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Production of white wine by *Saccharomyces cerevisiae* immobilized on grape pomace

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White wine was produced with *Saccharomyces cerevisiae* cells immobilized on grape pomace, by natural adsorption. The support, the main solid waste from the wine industry, consisted of the skins, seeds and stems. Immobilization was tested using different media, namely complex culture medium, raw grape must and diluted grape must. Grape pomace was revealed to be an appropriate support for yeast cell immobilization. Moreover, grape must was shown to be the most suitable medium as immobilized cells became adapted to the conditions in the subsequent alcoholic fermentation in the wine-making process. The wines produced, either with immobilized cells or with free cells, were subjected to chemical analysis by HPLC (ethanol, glycerol, sugars and organic acids) and by gas chromatography (major and minor volatile compounds); additionally, colour (CIELab) and sensory analysis were performed. The use of immobilized systems to conduct alcoholic fermentation in white wine production proved to be a more rapid and a more efficient process, especially when large amounts of SO_2 were present in the must. Furthermore, the final wines obtained with immobilized cells demonstrated improved sensory properties related to the larger amounts of ethanol and volatile compounds produced. The more intense colour of these wines could be a drawback, which could be hindered by the reutilization of the biocatalyst in successive fermentations. Copyright © 2012 The Institute of Brewing & Distilling

Keywords: grape pomace; immobilized yeasts; alcoholic fermentation; wine-making

Introduction

In recent years, cell immobilization systems have been explored for use in wine production, to conduct alcoholic fermentation *(1–3)*, as well as in malolactic fermentation *(4–6)*. Achievements in this area of research are very important for attempts to reduce operating costs, control the fermentation processes and increase the quality of the final product – the wine.

Cell immobilization systems utilized for alcoholic fermentations have technological and economic advantages when compared with free cell systems, such as increased productivity, higher cell concentrations in the reactors, possible reuse of the biomass in consecutive batch processes, greater tolerance of the cells to inhibitory substances and the possibility of operating the processes in a continuous mode *(7–10)*. The immobilization techniques can be divided into four categories: attachment to a support, entrapment in a porous matrix, cell aggregation and containment behind a barrier *(10–12)*.

The supports to be used in the alcoholic beverage industry should have high resistance and stability, should not damage the quality of the final product and have food-grade purity *(10,12)*. Some inorganic supports such as the mineral kissiris *(13)* and γ-alumina *(14)* have been used successfully for the immobilization of *Saccharomyces cerevisiae*. However, some of these supports may be undesirable, owing to the release of mineral residues into the final product *(14)*. Organic supports, mainly of natural origins, such as pieces of fruit, are a good alternative, where the cells adhere to the surface by natural adsorption. Apple *(15)*, quince *(16)*, pear *(17)*, watermelon *(18)*, grape skins *(19)* and dried raisin berries *(3)* have already been studied and have advantages on an industrial scale, as they are of food-grade purity and could reduce the cost of the process.

Grape pomace is the most plentiful solid waste of the wine industry. It results from the pressing of grapes and consists mostly of skins, seeds and stems. Traditionally, it is used to produce spirits or as fertilizer. It is also utilized to obtain value-added products *(20,21)*, such as enzymes *(22)*, extracts with antibacterial activity *(23)*, grape seed oil, anthocyanic dyes and tartaric acid *(24)*. As this is a by-product that is always extensively generated in wine production, it is important to find alternative uses.

From a consumer point of view, flavour is one of the most valuable attributes contributing to the overall quality of a wine. Aroma volatile compounds are the primary contributors to wine flavour, producing an effect on the sensory senses of the taster *(25)*. Colour is another parameter connected to the quality of the wine. It gives an idea of the evolution of the wine in time and of the existence of possible defects *(26)*. One valuable technique for distinguishing between wines is sensory evaluation. Sensory tests can discriminate between wines and estimate the quality of wine produced using different enological practices *(27)*.

The aim of the present study was to produce white wine, with *S. cerevisiae* immobilized on grape pomace by natural adsorption, and to compare this wine with wines produced using free cells. Sensory characteristics, colour and volatile aroma compounds were evaluated.

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Materials and methods

Inoculum preparation

A commercial *S. cerevisiae* strain (Lalvin QA23, Proenol) was used in the experiments. The inoculum was prepared by cultivation of the yeast in 500 mL Erlenmeyer flasks containing 200 mL of YPD medium with the following composition ($g\,L^{-1}$): yeast extract (10), peptone (20) and glucose (20). Cells were cultivated under static conditions, at 30 °C for 24 h, being subsequently recovered by centrifugation (7000 min^{-1}, 20 min), washed with distilled water and re-suspended in the fermentation medium to obtain an initial concentration of 1 $g\,L^{-1}$ (dry weight).

Support materials for cell immobilization

Grape pomace, constituted by stems, seeds and skins, picked randomly after crushing and pressing of indistinct white grapes, was used as support material for cell immobilization. This support material was supplied by a local wine-making company, being washed with distilled water and dried at 60 °C, until constant weight, before use.

Immobilization of cells

Saccharomyces cerevisiae cells (1 $g\,L^{-1}$; dry weight) and 2 g of dry grape pomace, previously sterilized at 121 °C for 20 min, were added to 200 mL of a complex culture medium composed of ($g\,L^{-1}$): glucose (120), yeast extract (4), $(NH_4)_2SO_4$ (1), KH_2PO_4 (1) and $MgSO_4$ (5). The mixture was left to ferment in 500 mL Erlenmeyer flasks under static conditions at 30 °C for 24 h (Fig. 1). To compare the effect of the medium composition on immobilization efficiency, the same procedure was performed in 200 mL of diluted grape must (~120 $g\,L^{-1}$ of total sugars, glucose and fructose).

The final immobilization experiments, carried out to produce white wine, were performed in 300 mL of diluted grape must (~120 $g\,L^{-1}$ of total sugars) and in 300 mL of raw grape must (~210 $g\,L^{-1}$ of total sugars) with 1 $g\,L^{-1}$ of *S. cerevisiae* cells (dry weight). In each broth, 50 g of dry sterilized grape pomace was added for cell immobilization at 25 °C for 78 h, with agitation (200 min^{-1}). The biocatalyst prepared in raw grape must was washed twice with grape must and reused for the subsequent batch fermentations.

Fermentation conditions

The alcoholic fermentations for the wine-making process were performed in two different series, each one including two consecutive batches with immobilized cells (batches 1 and 2) as depicted in Fig. 2. In the first series (series 1), 60 g of wet grape pomace with immobilized cells (corresponding to 0.75 g of dry weight of cells) was placed in 3 L of grape must, i.e. a cell concentration of 0.25 $g\,L^{-1}$. The density was monitored daily and the fermentation was stopped when it reached 0.995 $g\,mL^{-1}$. After that, the support was recovered and washed twice with grape must and reused for the second batch fermentation. Free cell fermentations, with the same cell concentration, were performed as controls. In the second fermentation series (series 2), the procedure was the same, but 400 g of wet grape pomace with immobilized cells was used (corresponding to 5.78 g dry weight of cells) in 2.75 L of grape must, i.e. a cell concentration of 2.10 $g\,L^{-1}$. All experiments were performed at room temperature (~22 °C), without agitation and in duplicate.

After the addition of sulphur dioxide (30 $mg\,L^{-1}$) and bentonite (600 $mg\,L^{-1}$), the produced wines were stabilized at 4 °C over 15 d. Then, they were filtered and the SO_2 concentration was again adjusted to 30 $mg\,L^{-1}$ before bottling. HPLC, gas-chromatography and colour, as well as immobilized cells concentration determinations were performed on the finished wines. Sensory analysis was performed only for wines from the second series.

HPLC analysis

Glucose, fructose, ethanol, glycerol and organic acids (citric, tartaric, malic, succinic lactic and acetic) concentrations were determined by high performance liquid chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index

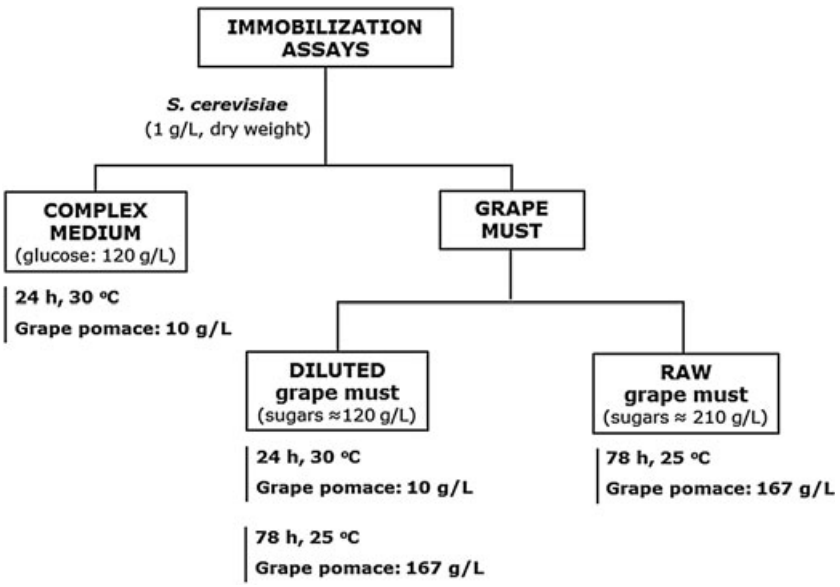

Figure 1. Flow chart for the immobilization assays.

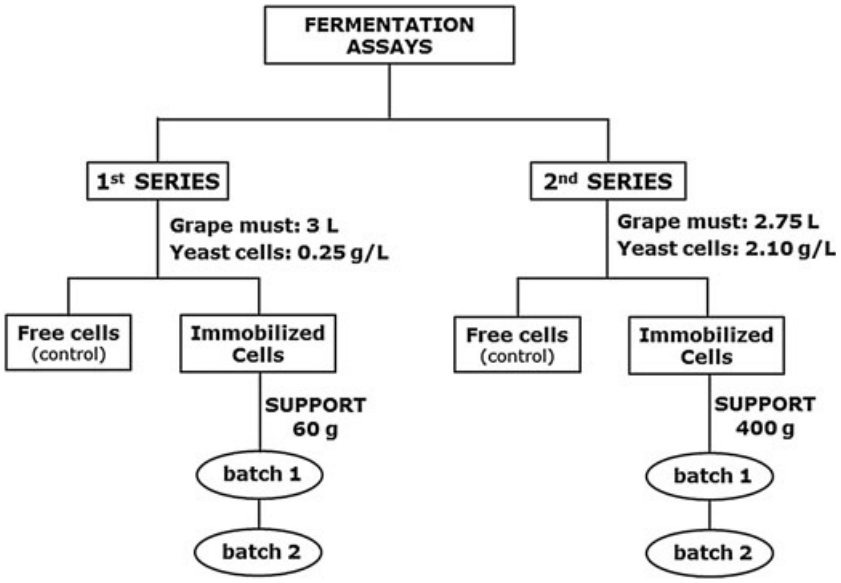

Figure 2. Flow chart for the fermentation assays.

detector (Jasco 830-RI), an ultraviolet detector and a Varian Metacarb 67 H column (300 × 6.5 mm) operated at 80 °C. A 5 mmol L^{-1} H_2SO_4 solution was used as eluent at a constant flow rate of 0.3 mL min^{-1}. Identification of metabolites was performed by comparing retention times with those of pure standard compounds and quantification was carried out after external standard calibration.

Gas-chromatographic analysis

Major volatile compounds were directly analyzed after adding 292.5 μg of 4-nonanol (internal standard, IS) to 5 mL of wine. Minor volatile compounds were analyzed after extraction of 8 mL of wine with 400 μL of dichloromethane, spiked with 2.91 μg of 4-nonanol (IS), according to the methodology proposed by Oliveira *et al*. *(28)*. All analyses of volatiles were carried out in triplicate.

A Chrompack CP-9000 gas chromatograph equipped with a split/splitless injector and a flame ionization detector (FID) with a capillary column, coated with CP-Wax 52 CB (50 m × 0.25 mm; 0.2 μm film thickness, Chrompack), was used. The temperatures of the injector and the detector were both set to 250 °C. The oven temperature was held at 60 °C, for 5 min, then programmed to rise from 60 to 220 °C, at 3 °C min^{-1}, and held at 220 °C for 10 min. The carrier gas was helium 55 (Praxair) at 120 kPa. Major volatile compounds were analyzed in split mode (13 mL min^{-1}) injecting 1 μL of sample, and the extracts containing minor volatile compounds were injected (3 μL) in splitless mode (for 15 s).

Identification of volatiles was performed with Varian MS Workstation software, version 6.6, by comparing retention indices with those of pure standard compounds. Minor volatile compounds were quantified as 4-nonanol equivalents only.

Colour analysis

The colour of the wines was assayed by the CIELab method, by measuring the absorbance between 380 nm and 770 nm (data pitch = 2 nm), using a Jasco UV–vis V-560 spectrophotometer. The recorded data were processed by an algorithm using the program Matlab version r2010a, developed by the Colour Laboratory, Department of Physics, University of Minho, to obtain the CIELab coordinates, L^*, a^* and b^*. These coordinates allowed the determination of three other parameters in the produced wines: saturation (C^*), variation in saturation (ΔC^*) and variation in lightness (ΔL^*), according to Almela *et al*. *(26)*. The following equations were used:

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$\Delta C^* = C_x^* - \bar{C}^* \quad (2)$$

$$\Delta L^* = L_x^* - \bar{L}^* \quad (3)$$

C_x^* and L_x^* are the saturation and lightness of the wines produced by immobilized cells, and $\bar{C}^*$ and $\bar{L}^*$ are the saturation and brightness, respectively, of the reference wines, i.e. wines produced with free cells.

Sensory analysis

The three wines produced in the second series of fermentations were subjected to sensory analysis, in dark glasses, using a triangular test *(29)*. Six sets of three glasses were prepared, of which two contained the same wine (Table 1). The glasses were identified on the basis of random numbers with three digits and contained 30 mL of wine. The tests were conducted using 35 panellists without significant experience, at the Laboratory of Food Science and Technology, Centre of Biological Engineering, University of Minho. The panellists were also asked to name a preference in each of the series of the three wines.

Immobilized cell determination

The immobilized cell concentration was determined at the fermentation's end by washing the biocatalyst with 30 g L^{-1} NaOH solution, for 24 h, at 30 °C and an agitation rate of

Table 1. Composition of each set of three glasses used in the sensory evaluation of the second series of fermentations

Set	Wines		
	Glass 1	Glass 2	Glass 3
1	Free cells	Free cells	Batch 1
2	Free cells	Free cells	Batch 2
3	Batch 1	Batch 1	Batch 2
4	Batch 1	Batch 1	Free cells
5	Batch 2	Batch 2	Free cells
6	Batch 2	Batch 2	Batch 1

120 min^{-1}, according to Genisheva *et al. (8)*. The free cell concentration in the fermentation medium was estimated by measuring the absorbance at 600 nm, which was correlated to a calibration curve (dry weight vs absorbance).

Statistical analysis

All of the fermentation experiments were conducted in duplicate. The results were analyzed by ANOVA, using FAUANL software *(30)*. Tukey's test was used to detect significant differences between samples.

Results and discussion

The ability of immobilized *S. cerevisiae* to ferment grape must was evaluated by measuring glucose and fructose consumption, ethanol, glycerol, major volatile and minor volatile compound production, sensory evaluation and chromatic characteristics.

Immobilization of *Saccharomyces cerevisiae*

The immobilization of the yeast cells was performed in three different immobilization media: complex culture medium, diluted grape must and raw grape must. Initially, a comparison was performed between fermentations with two different media: complex culture and diluted grape must. The quantities of the immobilized cells per mass of support, X_{im}, at the end of immobilization runs in complex medium and in diluted must were 14.90 mg g^{-1} and 16.10 mg g^{-1}, respectively; these results showed no significant differences ($p < 0.05$). Nevertheless the two assays showed a significant difference ($p < 0.05$) in terms of the free biomass produced. The free cell concentrations in the complex culture medium and diluted grape must were 6.35 g L^{-1} and 4.80 g L^{-1}, respectively. Therefore, the assays with the complex culture medium had a higher total concentration of cells than the assays with the diluted must, but showed a lower immobilization efficiency (data not showed). The composition of the diluted must may have favoured the stability of cells on the support, and even influenced the yeast's own metabolism, since it was rich in sugars, acids, amino acids, minerals and pectic substances, amongst others, some of which were absent in the complex medium. During the immobilization process in the complex medium, 127 g L^{-1} of initial glucose was almost completely consumed after 16 h (residual glucose was 5.5 g L^{-1}). In relation to immobilization in diluted must, the total initial concentration of sugars was 132 g L^{-1} (53 g L^{-1} glucose and 79 g L^{-1} of fructose) and after 16 h there were still 52.7 g L^{-1} of sugars remaining (15.3 g L^{-1} glucose and 37.4 g L^{-1} of fructose). This suggests that the yeast took longer to adapt to the environment and, therefore, to take up these sugars.

According to the previous results and with the purpose of producing larger amounts of immobilized support for further use in fermentations, immobilization runs were carried out using 50 g of support material and diluted or raw grape must (Fig. 1). As the initial total sugar concentration of the raw must was 210 g L^{-1}, the immobilization was conducted for a longer period (78 h). The amount of immobilized cells was measured throughout the process, as shown in Fig. 3. The concentration of immobilized yeast cells in the two musts varied greatly over the 78 h and was higher in raw grape must. The highest amount detected of immobilized cells per mass of support of 40 mg g^{-1} was after 46 h. The immobilizations with a large amount of support were carried out under agitation, unlike previous tests, to ensure that the support was always immersed in the medium, thus allowing maximum contact between the immobilized cells and the medium constituents. The media agitation during the immobilization process and the absence of barriers between cells and the medium possibly favoured the constant desorption and replacement of microorganisms in the media. The agitation may have had a negative effect on the stability of the biofilm *(8)*. However, it was necessary in order to facilitate contact between the cells and the support and to promote a more homogeneous distribution of the constituents of the must. Note that the tests were performed with only enough juice to involve the support material, in order to reduce the amount of spent must. The raw grape must appeared to be the best option for immobilizing the yeast cells, since it allowed for the immobilization of more cells, as well as prior adaptation of their metabolism to the fermentation medium.

Fermentation trials with immobilized cells

Saccharomyces cerevisiae cells, previously immobilized on grape pomace, were used for the fermentation of grape must. For comparison, fermentations under the same conditions but

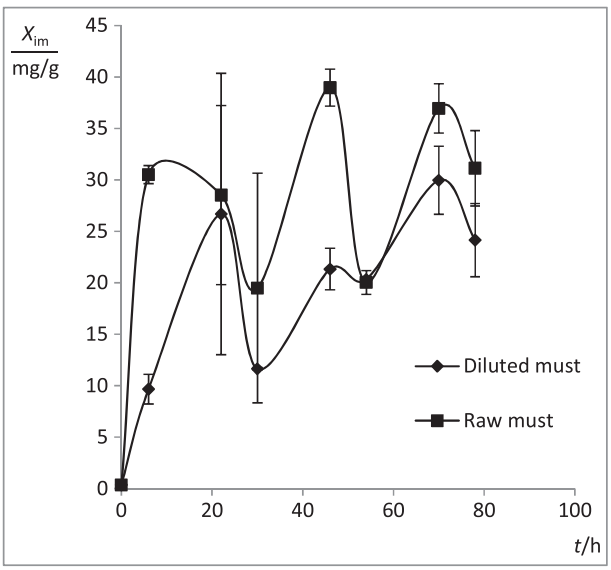

Figure 3. Variation of the mass of the immobilized cells per gram of support (X_{im}) over time (t) in raw and diluted grape must.

without the addition of the support were performed (Fig. 2). Two series of fermentation were conducted, each with two repeated batch runs (1 and 2). Batch 1 was carried out with the previously immobilized grape pomace, which was separated from the liquid at the end of the alcoholic fermentation, washed with grape must and reused in the batch 2 run. The two series were performed with different quantities of biocatalyst, 60 g and 400 g (wet weight), respectively.

The provided must had a high concentration of free SO_2 (54.4 mg L^{-1}). Therefore, the first series of fermentations were conducted under an inadequate environment for yeast development, causing a delay at the beginning of the process. The experiments with the free cells did not start until almost half of the SO_2 was removed and there was a supplementary addition of 0.5 g L^{-1} of the yeast cell suspension. However, the high concentration of free SO_2 did not appear to exert a negative influence over the fermentation assays with the immobilized yeast cells. This suggested that the immobilized yeast cells were more tolerant to the large quantities of free SO_2. This is important since SO_2 is one of the main inhibitors of yeast cell growth, thus helping in the conservation of the must for longer time periods *(31)*. Wine producers usually encounter problems when trying to ferment grape musts with high concentrations of SO_2. The use of immobilized cells may be a solution to this problem.

Free cell fermentations of the first series were complete on day 22, while fermentations with the immobilized cells, batches 1 and 2, were complete on days 14 and 11, respectively. Regarding the second series, fermentation runs with free cells were more rapid (4 d) than the two consecutive batches with immobilized cells (6 d and 7 d, respectively). This was probably due to substrate diffusion problems in the fermentation flask, which was full of the immobilized support. As a result of these diffusion problems, series 2 presented lower amounts of immobilized cells for batches 1 and 2. The immobilized biomass was 70.45 mg g^{-1} and 62.61 mg g^{-1}, respectively; this is in contrast to the first series, where the concentrations for batches 1 and batch 2 were 106.05 mg g^{-1} and 111.92 mg g^{-1}, respectively.

Since some cells would always be floating off the immobilization media, the fermentations were mostly likely a mix of both free and immobilized cells. Moreover, the support material used for immobilization – grape pomace – is not inert, and may affect the success and stability of the fermentation trials, as yeasts would metabolize some constituents and, concomitantly, colour compounds would be released into the wine. Nevertheless, the treatment carried out before immobilization and also the immobilization procedure itself certainly would soften these effects.

HPLC analysis

The concentrations of glucose, fructose, glycerol, ethanol and organic acids (citric, tartaric, malic, succinic, lactic and acetic) determined by HPLC can be seen in Table 2. The residual sugars concentration was low in all of the wines, varying between 0.10 g L^{-1} and 0.54 g L^{-1} for glucose and between 1.08 g L^{-1} and 8.76 g L^{-1} for fructose. In the fermentations with free cells, the glycerol concentration was higher than in fermentations with immobilized cells. Nevertheless the levels were in the usual range, i.e. 5 g L^{-1} to 15 g L^{-1} *(31)*. With regard to ethanol, only the fermentations with immobilized cells in batch 2 of the second series showed higher concentrations compared with the fermentations with free cells. Since immobilized cells are more tolerant to inhibitors *(32)*, they could maintain their fermentation activity even when the alcohol content was high. Ethanol affects the metabolic activity of yeasts, influencing the type and amount of volatile compounds produced, and also acts as a substrate for the formation of several ethyl esters *(33)*.

In all of the wines produced, the tartaric, malic and succinic acid concentrations were the highest of the six acids analyzed. Malic and tartaric acids are normally found in large amounts in grapes and musts, and do not undergo large changes during fermentation, while succinic acid is a by-product of the metabolism of yeasts *(31)*, which may explain the recorded values (between 2.22 g L^{-1} and 3.19 g L^{-1}). Citric acid is usually present at very low concentrations in wines *(34)* and this was

Table 2. Mean concentrations (*C*) of sugars, organic acids, ethanol and glycerol analyzed by HPLC at the end of the alcoholic fermentation

Compound	*C*/(g L^{-1})					
	First series*			Second series**		
	Free	Batch 1	Batch 2	Free	Batch 1	Batch 2
Glucose	0.26^{bc}	0.38^{ab}	0.41^{ab}	0.10^{c}	0.30^{b}	0.54^{a}
Fructose	5.37^{b}	4.96^{b}	5.45^{b}	1.08^{d}	2.49^{c}	8.76^{a}
Glycerol	5.73^{bc}	4.47^{c}	4.59^{c}	7.20^{a}	4.69^{c}	6.61^{ab}
Ethanol	77.06^{ab}	79.21^{ab}	80.63^{ab}	64.13^{b}	70.83^{b}	95.42^{a}
Citric acid	0.25^{a}	0.43^{a}	0.41^{a}	0.36^{a}	0.54^{a}	0.42^{a}
Tartaric acid	2.74^{bc}	3.20^{abc}	3.56^{ab}	2.32^{c}	2.37^{c}	3.93^{a}
Malic acid	4.58^{a}	4.47^{a}	4.79^{a}	2.95^{a}	3.68^{a}	4.38^{a}
Succinic acid	2.33^{a}	2.22^{a}	2.26^{a}	2.51^{a}	2.63^{a}	3.19^{a}
Lactic acid	2.07^{c}	1.84^{c}	1.97^{c}	3.03^{b}	2.81^{b}	3.69^{a}
Acetic acid	0.51^{ab}	0.29^{ab}	0.33^{ab}	0.58^{a}	0.16^{b}	0.49^{ab}

[a, b, c, d] Values with the same letters show no significant difference at the 95 % confidence level between fermentation assays.
* Cell concentration of 0.25 g L^{-1};
** Cell concentration of 2.10 g L^{-1}.

also true in the present study. The lactic acid concentration was similar in all of the fermentations. Acetic acid was found in higher concentrations in the free cell fermentations than in the fermentations with the immobilized cells. Nevertheless the values were always below the legal limit for white wines of 1.08 g L^{-1} *(35)*.

Major volatile compounds

The concentrations attained for the major volatile compounds, identified and quantified by GC-FID, are shown in Table 3. This group includes acetaldehyde, ethyl acetate, methanol and the higher alcohols (1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol).

Acetaldehyde reached concentrations up to 8.6 mg L^{-1}, values lower than its orthonasal perception threshold of 10 mg L^{-1} *(36)*. Although fermentations conducted by free cells for both series presented similar results, fermentations with immobilized cells in the first series (batches 1 and 2) produced larger amounts of acetaldehyde; for the second series, immobilized and free systems presented similar results. Usually, acetaldehyde is present in concentrations below 75 mg L^{-1} in young wines *(37)*. Kourkoutas *et al*. *(16,38)*, however, found amounts of 13 mg L^{-1} to 106 mg L^{-1} in wines produced with immobilized cells on quince and apple. This compound can confer fresh, green and even oxidized notes to wines *(37,39)*.

Ethyl acetate has a perception threshold of 7.5 mg L^{-1} *(36,40)*, contributing to the 'fruity' and 'solvent' character of wines *(41)*. It was found in higher concentrations in fermentations with immobilized cells. Moreover batch 2 presented larger amounts of ethyl acetate than batch 1, a fact observed for both series of fermentations. Batch 2 of series 2 achieved the highest levels for this compound. In all of the fermentations, this compound was found in concentrations higher than its perception threshold.

In regard to methanol, all fermentations presented lower levels (17.0 mg L^{-1} to 33.4 mg L^{-1}) than what has been published for Turkish white wines, 30.5 mg L^{-1} to 121.4 mg L^{-1} *(42)*. Wines produced with cells immobilized on quince fruit have a reported methanol content of under 100 mg L^{-1} *(16)*. The low concentration of methanol found in the wines produced in these experiments is a positive finding. Methanol concentration is under regulatory control owing to its toxic nature and the permitted limit, according to OIV *(35)*, is 250 mg L^{-1}. Methanol results from the pectin of the skin of the grapes, which undergoes an enzymatic conversion *(31)*. Since the fermenting must was in contact with the grape skins for a long time period, this could lead to elevated amounts of methanol in the product. However, this study has shown only small amounts of methanol to be present, even when a larger amount of grape pomace was used as the support in the immobilized system (series 2).

From the identified higher alcohols, 3-methyl-1-butanol showed the highest concentration (between 113.9 mg L^{-1} and 193.6 mg L^{-1}), and was above its perception threshold of 30 mg L^{-1} *(40)*. This alcohol may contribute to the 'sweet' and 'fusel' odour descriptors of wines *(43)*. Although higher alcohols, individually, do not give pleasant notes to the wine (except 2-phenylethanol), together they can contribute positively to the overall aroma. Some authors have stated that 300 mg L^{-1} is the limit for a positive contribution *(44)*. Higher concentrations can bring strong and pungent notes to the wine *(45)*. Nevertheless, the particular impact of each volatile component, or group of components, to the overall aroma of wine depends on its composition and on the concentration and the perception thresholds *(27)*. Only batch 1 of the second fermentation series presented more than 300 mg L^{-1} for the sum of the higher alcohols. Comparable results were observed by Kourkoutas *et al*. *(38)* when batch fermentations were conducted at low temperatures with immobilized *S. cerevisiae*. An interesting higher alcohol was 2-phenylethanol, which presented concentrations between 19.5 mg L^{-1} and 34.9 mg L^{-1}, always above the perception threshold of 10 mg L^{-1}, thus giving 'rose' and 'sweetish' nuances to the wine *(40,46)*. Oliveira *et al*. *(47)* reported similar concentrations for this compound in *Loureiro* (31.3 mg L^{-1}) and *Alvarinho* (21 mg L^{-1}) wines. The levels of 2-phenylethanol in wine are mainly related to the amino acids metabolism of the yeast during the fermentation *(48)*.

Table 3. Mean concentrations (*C*) and confidence limits ($p = 0.05$), of the major volatile compounds at the end of alcoholic fermentation

Compound	First series *						Second series **					
	Free cells		Batch 1		Batch 2		Free cells		Batch 1		Batch 2	
	C/(mg L^{-1})	±	*C* /(mg L^{-1})	±	*C* /(mg L^{-1})	±	*C*/(mg L^{-1})	±	*C*/(mg L^{-1})	±	*C*/(mg L^{-1})	±
Acetaldehyde	$2.6^{b,c}$	1.7	8.6^{a}	5.6	5.1^{b}	3.3	$2.6^{b,c}$	2.2	1.4^{c}	0.4	$2.4^{b,c}$	2.1
Ethyl acetate	$28.3^{c,d}$	0.9	30.2^{c}	3.0	37.7^{b}	2.5	23.2^{d}	5.8	$27.9^{c,d}$	3.1	50.4^{a}	8.3
Methanol	18.1^{b}	6.5	20.7^{b}	6.6	16.9^{b}	1.7	17.0^{b}	4.8	32.8^{a}	6.5	33.4^{a}	4.2
1-Propanol	16.2^{c}	5.0	$18.0^{b,c}$	1.4	$20.2^{b,c}$	4.2	16.1^{c}	1.1	21.9^{b}	0.9	41.5^{a}	6.9
2-Methyl-1-propanol	21.2^{d}	4.4	36.1^{b}	5.8	37.9^{b}	4.1	$25.8^{c,d}$	3.2	29.9^{c}	1.6	44.5^{a}	7.0
2-Methyl-1-butanol	31.1^{a}	5.7	$26.8^{a,b}$	4.0	$21.8^{b,c}$	3.7	$26.6^{a,b}$	2.8	32.8^{a}	2.2	18.4^{c}	12.8
3-Methyl-1-butanol	162.3^{b}	16.9	163.4^{b}	18.6	$133.0^{c,d}$	10.0	113.9^{d}	8.6	193.6^{a}	7.2	$144.5^{b,c}$	44.6
2-Phenylethanol	34.9^{a}	13.7	$26.3^{a,b,c}$	4.7	19.5^{c}	4.1	$23.1^{b,c}$	7.1	$33.1^{a,b}$	15.4	$25.5^{a,b,c}$	2.3

$^{a, b, c, d}$ Values with the same letters show no significant difference at the 95 % confidence level between fermentation assays.
* Cell concentration of 0.25 g L^{-1};
** Cell concentration of 2.10 g L^{-1}.

Minor volatile compounds

In total, 14 minor volatile compounds were identified and quantified, including seven esters, three alcohols, three volatile fatty acids and one C_{13}-norisoprenoid. The respective concentrations and level of significance ($p < 0.05$) are shown in Table 4. In general, fermentations with immobilized cells had larger amounts of the minor volatile compounds, with the resulting wines having a more rich and pleasant aroma profile. Moreover the concentration increased from batch 1 to batch 2 (Table 4).

The statistical analysis regarding concentrations of the minor volatile compounds showed no difference between the fermentations with reference to the five following compounds: ethyl lactate, hexan-1-ol, *E*-3-hexen-1-ol, *Z*-3-hexen-1-ol and β-damascenone. However, all the concentrations found for β-damascenone were above the perception threshold of 0.05 $\mu g\ L^{-1}$ *(40)*, thus bringing sweet, apple and dry plum nuances to the wines *(46,49)*. For the other nine analyzed compounds, significant differences were found ($p < 0.05$). The free cell fermentations were found to differ from the immobilized cell fermentations regarding the following aromatic compounds: ethyl butyrate, isoamyl acetate, ethyl hexanoate, hexyl acetate, ethyl octanoate, 2-phenylethyl acetate, hexanoic acid, octanoic acid and decanoic acid. Ethyl butyrate and octanoic acid, bring fruity and fatty characteristic *(46)*, respectively, and were present in all of the wines in concentrations above their perception thresholds of 20 $\mu g\ L^{-1}$and 500 $\mu g\ L^{-1}$ respectively *(36,50)*. For isoamyl acetate (3-methylbutyl acetate), no differences were observed for the free cell fermentations (both series), but in contrast to the previous observations the assays with immobilized cells were different between each other. Isoamyl acetate was found in concentrations higher than its perception threshold, 30 $\mu g\ L^{-1}$ *(36,40)*, for all fermentations assays, bringing banana descriptors to the overall aroma of wine *(46)*. Similarly, concentrations of ethyl octanoate with free cells (both series) were different from each other and from the immobilized cells. However the assays with immobilized cells were equal to each other. All the wines produced had concentrations of ethyl octanoate above the perception threshold of 5 $\mu g\ L^{-1}$ *(50)*, bringing fruity and fresh notes to wines *(46)*. The concentrations of ethyl hexanoate (fruity and flowery notes according to Escudero *et al.* *(46)* and López *et al.* *(49)*) were found to be different for all the fermentations ($p < 0.05$); moreover, with immobilized cells, the obtained concentrations were above the perception threshold of 250 $\mu g\ L^{-1}$ *(36)*. The concentrations for 2-phenylethyl acetate in batch 2 from both series were similar (batch 2, series 1) or even above (batch 2, series 2) the perception threshold of 250 $\mu g\ L^{-1}$ *(36,40)* thus contributing flowery notes *(49)*. Decanoic acid (fatty), in wine produced in batch 2 from series 1, was found in concentration above the perception threshold of 1000 $\mu g\ L^{-1}$ *(50)*.

Colour analysis

The colour of the wine is another important characteristic from the consumer's point of view. For this reason, colour analysis of the wines was carried out using the CIELab method, with the determined coordinates L^*, a^* and b^*. Furthermore, in order to compare the wines, variation in saturation, ΔL^*, and variation in lightness, ΔC^*, were also calculated (Table 5).

The results obtained for the coordinates L^*, a^* and b^* showed significant differences between wines in terms of the colour parameters (Tukey's test). Exceptions were the fermentations with the free cells and batch 2 from series 1 (Table 5). These two wines were alike in terms of the colour parameters. Since it was found that the wines were indeed different in respect to the parameters L^*, a^* and b^*, the average values for each parameter were compared. Wines produced with a greater amount of support (batches 1 and 2, series 2) presented the lower values of L^* (lower brightness and higher opacity), which suggests that these wines have a higher colour intensity. These results suggest that increasing the amount of support used in the fermentation process directly influenced the intensity of the colour of the wines. The parameter C^* was higher for the wines in batch 1 and batch 2 (series 2), indicating a higher colour vividness. All the wines showed the coordinate values a^* below zero and the coordinate b^* greater than zero, indicating a shift towards the green and yellow colour, respectively.

The wines from batch 1 with immobilized cells from both series had a higher colour intensity (lower values of L^*) as well as increased colour saturation (higher values of C^*), compared with the wines produced with immobilized cells in batch 2. Figure 4 shows the differences in colour of the produced wines, using a graphical representation of ΔL^* as function of ΔC^*, which reduces the CIELab coordinates into a two-dimensional colour space *(26)*. Thus, the deviations in the colour of the wines produced by the immobilized cells, compared with those produced with free cells, could be observed. It was found that the wines produced in the first fermentation (batch 1) with immobilized cells had a darker colour than those produced in the second fermentation (batch 2), due to the lower values of ΔL^*. This fact could be attributed to the release of some coloured compounds from the grape pomace; however, as the number of batches increased, the colour tended to stabilize.

Sensory analysis

A triangular test was used to evaluate possible differences between the two products, based on the analysis of three samples, in which the taster had to decide which one of the three samples was different. In the present study, sensory analysis was performed by an olfactory triangular test, by comparing the three wines from the second series of fermentations. Table 1 shows the six sets of glasses used in the evaluation, where each wine appeared twice in the sets. Since there were two sets to compare the same wines, the responses for homologous sets were grouped, and the number of correct responses for each wine was two per taster. In this case, the total number of correct responses for each wine was 70 (35 tasters 2 responses). According to ASTM E1885–04 norm *(29)*, the differences were considered statistically significant ($p < 0.05$) only when the number of correct responses was higher than 31. As can be seen in Table 6, all of the wines showed significant differences.

During the sensory evaluation of wines, the panellists were also questioned as to their preference in each set of three glasses. In the six sets, each wine appeared four times; the maximum number of preferences was 140 (35 tasters $\times$ 4 possible responses). Wine produced in batch 2 recorded the greatest number of preferences, accounting for 83 votes. The wine made with free cells was the second preferred wine (70 votes), followed by the wine produced in batch 1 (60 votes). It should be noted that the preferred wine was the one with the higher concentration of the minor volatile compounds, indicating their contribution to olfactory quality.

Table 4. Mean concentrations (C) and confidence limits ($p = 0.05$) of the minor volatile compounds at the end of alcoholic fermentation

Compound	First series *						Second series **					
	Free cells		Batch 1		Batch 2		Free cells		Batch 1		Batch 2	
	$C/(\mu g\,L^{-1})$	±	$C/(\mu g\,L^{-1})$	±	$C/(\mu g\,L^{-1})$	±	$C/(\mu g\,L^{-1})$	±	$C/(\mu g\,L^{-1})$	±	$C/(\mu g\,L^{-1})$	±
Ethyl butyrate	35.8^{a}	54.8	91.5^{b}	35.7	119.4^{b}	92.0	32.8^{a}	46.7	129.4^{b}	10.8	120.9^{b}	9.5
Isoamyl acetate	140.1^{d}	199.3	621.0^{c}	424.3	1032.4^{b}	523.8	281.0^{d}	27.3	1035.2^{b}	78.2	1596.3^{a}	162.6
Ethyl hexanoate	85.2^{e}	98.5	290.2^{c}	99.2	327.9^{b,c}	23.0	166.6^{d}	19.6	548.8^{a}	9.8	360.0^{b}	17.4
Hexyl acetate	1.7^{c}	3.8	25.4^{a,b}	42.2	33.2^{a}	4.8	6.1^{b,c}	0.4	27.9^{a}	1.8	39.0^{a}	4.7
Ethyl lactate	336.7^{a}	473.0	343.4^{a}	345.6	224.9^{a}	340.6	150.2^{a}	33.5	131.6^{a}	33.0	279.2^{a}	42.1
Hexan-1-ol	314.8^{a}	388.6	240.0^{a}	160.9	212.6^{a}	189.3	189.3^{a}	24.9	182.3^{a}	23.6	246.3^{a}	35.0
E-3-Hexen-1-ol	5.1^{a}	7.2	10.1^{a}	10.0	9.9^{a}	9.8	7.4^{a}	6.4	6.8^{a}	0.9	10.6^{a}	0.9
Z-3-Hexen-1-ol	7.0^{a}	5.5	8.2^{a}	4.7	10.0^{a}	8.2	8.0^{a}	1.4	7.6^{a}	5.6	7.2^{a}	4.1
Ethyl octanoate	15.5^{c}	11.6	168.8^{a}	27.0	207.2^{a}	81.1	79.1^{b}	7.2	190.2^{a}	6.5	191.8^{a}	18.7
2-Phenylethyl acetate	166.5^{c}	93.6	173.2^{c}	80.0	247.6^{b}	79.4	80.3^{d}	5.4	209.0^{b,c}	11.0	434.6^{a}	3.4
β-Damascenone	1.7^{a}	7.3	1.6^{a}	3.7	4.2^{a}	3.8	3.3^{a}	0.3	2.8^{a}	5.9	1.4^{a}	0.7
Hexanoic acid	871.2^{a,b}	768.0	944.5^{a,b}	585.9	727.6^{a,b}	389.6	563.6^{b}	46.3	1079.9^{a}	216.7	722.4^{a,b}	70.2
Octanoic acid	1658.4^{d}	877.5	4104.9^{a}	1021.4	3938.5^{a}	1323.5	2367.3^{c,d}	178.8	3247.3^{a,b}	150.3	2845.6^{b,c}	80.0
Decanoic acid	173.3^{b}	175.7	793.3^{a,b}	490.3	1184.8^{a}	834.6	360.9^{a}	40.9	364.8^{b}	24.1	528.8^{b}	48.1
Total	3530.0		7816.1		8280.2		4295.9		7163.6		7384.1	

$^{a, b, c, d}$ Values with the same letters show no significant difference at the 95 % confidence level between fermentation assays.

* Cell concentration of 0.25 g L^{-1};

** Cell concentration of 2.10 g L^{-1}.

Table 5. CIELab coordinates and the calculated values for C^*, ΔL^* and ΔC^*

Parameter	First series						Second series					
	Free cells		Batch 1		Batch 2		Free cells		Batch 1		Batch 2	
		±		±		±		±		±		±
L^*	$95.92^{a,b}$	0.03	$95.64^{b,c}$	0.24	$95.96^{a,b}$	0.34	96.18^{a}	0.21	94.94^{d}	0.33	95.43^{c}	0.73
a^*	-0.43^{d}	0.01	-0.62^{c}	0.02	-0.40^{d}	0.01	-0.45^{d}	0.04	-1.48^{a}	0.02	-1.06^{b}	0.14
b^*	$0.03^{d,e}$	0.07	0.11^{c}	0.28	0.12^{d}	0.30	0.07^{e}	0.18	0.06^{b}	0.15	0.27^{a}	0.66
C^*	2.03	0.06	2.84	0.28	2.06	0.29	1.69	0.17	6.63	0.14	4.99	0.62
ΔL^*	0.00		−0.28		0.03		0.00		−1.24		−0.75	
ΔC^*	0.00		0.81		0.03		0.00		4.94		3.29	

[a, b, c, d] Values with the same letters show no significant difference at the 95% confidence level.
L^*, a^*, b^* CIELab coordinates; C^*, saturation of colour; ΔC^*, variation of saturation; ΔL^*, variation of lightness.

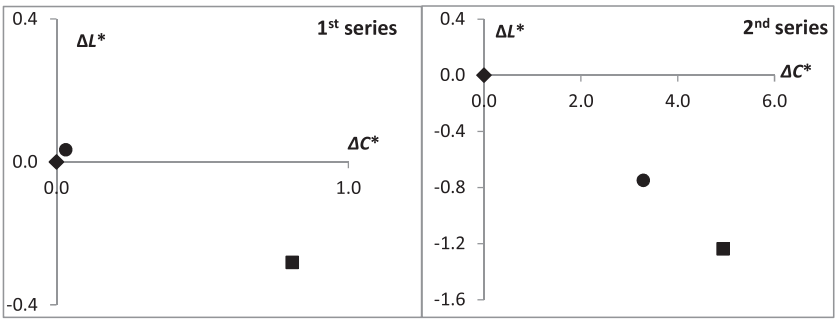

Figure 4. Variation of saturation ΔC^*, and variation of lightness, ΔL^*, of wines produced in both fermentation series (♦ free cells, ■ batch 1, ● batch 2).

Table 6. Number of correct answers, from a total of 70, recorded during the sensory test comparisons of the second series of fermentations

Combinations	Correct answers
Free cells and batch 1	48
Free cells and batch 2	38
Batch 1 and batch 2	43

Conclusions

Grape pomace was shown to be a suitable support for yeast immobilization and can be used for alcoholic fermentation in wine production. The duration of the fermentations was influenced mainly by the amount of the immobilization support used in each assay and also by the concentration of the SO_2 initially present in the must. However, the fermentation with the immobilized cells proved to be more rapid and efficient than the fermentation with the free cells, especially in musts with high concentrations of SO_2. Moreover it was possible to identify significant differences between the analyzed wines with respect to the volatile aroma compounds.

The wines obtained with the immobilized cells showed, generally, higher concentrations of ethanol, major volatile compounds and minor volatile compounds and a higher colour intensity compared with the wines produced with the free cells. However, since the intensity of the colour decreased with the increasing number of batches, there tended to be a stabilization. The sensory test suggested that the technique of cell immobilization on grape pomace applied to wine production could influence the quality of the final product. Also, the composition of grape pomace, used as immobilization support, is one of the factors that could influence the overall process.

The sensory analysis showed noticeable olfactory differences between the wines. Those produced by immobilized cells were not compromised, since the preference of the panellists was towards the wine produced with the immobilized cells.

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