equation [$k = A \exp(-E_a/RT)$] and substituting $_{Ea}$ and A, the reaction rate constants for free and immobilized cells at temperatures between 15 and 30 °C were calculated (**Table 6**). Immobilization and temperature affected significantly the reaction rate constants ($p < 0.01$ in both cases), and a strong interaction between them ($p < 0.01$) was also observed. More specifically, the reaction rate constant at 30 °C, k_{30}, was 1.8-fold higher than that of free cells (0.808 and 0.454 h^{-1}, respectively), whereas a decrease of fermentation temperature made, making the reaction rate constant of immobilized cells 3.6-fold higher than that of free cells at 15 °C (0.223 and 0.061 h^{-1}, respectively). These results prove that starch gel acts as a catalyst.

Scientific and Technological Consideration of Results. Corn starch gel is a very interesting food grade purity support for yeast cell immobilization. It was proved to be suitable for dry and semisweet winemaking at 27 °C, whereas it was also capable for low-temperature winemaking. Using yeast cells immobilized on corn starch gel, the formation of esters was accelerated, giving higher numbers and concentrations compared to wine produced by free cells. The decrease of fermentation temperature (even to 5 °C) led to further increase in the percentages of total esters compared to total alcohols, especially in the case of immobilized cells. This increase of the concentration of ethyl acetate and esters in general at extremely low-temperature fermentation may be attributed to the increase of the solubility of these compounds with the drop of temperature. This can be explained from the less vigorous fermentations occurring at low temperatures because the CO_2 release is low. The vigorous fermentations in the range of 25−30 °C are responsible for the removal of volatile esters. On the other hand, the reduction of amyl alcohols can be attributed to the reduction of enzymatic activity at extremely low-temperature fermentations. Furthermore, this study examined whether the increase of the rate of fermentation could be attributed to immobilized cells or was a result of the catalytic activity of starch. For this reason, the activation energy E_a and reaction rate constant of both free and immobilized cells were calculated, showing a reduction of the activation energy and an increase in reaction rate constants in the case of immobilized cells, especially at low temperatures (**Table 6**). This is in accordance with other previous theoretical and experimental investigations (*8, 14*). Therefore, starch reducing the E_a behaves as a catalyst or as a promoter of the enzymes involved in the process. Furthermore, any improvement of cell physiology by cell immobilization related to the improvement of the rate of fermentation could affect the glucose uptake rate. This increase of glucose uptake rate can be attributed more to the increase of glucose bioconversion rate than to the effect of cell physiology improvement. All of these show that corn starch gel is a very promising support for yeast cell immobilization in winemaking that reduces the fermentation time and improves the general quality of wines but also acts as a catalyst for the process, explaining the capability of immobilized cells for low-temperature fermentations. The catalytic effect of corn starch gel opens new perspectives in the research of immobilized cells, showing that the increased fermentation rates obtained in general by immobilized cells may be due to the catalytic activity of the support. However, each support needs to be examined separately whether it reduces the activation energy or not. This reduction of activation energy led to an increase of the rate of fermentation, making the extremely low-temperature fermentation, by some supports, attractive for industrial applications in food production.

ABBREVIATIONS USED

GC-MS, gas chromatography−mass spectrometry; SPME, solid phase microextraction; E_a, activation energy (kJ/mol); P, alcohol concentration (g/L); T, temperature (K); k, reaction rate constant (h^{-1}); t, fermentation time (h); X, cell mass concentration (g/L); R, ideal gas constant (kJ/mol K); A, Arrhenius pre-exponential factor of fermentation (h^{-1}); RI, retention index.

NOTE ADDED AFTER PRINT PUBLICATION

Figures 2 and **3** were incorrect in the paper published on the Web on November 26, 2008, and in the December 24, 2008, print issue (Vol. 56, Issue 24). The corrected electronic version was published on the Web on February 18, 2009, and an Addition/Correction appeared in the February 25, 2009, print issue (Vol. 57, Issue 4).

LITERATURE CITED

(1) Kourkoutas, Y.; Bekatorou, A.; Banat, I. M.; Marchant, R.; Koutinas, A. A. Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiol.* **2004**, *21*, 377–397.

(2) Bakoyiannis, V.; Kanellaki, M.; Kalliafas, A.; Koutinas, A. A. Low temperature wine-making by immobilized cells on mineral kissiris. *J. Agric. Food Chem.* **1992**, *40*, 1293–1296.

(3) Loukatos, P.; Kiaris, M.; Ligas, I.; Bourgos, G.; Kanellaki, M.; Komaitis, M.; Koutinas, A. A. Continuous wine-making by γ-alumina-supported biocatalyst. Quality of the wine and distillates. *Appl. Biochem. Biotechnol.* **2000**, *89*, 1–13.

(4) Suzzi, G.; Romano, P.; Vannini, L.; Turbanti, L.; Domizio, P. Cell-recycle batch fermentation using immobilized cells of flocculent *Saccharomyces cerevisiae* wine strains. *World J. Microbiol. Biotechnol.* **1996**, *12*, 25–27.
(5) Van Iersel, M. F. M.; Van Dieren, B.; Rombouts, F. M.; Abee, T. Flavor formation and cell physiology during the production of alcohol-free beer with immobilized *Saccharomyces cerevisiae*. *Enzyme Microb. Technol.* **1999**, *24*, 407–411.
(6) Kosseva, M.; Beschkov, V.; Kennedy, J. F.; Lloyd, L. L. Malolactic fermentation in Chardonnay wine by immobilized *Lactobacillus casei* cells. *Process Biochem.* **1998**, *33*, 793–797.
(7) Crapisi, A.; Spettoli, P.; Nuti, M. P.; Zamorani, A. Comparative traits *of Lactobacillus brevis*, *Lactobacillus fructivorans* and *Leuconostoc oenos* immobilized cells for the control of malolactic fermentation in wine. *J. Appl. Bacteriol.* **1987**, *63*, 513–521.
(8) Bardi, E.; Koutinas, A. A. Immobilization of yeast on delignified cellulosic material for room temperature and low-temperature wine making. *J. Agric. Food Chem.* **1994**, *42*, 221–226.
(9) Iconomopoulou, M.; Psarianos, K.; Kanellaki, M.; Koutinas, A. A. Low temperature and ambient temperature wine making using freeze dried immobilized cells on gluten pellets. *Process Biochem.* **2002**, *37*, 707–717.
(10) Kopsahelis, N.; Kanellaki, M.; Bekatorou, A. Low temperature brewing using cells immobilized on brewer's spent grains. *Food Chem.* **2007**, *104*, 480–488.
(11) Bekatorou, A.; Sarellas, A.; Ternan, N. G.; Mallouchos, A.; Komaitis, M.; Koutinas, A. A.; Kanellaki, M. Low-temperature brewing using yeast immobilized on dried figs. *J. Agric. Food Chem.* **2002**, *50*, 7249–7257.
(12) Tsakiris, A.; Bekatorou, A.; Psarianos, C.; Koutinas, A. A.; Marchant, R.; Banat, I. M. Immobilization of yeast on dried raisin berries for use in dry white wine-making. *Food Chem.* **2004**, *87*, 11–15.
(13) Kourkoutas, Y.; Kanellaki, M.; Koutinas, A. A.; Tzia, C. Effect of fermentation conditions and immobilization supports on the wine making. *J. Food Eng.* **2005**, *69*, 115–123.
(14) Kandylis, P.; Koutinas, A. A. Extremely low temperature fermentations of grape must by potatoes supported yeast-strain AXAZ-1. A contribution is performed to catalysis of alcoholic fermentation. *J. Agric. Food Chem.* **2008**, *56*, 3317–3327.
(15) Bardi, E.; Koutinas, A. A.; Soupioni, M.; Kanellaki, M. Immobilization of yeast on delignified cellulosic material for low temperature brewing. *J. Agric. Food Chem.* **1996**, *44*, 463–467.
(16) Argiriou, T.; Kaliafas, A.; Psarianos, K.; Kana, K.; Kanellaki, M.; Koutinas, A. A. New alcohol resistant strains of *S. cerevisiae* species for potable alcohol production using molasses. *Appl. Biochem. Biotechnol.* **1992**, *36*, 153–161.
(17) Zoeklein, B.; Fugelsang, K.; Gump, B.; Nury, F. Volatile acidity. In *Production Wine Analysis*; Van Nostrand Reinhold: New York, 1990; pp 105−110.
(18) Klein, J.; Kressdorf, B. Improvement of productivity and efficiency in ethanol production with Ca-alginate immobilized *Z. mobilis*. *Biotechnol. Lett.* **1983**, *5*, 497–502.
(19) Converti, A.; Bargagliotti, C.; Cavanna, C.; Nicolella, C.; Del Borghi, M. Evaluation of kinetic parameters and thermodynamic quantities of starch hydrolysate alcohol fermentation by *Saccharomyces cerevisiae*. *Bioprocess Eng.* **1996**, *15*, 63–69.
(20) Hoover, R. Composition, molecular structure, and physicochemical properties of tuber and root starches: a review. *Carbohydr. Polym.* **2001**, *45*, 253–267.
(21) Parker, R.; Ring, S. G. Aspects of the physical chemistry of starch. *J. Cereal Sci.* **2001**, *34*, 1–17.
(22) Ribérau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. The chemistry of wine stabilization and treatments. In *Hanbook of Enology*; Wiley: Chichester, U.K., 2000; Vol. 2, pp 41−54, 189, 193− 196.
(23) Antonelli, A.; Castellari, L.; Zambonelli, C.; Carnacini, A. Yeast influence on volatile composition of wines. *J. Agric. Food Chem.* **1999**, *47*, 1139–1144.
(24) Jackson, R. S. *Wine Science: Principles and Applications*; Academic Press: San Diego, CA, 1994; pp 184−187.
(25) Regodón Mateos, J. A.; Pérez-Nevado, F.; Ramírez Fernández, M. Influence of *Saccharomyces cerevisiae* yeast strain on the major volatile compounds of wine. *Enzyme Microb. Technol.* **2006**, *40*, 151–157.
(26) Rapp, A.; Mandery, H. Wine aroma. *Experentia* **1986**, *42*, 873–884.
(27) Etievant, X. P. Wine. In *Volatile Compounds in Foods and Beverages*; MaarseH., Ed.; Marcel Dekker: New York, 1991; pp 483−533.
(28) Vidrih, R.; Hribar, J. Synthesis of higher alcohols during cider processing. *Food Chem.* **1999**, *67*, 287–294.
(29) Mallouchos, A.; Komaitis, M.; Koutinas, A. 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.
(30) Miranda-Lopez, R.; Libbey, L. M.; Watson, B. T.; McDaniel, M. R. Odor analysis of Pinot Noir wines from grapes of different maturities by gas chromatography−olfactrometry technique (Osme). *J. Food Sci.* **1992**, *57* (985−993), 1019.
(31) Pisarnitskii, A. F. Formation of wine aroma: tones and imperfections caused by minor components. *Appl. Biochem. Microbiol.* **2001**, *37*, 552–560.
(32) Vilanova, M.; Sieiro, C. Determination of free and bound terpene compounds in Albariño wine. *J. Food Compos. Anal.* **2006**, *19*, 694–697.
(33) Peña-Alvarez, A.; Capella, S.; Juárez, R.; Labastida, C. Determination of terpenes in tequila by solid phase microextraction−gas chromatography−mass spectrometry. *J. Chromatogr., A* **2006**, *1134*, 291–297.

Received for review August 9, 2008. Revised manuscript received October 31, 2008. Accepted November 6, 2008. This work is part of the 03ED375 research project, implemented within the framework of the "Reinforcement Programme of Human Research Manpower" (PENED) and cofinanced by National and Community Funds (20% from the Greek Ministry of Development−General Secretariat of Research and Technology and 80% from the EU−European Social Fund).

JF802476W