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Ambient and low temperature winemaking by immobilized cells on brewer's spent grains: Effect on volatile composition

Mallouchos Athanasios [a], Loukatos Paul [a], Bekatorou Argyro [b], Koutinas Athanasios [b], Komaitis Michael [a,*]

[a] *Agricultural University of Athens, Food Science and Technology Department, Iera Odos 75, 118 55 Athens, Greece*
[b] *University of Patras, Department of Chemistry, Section of Analytical, Environmental and Applied Chemistry, 26500 Patras, Greece*

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Abstract

Repeated batch wine fermentations were conducted using immobilized yeast cells on brewer's spent grains as well as free cells, at 25, 20, 15 and 10 °C. The operational stability of the biocatalyst was good and no decrease of its activity was observed, even at 10 °C. Ethanol and wine productivities were high, showing the suitability of the biocatalyst for low temperature winemaking. The interaction effect of immobilization and temperature was statistically significant in most cases. Immobilized cells produced wines with a higher content of ethyl and acetate esters as well as volatile fatty acids, at temperatures of 15 and 10 °C, whereas the opposite was observed for free cells at higher temperatures. Same amounts of higher alcohols were produced by both immobilized and free cells. These amounts decreased dramatically with temperature drop. Wines produced by immobilized cells at low fermentation temperatures (10–15 °C) were characterized by a potentially better flavour due to a better ratio of esters to alcohols.

Keywords: Immobilization; Yeast; Wine; Volatiles; Esters; Acetates; Higher alcohols; Acids; Temperature; Fermentation; GC/MS; Spent grains

1. Introduction

Cell immobilization in alcoholic fermentation has been extensively studied during the past three decades because of its technical and economical advantages over those of free cell systems (Dervakos & Webb, 1991; Lu, Lu, Bie, & Fujimura, 2002; Sakurai, Nishida, Saito, & Sakakibara, 2000; Shindo, Takata, Taguchi, & Yoshimura, 2001; Sree, Sridhar, Suresh, Banat, & Rao, 2000). However, for application in the wine industry, research is needed to find out an immobilization support that meets the prerequisite of food grade purity, low cost and a final product of improved aroma and taste. Particles of various synthetic or natural materials, organic and inorganic, have been used as supports for cell immobilization in wine fermentations (Divies, Cachon, Cavin, & Prevost, 1994; Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004).

Recently, brewer's spent grains (BSG), a by-product of the brewing industry, were used as a carrier, after treatment with acid and alkali, in order to immobilize yeast cells for pectinase production (Almeida, Brányik, Moradas-Ferreira, & Teixeira, 2003). Brányik and coworkers used the aforementioned support for continuous primary fermentation of beer (Brányik, Vicente, Cruz, & Texeira, 2004; Brányik, Vicente, Machado Cruz, & Teixeira, 2001) and suggested that it is a very efficient immobilization carrier due to its high yeast loading capacity, easy preparation, reusability, availability and inert, non-toxic nature.

Wine aroma is the result of a complex combination of components that give each wine its distinctive character.

Abbreviations: GC/MS, gas chromatography–mass spectrometry; BSG, brewer's spent grains; IC, immobilized cells; FC, free cells; SPME, solid phase microextraction; EERI, ethyl ester retention index; LRI, linear retention index; MCFA, medium chain length acids.

* Corresponding author. Tel./fax: +30 210 5294681.
E-mail address: achem@aua.gr (M. Komaitis).

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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 (Nykanen & Suomalainen, 1983; Rapp & Mandery, 1986). 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 (Etievant, 1991; Jackson, 1994). Several authors have reported the combined effect of temperature and immobilization on the formation of the major volatiles produced during wine fermentations (Bakoyianis, Kana, Kaliafas, & Koutinas, 1993; Bardi, Koutinas, Psarianos, & Kanellaki, 1997; Kourkoutas, Kanellaki, Koutinas, & Tzia, 2005; Tsakiris et al., 2004; Yajima & Yokotsuka, 2001). However, publications concerning the analysis of the volatile components in wines produced by immobilized cells are scarce in the literature (Mallouchos, Komaitis, Koutinas, & Kanellaki, 2003; Mallouchos et al., 2003).

The aim of the present work was to investigate the suitability of yeast cells immobilized on untreated BSG as a biocatalyst for wine making at ambient and low temperatures. The influence of the immobilization and temperature on the volatile composition of the produced wines was also investigated.

2. Material and methods

2.1. Yeast strain and fermentation media

The cryotolerant, alcohol resistant yeast strain VIS-ANTO-1 (Yeast collection of Food Biotechnology Group, University of Patras, Greece) of *Saccharomyces cerevisiae* was grown on a semi-synthetic medium containing glucose (20 g/l), yeast extract (4 g/l), $(NH_4)_2SO_4$ (1 g/l), KH_2PO_4 (1 g/l) and $MgSO_4 \cdot 7H_2O$ (5 g/l) (Merck E.P.E, Alimos, Greece). Glucose media of the above composition of nutrient salts were also used for cell immobilization, where the respective amount of glucose was 120 g/l. The strain was cultivated in static flasks under semi-aerobic conditions at 25 °C for 24 h and separated by centrifugation at 1310 g for 10 min. Grape must of cultivar Roditis and concentrated must was obtained from Achaia Clauss winery (Patras, Greece). The initial density of natural must was 11.8 °Be. All media were sterilized at 121 °C for 20 min.

2.2. Immobilization

Brewer's spent grains were obtained from the Athenian Brewery SA (Athens department, Greece) and were used after exhaustive washing with tap-water. Two hundred grams of wet BSG were placed in a glass-tower bioreactor (1.0 l) (Tsakiris, Sipsas, Bekatorou, Mallouchos, & Koutinas, 2004) and sterilized at 121 °C for 20 min. Cell immobilization followed by adding 600 ml of the semi-synthetic medium (containing glucose, 120 g/l), in which 12 g wet weight cells of the *S. cerevisiae* had been suspended. The system was allowed to ferment for about 6–8 h, until the density of the fermented liquid reached a final value of 0–1 °Be. The supernatant liquid was decanted and the support was washed twice with 300 ml of the semi-synthetic medium. The prepared biocatalyst was used directly for fermentations of concentrated and natural grape must.

2.3. Determination of immobilized cells

Samples of 10 g wet spent grains (2 g approximately dry weight) were taken during fermentation and homogenized for 4 min in a Stomacher with 90 ml 1/4 strength Ringer solution (De Pina & Hogg, 1999). After appropriate decimal dilution of the homogenates, the yeast cells were enumerated using a Neubauer improved hemacytometer. The immobilized cells population was at the level of 10^{10} g cells/g wet weight carrier corresponding to 0.33 g immobilized cells (dry weight)/g dry weight carrier.

2.4. Grape must fermentations

Ten repeated batch fermentations of 600 ml concentrated grape must having different initial sugar concentrations, were carried out using the aforementioned biocatalyst at 30 °C. At the end of each fermentation batch, the support was washed with 300 ml of concentrated grape must to remove free cells and residual sugars. The fermented liquids were analyzed for ethanol, residual sugar and major volatile by-products. Repeated fermentation batches of natural grape must followed at temperatures of 25, 20, 15 and 10 °C (three batches at each temperature). The fermentation was monitored by measuring Baume density and stopped when it reached the value of 0–0.5 °Be. At the end of each fermentation batch, the produced wines were centrifuged at 1310*g* for 10 min and stored at low temperature until further use. The support was washed with 300 ml of fresh grape must and the biocatalyst was used for the next fermentation batch. Repeated batch fermentations using free yeast cells were also carried out as controls with the same initial yeast concentration (22 g dry wt cells/l). The inoculum used in free cell fermentations was taken from the previous fermentation batch conducted. The first batch was inoculated with free cells prepared as described in Section 2.1.

2.5. Assays

The ethanol concentration in the final products was determined in a Jasco PU-980 HPLC using an RI detector (Jasco RI-930) and a Fast Fruit Juice polymeric column (Waters, USA). The eluent was 0.1% (v/v) H_3PO_4 at a flow rate of 1.0 ml/min. Column and detector temperature was set at 60 and 35 °C, respectively. Residual sugars were determined using a Pinnacle II Amino 5 μm column (250 × 4.6 mm, Restek, USA) and acetonitrile/water (75:25, v/v) as eluent at a flow rate of 1.0 ml/min. Column

temperature was set at 35 °C. Ethanol productivity was calculated as grams of ethanol per liter liquid volume produced per day. Wine productivity was calculated as grams of wine per liter total volume produced per day.

Total acidity (expressed as tartaric acid, g/l) and volatile acidity (expressed as acetic acid, g/l) were determined according to the official methods for wine analysis proposed by the European Union (Official Journal of the European Communities, 1990).

The major volatile by-products (acetaldehyde, ethyl acetate, propanol, isobutanol, amyl alcohols [3-methyl- and 2-methyl-1-butanol], Sigma–Aldrich, Athens, Greece) were determined by direct injection of wine samples in a gas chromatograph of Fisons Instruments (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, Varian Inc., USA). Helium was used as 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 100 °C at a rate of 4.0 °C/min. Then, it reached 250 °C at a rate of 20 °C/min. It remained at this temperature for 5 min. The injector temperature was maintained at 230 °C, whereas the detector temperature at 250 °C. The concentrations of the above compounds were determined using calibration curves covering the normal concentration range of these substances in wines and 3-pentanol was used as internal standard. All samples were analyzed in triplicate.

2.6. Headspace SPME-GC/MS

The minor volatile constituents of the produced wines were determined by means of gas chromatography–mass spectroscopy. The volatiles were isolated by the headspace solid phase micro-extraction method. The fiber used for the absorption of volatiles was a CAR-PDMS-DVB (50/30/30 μm, Supelco, USA). The conditions of headspace-SPME sampling were as follows: 20 ml liquid sample, 6 g NaCl and 100 μl internal standard (2-pentanol, 2470 mg/l) were transferred into a 40-ml screw-capped glass vial with a mininert valve (Sigma–Aldrich, Athens, Greece). The contents were magnetically stirred for 5 min at 30 °C, and then the fiber was exposed to the headspace for 30 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 250 °C for 5 s. 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 Fisons 8000 series gas chromatograph (Model 8060) coupled to a Fisons MD-800 quadrupole mass spectrometer. Helium was used as carrier gas (1.0 ml/min). Separation of compounds was performed on a DB-Wax column (30 m × 0.25 mm, df = 0.25 μm, Agilent J& W, USA). Oven temperature was programmed from 40 to 250 °C with a rate of 4 °C/min. It was held at 250 °C for 5 min. The injector, ion source and interface temperatures were set at 250, 200 and 260 °C, respectively. The mass spectrometer was operated in the electron impact mode with the electron energy set at 70 eV and scan range 30–400 m/z. Identification of the compounds was effected by comparing: (i) the linear retention indices based on the homologous series of even ethyl esters (C2–C18) and even *n*-alkanes (C10–C22) 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. The determination of ethyl ester retention indices was effected by the AMDIS software (NIST) using the retention times of the ethyl esters already present in wine samples. The *n*-alkanes retention indices determination was carried out by extrapolation from chromatograms of liquid injections of a solution (CH_2Cl_2) containing the even *n*-alkanes series, even ethyl esters series and reference compounds. Semi-quantitative 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 (12.35 mg/l). The peak area was measured either by full scanning or by choosing specific fragments (Masslab 1.2.7 software, Fisons). All standard compounds were bought from Sigma–Aldrich (Athens, Greece).

2.7. Statistical analysis

Analysis of variance (ANOVA) was carried out on volatile compounds' data. The means were compared by Tukey's honestly significant difference (HSD) procedure (STATGRAPHICS Plus).

3. Results and discussion

3.1. Immobilization and fermentations

BSG meets a lot of the prerequisites for cost effective industrial immobilization, such as low cost, food-grade purity, inert under fermentation conditions and easy preparation. It has been recently used in a modified form (treatment with acid and alkali) for continuous primary fermentation of beer. However, according to Brányik et al. (2001) and our results (not shown), the modified BSG cannot support cell adhesion and cannot act as a promoter of alcoholic fermentation under batch conditions. The biocatalyst loses its operational stability and leads to sluggish fermentations after a few batches. Thus, we proceeded to a step-by-step investigation of the raw BSG (no chemical treatment) to evaluate its efficiency for repeated batch fermentations of grape must, at ambient and low temperatures, in terms of productivity and formation of volatile by-products. These are factors directly related to commercial application and consumer acceptance. Furthermore, comparison with the traditional free cell fermentations was attempted.

To confirm that the immobilization of VISANTO-1 cells on raw BSG is possible and that the immobilized biocatalyst can be successfully used for wine making, 10 repeated batch fermentations of concentrated grape must of various sugar content were carried out at 30 °C.

Fermentation times were low and constant from batch to batch, varying from 13.5 to 35.5 h, depending on the initial sugar concentration. Ethanol productivities were very high (Table 1). Subsequently, the same biocatalyst was used in repeated batch fermentations of natural grape must at ambient and low temperatures. The operational stability of the immobilized biocatalyst was retained, even at low temperatures. The fermentations times ranged from 2 days at 25 °C to 5 days at 10 °C, which are considerably lower than those of free cell fermentations (Table 1). This resulted in significant differences of ethanol and wine productivities between the immobilized and free cells, which were more pronounced at low fermentation temperatures (15 and 10 °C). In both cases, ethanol content, total and volatile acidity of the produced wines were within the normal ranges. Residual sugars were found in low levels in all wines showing the possibility for dry wine making by the immobilized cells, even at low temperatures. The successful immobilization of yeast on BSG was also confirmed by electron microscopy. Fig. 1 shows the morphology of spent grains' surface after the attachment of cells and the formation of yeast biofilm. Local biomass accumulations were observed on the surface of the carrier while other large zones were not colonized by yeast cells. Similar electron micrographs were obtained for the modified BSG by other researchers (Brányik, Vicente, Oliveira, & Teixeira, 2004).

3.2. Major volatile by-products

Acetaldehyde is one of the most important carbonyl compounds formed during degradation of sugars by yeasts and constitutes more than 90% of the total aldehyde content in wine. At low levels, it gives a pleasant fruity aroma, but at high concentrations it possesses a pungent irritating odor, which is undesirable for table wines (Etievant, 1991; Jackson, 1994; Liu & Pilone, 2000). The interaction of fermentation type (immobilized or free cells) with temperature had a statistically significant effect on the final content of acetaldehyde (Table 2). The combined use of immobilized cells and low temperature resulted in the production of small amounts of this compound, whereas the opposite was observed in the case of fermentations with free cells. Similar results have been reported previously by other researchers (Kourkoutas et al., 2005; Mallouchos, Komaitis, et al., 2003; Mallouchos et al., 2003; Yajima and Yokotsuka, 2001). The differences observed may reflect the effect of temperature and immobilization on the activity of pyruvate decarboxylase and alcohol dehydrogenase, which are implicated in the biosynthesis of acetaldehyde by yeasts. Generally, the concentration of acetaldehyde was in levels similar to those found in commercial wines and below the odor threshold value (100 mg/l).

Table 1
Kinetic and physicochemical parameters of the repeated batch fermentations of concentrated and natural grape must using cells immobilized on brewer's spent grains and free cells at different temperatures

Grape must	Temperature (°C)	Batch	Initial density (°Be)	Fermentation time (h)	Residual sugar (g/l)	Ethanol (% v/v)	Ethanol productivity (g/l/day)	Wine productivity (g/l/day)	Volatile acidity (acetic acid g/l)	Total acidity (tartaric acid g/l)
Immobilized cells										
Concentrated	30	1–3	7.7	13.5 ± 0.7	2.9 ± 1.1	6.6 ± 0.1	92.2 ± 4.2	–	–	–
Concentrated	30	4–6	10.6	21.5 ± 0.5	2.7 ± 2.1	10.0 ± 0.2	88.2 ± 5.9	–	–	–
Concentrated	30	7–10	12.5	35.5 ± 0.5	2.4 ± 0.9	12.4 ± 0.3	55.7 ± 1.0	–	–	–
Natural	25	11–13	11.8	45.0 ± 2.8	7.0 ± 2.8	11.6 ± 0.1	48.2 ± 2.8	294 ± 18.5	0.28 ± 0.07	6.8 ± 0.5
Natural	20	14–16	11.8	64.2 ± 5.9	8.8 ± 3.1	11.7 ± 0.2	34.4 ± 2.5	207 ± 18.1	0.26 ± 0.04	6.3 ± 0.3
Natural	15	17–19	11.8	100.0 ± 4.2	5.9 ± 1.3	11.8 ± 0.2	22.5 ± 0.8	132 ± 5.6	0.20 ± 0.05	6.1 ± 0.2
Natural	10	20–22	11.8	118.5 ± 1.4	5.0 ± 1.4	11.7 ± 0.1	18.5 ± 0.4	111 ± 1.3	0.22 ± 0.06	5.9 ± 0.3
Free cells										
Natural	25	1–3	11.8	70.5 ± 1.8	6.6 ± 1.7	11.7 ± 0.1	30.5 ± 2.1	205 ± 10.5	0.88 ± 0.09	6.6 ± 0.4
Natural	20	4–6	11.8	120.5 ± 2.3	6.9 ± 2.2	11.4 ± 0.2	18.4 ± 0.6	120 ± 5.1	0.73 ± 0.07	6.5 ± 0.3
Natural	15	7–9	11.8	188.0 ± 5.7	6.4 ± 1.5	11.5 ± 0.1	11.0 ± 0.5	77 ± 3.2	0.84 ± 0.07	6.4 ± 0.3
Natural	10	10–12	11.8	624.5 ± 10.5	9.8 ± 3.4	11.2 ± 0.3	3.3 ± 0.4	23 ± 1.1	0.93 ± 0.03	6.2 ± 0.4

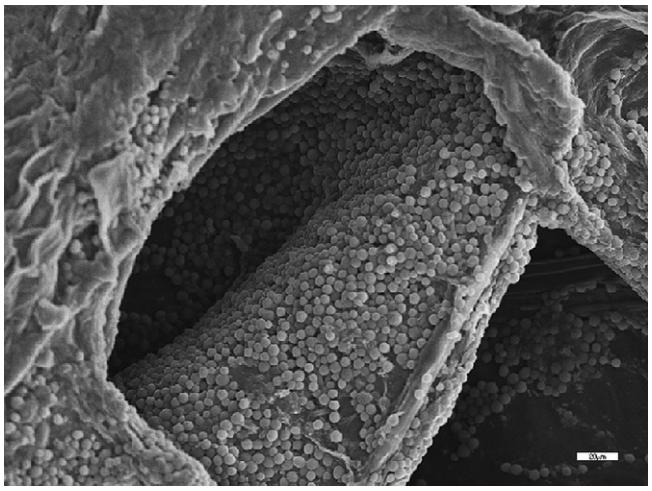

Fig. 1. Electron micrograph showing the morphology of the brewer's spent grains after the immobilization of VISANTO-1 cells.

Immobilized cells produced significant higher quantities of ethyl acetate than free cells at every temperature studied ($p < 0.05$) (Table 2). These contribute positively to the wine aroma because they are above the threshold value (12.3 mg/l) and lower than the value which is considered to have a negative impact on aroma (Etievant, 1991; Jackson, 1994). Similar results have been reported in studies with immobilized cells on various supports (Bakoyianis et al., 1993; Bardi et al., 1997; Iconomopoulou, Psarianos, Kanellaki, & Koutinas, 2002). The synthesis of acetate esters in *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 by the addition of unsaturated fatty acids (Fujii, Kobayashi, Yoshomoto, Furukawa, & Tamai, 1997). Temperature drop to 10 °C resulted in a significant decrease of ethyl acetate concentration, for both immobilized and free cells. However, the impact of this factor was not very strong, as can be seen from the *F*-ratio (Table 2).

Propanol, isobutanol and amyl alcohols were the most abundant higher alcohols found in wines. Their odors are rather unpleasant and most authors suggest that they contribute more to the intensity of the odor of the wine than to its quality (Etievant, 1991; Jackson, 1994; Nykanen & Suomalainen, 1983). Propanol was produced in similar amounts by both free and immobilized cells at the temperatures of 25, 20 and 15 °C. Significant higher amounts were produced by the immobilized biocatalyst at 10 °C. A strong interaction between fermentation type and temperature affecting isobutanol concentration was observed. The effect was more pronounced for immobilized cells, which produced at 10 °C the quarter of the amount produced at 25 °C. A remarkable decrease of amyl alcohols with the decrease of temperature was also observed by both immobilized and free cells. Their amount was similar at every temperature (fermentation type was not statistically significant). The decrease of the amount of higher alcohols

Table 2
Major volatile by-products obtained by the repeated batch fermentations of concentrated and natural grape must using cells immobilized on brewer's spent grains and free cells at different temperatures

Grape must	Temperature (°C)	Batch	Initial density (°Be)	Acetaldehyde (mg/l)	Ethyl acetate (mg/l)	Propanol (mg/l)	Isobutanol (mg/l)	Amyl alcohols (mg/l)	Total volatiles (mg/l)
Immobilized cells									
Concentrated[A]	30	1–3	7.7	21.2 ± 2.1a	16.4 ± 2.4a	22.1 ± 1.6a	23.4 ± 3.5a	98.0 ± 11.2a	181.1 ± 17.9a
Concentrated	30	4–6	10.6	40.8 ± 2.7b	30.2 ± 2.0b	26.2 ± 1.5b	34.5 ± 2.4a	127.4 ± 7.6b	259.1 ± 8.5b
Concentrated	30	7–10	12.5	56.4 ± 3.2c	44.8 ± 1.4c	32.6 ± 1.6c	63.8 ± 9.9b	137.8 ± 5.5b	335.4 ± 16.3c
Natural[B]	25	11–13	11.8	39.0 ± 3.1	43.2 ± 6.9	24.4 ± 3.2	81.0 ± 3.5	140.1 ± 6.7	327.7 ± 17.3
Natural	20	14–16	11.8	34.9 ± 2.5	53.0 ± 7.3	25.2 ± 2.9	49.1 ± 2.7	149.8 ± 9.5	312.1 ± 16.1
Natural	15	17–19	11.8	30.4 ± 1.4	37.5 ± 5.1	25.5 ± 3.3	35.0 ± 5.2	137.3 ± 7.5	266.7 ± 21.6
Natural	10	20–22	11.8	25.1 ± 1.6	36.0 ± 4.1	43.4 ± 3.9	20.6 ± 1.3	80.8 ± 1.2	205.9 ± 4.6
Free cells									
Natural	25	1–3	11.8	38.8 ± 2.8	27.2 ± 5.2	20.1 ± 1.8	25.2 ± 3.8	152.7 ± 8.1	261.1 ± 9.8
Natural	20	4–6	11.8	36.6 ± 1.5	24.3 ± 3.5	25.1 ± 3.2	24.1 ± 2.6	144.3 ± 6.7	254.6 ± 5.4
Natural	15	7–9	11.8	38.2 ± 3.0	25.3 ± 5.0	28.2 ± 3.7	21.5 ± 2.2	115.5 ± 4.5	228.7 ± 14.9
Natural	10	10–12	11.8	40.5 ± 3.5	17.3 ± 4.0	29.5 ± 2.7	12.5 ± 2.4	80.6 ± 2.3	180.4 ± 13.5
F-ratio	Fermentation type (*Z*)			**35.42**	**76.38**	**9.10**	**403.34**	1.99	**64.80**
	Temperature (*T*)			**6.32**	**5.67**	**22.85**	**142.24**	**141.17**	**64.43**
	Z × T			**11.40**	2.64	**7.87**	**67.48**	**7.42**	2.35

[A] One-way ANOVA between repeated batch fermentations of concentrated grape must at three levels of initial density. Different letters (a, b, c) indicate significant differences ($p < 0.05$).
[B] Two-way ANOVA between repeated batches of natural grape must using free or immobilized cells under different temperatures. Z, fermentation type (immobilized or free cells); T, temperature; Z × T, interaction. Significant *F*-ratios are in bold ($p < 0.05$).

produced with temperature decrease has been reported by other workers (Erten, 2002; Girard, Kopp, Reynolds, & Cliff, 1997; Jackson, 1994). Similar results have been also observed in fermentations using immobilized cells on various supports (Bakoyianis et al., 1993; Bardi et al., 1997; Kourkoutas et al., 2005; Tsakiris, Bekatorou, et al., 2004; Tsakiris et al., 2004). As regards the fermentations with concentrated grape must, increasing the content of initial sugars had a significantly positive effect on the amount of all volatiles produced.

3.3. Minor volatiles – qualitative composition

Detailed investigation of the minor volatile components of the wines produced by free and immobilized cells was effected by GC/MS. For identification purposes, the linear retention index based on the homologous series of C2–C18 ethyl esters (EERI) was used. This series of compounds was chosen, instead of the *n*-alkane series, for two reasons. First, wine contains itself, in most cases, all these esters. Thus, there was not necessary to spike the samples with a standard mixture of these components, introducing in this way, one more step and error to the whole procedure. Second, most of the *n*-alkanes, especially those of high molecular weight, cannot be extracted with the headspace SPME technique. For comparison with the literature data, the linear retention indices (LRI) based on the even *n*-alkanes series (C8–C22), were estimated by extrapolation using liquid injection of a standard mixture consisting of the *n*-alkanes series and reference compounds available in the lab. In most cases, the experimentally calculated indices were in good agreement with the literature values. A large number of volatile compounds were identified in all wines, most of them with high degree of certainty (Table 3). The majority of the components were ethyl esters of fatty acids and acetate esters of higher alcohols. They are usually found to most wines, have very low threshold values and are responsible for the fruity character of wine aroma. Higher alcohols and volatile fatty acids followed esters in abundance. Most of them are mainly produced by yeast metabolism and will be discussed in the next section. Traces of carbonyl compounds were also identified. Finally, a number of miscellaneous compounds, some groups of which are known to contribute to the complexity of wine aroma, such as terpenoids, acetals and norisoprenoids were also detected in wine samples.

The wines produced by either free or immobilized cells on brewer's spent grains contained nearly 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 previous studies (Kourkoutas et al., 2003; Mallouchos, Komaitis, et al., 2003; Mallouchos et al., 2003; Tsakiris, Bekatorou, et al., 2004; Tsakiris et al., 2004). However, the differences revealed by the sensory evaluation of the samples (results not shown) were attributed to quantitative differences of the volatile compounds caused by the different yeast metabolism due to the combined effect of immobilization and temperature. Thus, the next step was to proceed in the determination of the relative concentrations of the three most important groups of volatiles produced by yeast, e.g. esters, alcohols and acids.

3.4. Minor volatiles – quantitative composition

As shown in Table 4, the main ethyl esters produced by either free or immobilized cells, were those of hexanoic, octanoic, decanoic and dodecanoic acid. The main acetate esters were those of amyl alcohols and 2-phenylethanol. The interaction of temperature with the fermentation type (IC or FC), as well as the main effects on the total content of esters were highly significant (Table 5). In most cases, FC produced wines with a higher amount of esters at the temperatures of 25 and 20 °C. Temperature drop to 15 °C resulted in a significant decrease of the amount of esters produced by FC. Further decrease of temperature seems to have no significant effect on the total concentration of esters. The opposite was observed for IC. Temperature decrease to 15 °C resulted in the production of greater amounts of esters by IC (2.5 times higher than FC). Further decrease of temperature at 10 °C resulted to a small decrease of ethyl esters amount. Similar results have been reported in other studies (Mallouchos, Komaitis, et al., 2003; Mallouchos et al., 2003). To our knowledge, this is the first study reporting the big influence of immobilization and temperature that leads to an inverse formation pattern of esters. However, it must be noted that apart from the different immobilization support that was used here (spent grains), also a different yeast strain was implicated in fermentations. The influence of yeast strain on the volatile constituents of wines (Antonelli, Castellari, Zambonelli, & Carnacini, 1999; Etievant, 1991; Jackson, 1994; Mateo, Jiménez, Pastor, & Huerta, 2001; Nykanen & Suomalainen, 1983) and the different response of each strain (as regards volatiles) on temperature effect is well known (Erten, 2002; Torija et al., 2003). The differences observed between IC and FC could be attributed to a mass transfer phenomenon or to different enzymic activities. In addition, because oxygen represses the synthesis of esters (Boulton, Singleton, Bisson, & Kunkee, 1996), a favorable microenvironment for ester synthesis in the vicinity of the biocatalyst might have been created due to limited oxygen diffusion.

Fatty acids are the only acids that were considered for possible flavor impact in wines because they have low odor threshold values, rather high concentrations in wines and enough volatility at room temperature (Etievant, 1991). As shown in Table 4, the relative concentration of the volatile fatty acids produced by IC increased with temperature decrease. At low temperatures (15–10 °C), this amount was approximately the double from that produced at higher temperatures (25–20 °C). The opposite was observed in

Table 3
Volatile compounds identified in wines produced by free cells and immobilized cells on brewer's spent grains

Compound	EERI[a]	LRI[b]	Fit[c]	Compound	EERI	LRI	Fit
Acetaldehyde	89	905	A	3-Methylbutyl hexanoate	821	1462	C
Acetone	142	922	A	Heptanol	824	1465	B
Ethyl formate	148	925	C	2-Furaldehyde	826	1466	B
Ethyl acetate	201	945	A	Octyl acetate	839	1479	B
1,1-Diethoxy ethane	206	947	C	Ethyl 7-octenoate	849	1489	C
Methanol	219	952	A	Decanal	861	1500	A
2-Methyl butanal	230	956	C	Benzaldehyde	883	1522	A
3-Methyl butanal	236	958	B	Ethyl 3-hydroxybutanoate	887	1526	C
Ethyl propanoate	301	981	A	Ethyl nonanoate	901	1540	A
Ethyl 2-methylpropanoate	310	986	A	Propanoic acid	905	1544	A
2,3-Butanedione	319	991	B	2,3-Butanediol	911	1551	A
Decane	342	1003	A	α-Linalool	915	1555	A
2-Methylpropyl acetate	366	1015	A	Butyl caprylate	916	1556	C
Ethyl butanoate	400	1034	A	1-Octanol	925	1567	A
Toluene	401	1035	B	2-Methyl propanoic acid	932	1573	A
1-Propanol	407	1040	A	1,3-Butanediol	944	1587	C
1,1-Diethoxy-3-methyl-butane	432	1067	C	Butyrolactone	979	1627	C
Hexanal	436	1071	A	Butanoic acid	984	1633	A
1-(1-Ethoxyethoxy)-pentane	457	1092	C	Ethyl decanoate	995	1645	A
2-Methyl-1-propanol	462	1098	A	3-Methylbutyl octanoate	1012	1663	C
3-Methylbutyl acetate	473	1110	A	2-Furanmethanol	1019	1669	A
p-Xylene	479	1116	C	3-Methyl butanoic acid	1025	1676	A
(*R*)-(−)-2-pentanol (IS)	490	1128	A	2-Methyl butanoic acid	1026	1677	B
1-Butanol	515	1153	A	Diethyl butanedioate	1032	1683	A
2-Hexanol	536	1176	C	Ethyl 9-decenoate	1046	1697	B
Limonene	548	1188	A	α-Terpineol	1054	1705	A
3- and 2-Methyl-1-butanol	583	1218	A	3-(Methylthio)-propanol	1075	1725	A
Ethyl hexanoate	600	1234	A	Naphthalene	1087	1737	A
Styrene	620	1253	B	2-Undecenal	1105	1754	B
1-Pentanol	626	1259	A	Ethyl benzeneacetate	1139	1787	C
Hexyl acetate	638	1272	A	2-Phenylethyl acetate	1169	1818	A
3-Hydroxy-2-butanone	655	1289	B	β-Damascenone	1174	1825	A
1-Hydroxy-2-propanone	668	1303	B	Anethole	1177	1827	A
4-Penten-1-ol	674	1309	A	Ethyl dodecanoate	1200	1849	A
4-Methyl-1-pentanol	687	1322	A	Hexanoic acid	1202	1851	A
Ethyl heptanoate	697	1334	A	Geranyl acetone	1208	1859	B
3-Methyl-1-pentanol	698	1335	A	3-Methylbutyl decanoate	1216	1867	C
Ethyl 2-hexenoate	708	1344	C	2-Phenylethanol	1268	1919	A
2-Methylpropyl hexanoate	716	1352	C	Ethyl tetradecanoate	1400	2054	A
1-Hexanol	724	1361	A	Octanoic acid	1411	2067	A
Heptyl acetate	737	1375	B	1,3-Dihydroxy-2-propanone	1435	2091	C
2-Nonanone	751	1390	B	2-Phenylethyl hexanoate	1518	2174	C
Nonanal	754	1392	B	Decanoic acid	1617	2281	B
Butyl hexanoate	775	1415	C	Ethyl 9-Hexadecenoate	1623	2288	C
Hexyl 2-methylpropanoate	778	1418	C	Glycerin	1658	2326	C
Ethyl octanoate	800	1441	A	10-Undecenoic acid	1673	2342	B
cis-Linalool oxide B (*cis*-5 ring)	809	1448	B	Benzoic acid	1757	2434	C
Acetic acid	814	1455	A	Dodecanoic acid	1806	2488	B

[a] EERI: ethyl ester retention index.
[b] LRI: *n*-alkane linear retention index.
[c] A: MS data and retention index in agreement with those of authentic compound, B: MS data and retention index in agreement with those in literature, C: MS data in agreement with those in NIST and WILEY libraries.

the case of FC. Similar findings have been reported in a previous work with cells immobilized on grape skins (Mallouchos et al., 2003). ANOVA analysis on the sum of acids showed a highly significant interaction effect of fermentation type (IC-FC) and temperature, as in the case of esters (Table 5). The increase in the concentration of the medium chain length acids (MCFA) with the decrease of temperature has been also reported by Torija et al. (2003). They related the increased synthesis of MCFA with the ability of each yeast strain to modulate its membrane fluidity, under different growth temperatures.

Linear regression analysis of the total amount of fatty acids versus the total amount of ethyl esters gave a straight correlation between them, for both IC ($r = 0.997$, $p < 0.01$) and FC ($r = 0.994$, $p < 0.01$). This relationship can be concluded from Fig. 2, which shows the similarity between the

Table 4
Relative amounts (mg/l) of volatile compounds determined by GC/MS in wines produced by free cells (FC) and immobilized cells (IC) on spent grains at 25, 20, 15 and 10 °C

Compound	FC				IC			
	25	20	15	10	25	20	15	10
Ethyl esters								
Ethyl propanoate	0.083	0.263	0.332	0.346	0.055	0.091	0.155	0.186
Ethyl butanoate	0.289	0.320	0.347	0.443	0.187	0.210	0.381	0.428
Ethyl hexanoate	69.7	62.8	55.2	82.8	35.6	50.3	109	89.1
Ethyl heptanoate	0.070	0.071	0.044	0.120	0.089	0.098	0.308	0.226
Ethyl 2-hexenoate	0.036	0.022	0.021	0.022	0.024	0.022	0.038	0.036
Ethyl octanoate	453	287	191	211	194	170	546	436
Ethyl nonanoate	0.159	0.104	0.053	0.077	0.048	0.052	0.104	0.058
Ethyl decanoate	622	367	67.0	53.4	69.1	74.2	138	129
Diethyl butanedioate	0.033	0.028	0.022	0.019	0.015	0.018	0.021	0.010
Ethyl 9-decenoate	24.5	17.4	6.37	5.51	0.914	1.072	2.86	3.07
Ethyl dodecanoate	9.12	5.95	0.511	1.28	4.81	2.51	2.00	3.81
Ethyl tetradecanoate	0.062	0.025	0.004	0.013	0.018	0.023	0.021	0.076
Acetates								
2-Methylpropyl acetate	0.148	0.134	0.124	0.050	0.397	0.223	0.263	0.300
3- and 2-Methylbutyl acetate	40.7	32.7	16.0	7.64	23.5	25.9	36.0	38.4
Hexyl acetate	0.031	0.031	0.021	0.023	0.013	0.013	0.024	0.078
Heptyl acetate	0.076	0.094	0.048	0.108	0.069	0.068	0.335	0.324
2-Phenylethyl acetate	3.95	2.57	1.31	1.06	1.99	2.14	3.65	1.60
Miscellaneous esters								
3-Methylbutyl hexanoate	0.084	0.050	0.022	0.023	0.048	0.031	0.075	0.057
3-Methylbutyl octanoate	0.142	0.064	0.014	0.013	0.044	0.034	0.032	0.032
3-Methylbutyl decanoate	0.925	0.498	0.046	0.066	0.163	0.109	0.098	0.067
2-Phenylethyl hexanoate	0.179	0.070	0.018	0.048	0.072	0.045	0.088	0.083
Total ester content	**1225**	**777**	**339**	**364**	**332**	**327**	**841**	**703**
Alcohols								
Butanol	0.194	0.220	0.178	0.166	0.159	0.114	0.089	0.067
4-Pentenol	0.036	0.026	0.014	0.005	0.019	0.029	0.041	0.018
4-Methyl-1-pentanol	0.063	0.041	0.039	0.004	0.033	0.044	0.057	0.026
3-Methyl-1-pentanol	0.085	0.085	0.077	0.028	0.062	0.104	0.123	0.056
Hexanol	0.125	0.157	0.140	0.183	0.100	0.074	0.078	0.153
2,3-Butanediol	3.00	3.54	2.20	2.90	3.08	2.50	3.22	4.02
Octanol	0.240	0.222	0.143	0.097	0.045	0.034	0.040	0.033
1,3-Butanediol	0.857	0.857	0.546	0.783	0.895	0.703	0.883	1.156
3-(Methylthio)-1-propanol	0.220	0.148	0.059	0.039	0.151	0.204	0.250	0.084
2-Phenylethanol	70.3	60.8	27.5	24.5	42.1	47.6	52.4	16.3
Total alcohol content	**75.4**	**66.3**	**31.1**	**29.0**	**46.9**	**51.7**	**57.5**	**22.2**
Acids								
Acetic	3.85	1.16	1.12	1.34	1.15	1.14	2.08	1.88
Propanoic	0.032	0.019	0.013	0.013	0.010	0.011	0.015	0.011
2-Methyl propanoic	0.186	0.163	0.114	0.081	0.162	0.125	0.109	0.048
Butanoic	0.119	0.106	0.085	0.109	0.058	0.078	0.105	0.095
3-Methyl butanoic	0.163	0.114	0.080	0.049	0.062	0.087	0.104	0.054
2-Methyl butanoic	0.286	0.231	0.136	0.105	0.045	0.057	0.069	0.026
Hexanoic	0.212	0.191	0.111	0.164	0.065	0.099	0.222	0.201
Octanoic	6.63	4.84	1.93	2.88	2.32	2.29	5.33	4.94
Decanoic	6.26	4.78	0.785	1.67	0.546	0.662	1.38	1.16
Dodecanoic	0.110	0.058	0.009	0.023	0.010	0.011	0.017	0.026
Total acid content	**17.8**	**11.7**	**4.38**	**6.44**	**4.42**	**4.55**	**9.43**	**8.45**

formation patterns of acids and their respective ethyl esters at the temperature range investigated. These finding supports the well known mechanism of ethyl ester synthesis from their corresponding fatty acid proposed by Nordstrom (1964). Similar results have been reported by other authors in traditional wine fermentations (Antonelli et al., 1999; Etievant, 1991).

Except from the major alcohols already studied before, few others were also detected and determined in the headspace of the wines. However, the majority of them were present in very low levels. 2-Phenylethanol was the only exception (Table 4). The total alcohol content was significantly affected by the interaction of immobilization and temperature (Table 5). It decreased with temperature

Table 5
Results of the ANOVA analysis related to the significance of the parameters: fermentation type and temperature on the total content of esters, alcohols and acids produced during repeated batch fermentations of natural grape must

Effect	Df	Esters		Alcohols		Acids	
		MS	*F*	MS	*F*	MS	*F*
Z	1	94345.9	72.53[a]	208.483	3.23[b]	67.9161	9.08[a]
T	3	76648.3	58.93[a]	1619.42	25.09[a]	21.2397	2.84[b]
Z × T	3	654691.0	503.32[a]	817.684	12.67[a]	107.571	14.38[a]

Df: degree of freedom, MS: mean square, *F*: MS/error ratio, Z: fermentation type (immobilized or free cells), T: fermentation temperature.
[a] Significant for $p < 0.01$.
[b] Significant for $p < 0.1$.

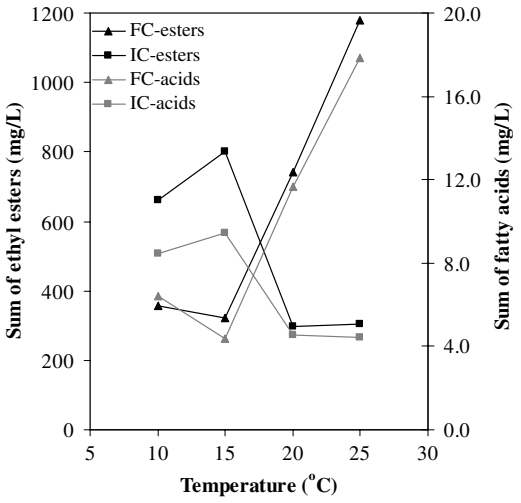

Fig. 2. Total relative amount of ethyl esters (black) and acids (grey) in wines produced by free cells (triangle) and immobilized cells (square) on brewer's spent grains at temperatures of 25, 20, 15 and 10 °C.

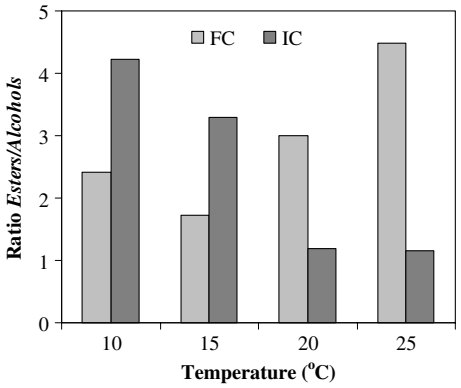

Fig. 3. Ratio of total esters (ethyl acetate excluded) to total alcohols (major alcohols included) in wines produced by free and immobilized cells on brewer's spent grains at various temperatures.

decrease in fermentations with FC, as expected (Etievant, 1991; Mallouchos, Komaitis, et al., 2003; Mallouchos et al., 2003), whereas for IC, the concentrations of higher alcohols increased from 25 to 15 °C and then decreased significantly at 10 °C. This increase is solely attributed to 2-phenylethanol and not to the rest of fusel alcohols (Etievant, 1991). Finally, the wines produced by IC were characterized by better ratios than those produced by FC, at low fermentation temperatures and had a potentially better fruity aroma (Fig. 3).

From the above results it can be concluded that the untreated brewer's spent grain is an interesting support for cell immobilization to be used in winemaking. Fermentation kinetics and operational stability were more than satisfactory. Furthermore, raw BSG is a cheap, abundant support of food grade purity and needs no more elaboration in order to be used as a yeast immobilization support, leading thus to improvement of the economics of the whole process. All these, in combination with the better aroma character of the produced wines due to the increased ratios of esters to alcohols, render raw BSG as a promising biocatalyst for batch winemaking at low fermentation temperatures.

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