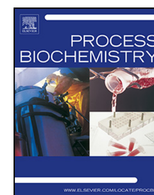
Process Biochemistry 49 (2014) 1–9

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Integrated continuous winemaking process involving sequential alcoholic and malolactic fermentations with immobilized cells

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ARTICLE INFO

Article history:
Received 17 July 2013
Received in revised form
25 September 2013
Accepted 12 October 2013
Available online 22 October 2013

Keywords:
Continuous winemaking
Immobilized cells
Aroma compounds
Packed bed reactor

ABSTRACT

An integrated winemaking process – including sequential alcoholic and malolactic fermentations operated continuously – was developed. For the continuous alcoholic fermentation, yeast cells (*Saccharomyces cerevisiae*) were immobilized either on grape stems or on grape skins, while bacterial cells (*Oenococcus oeni*) used for conducting continuous malolactic fermentation were immobilized on grape skins only. The produced wines were subjected to chemical analysis by HPLC (ethanol, glycerol, sugars and organic acids) and by gas chromatography (major and minor volatile compounds). The final proposed integrated continuous process permitted the production of 960 mL/d of a dry white wine, with an alcoholic strength of about 13 vol%, by using two 1.5 L tower bed reactors packed with 260 g of grape skins. The produced wines revealed a good physicochemical quality. Moreover, 67% of the malic acid concentration could be reduced in the second reactor. Both fermentative processes proved to be much more efficient than those conducted traditionally with free cells or even with immobilized cells, but in the batch mode of operation.

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1. Introduction

The two most important processes in wine production are alcoholic fermentation (AF), conducted by yeasts, and malolactic fermentation (MLF), conducted by bacteria. During the alcoholic fermentation the sugars of grape must are transformed mainly to ethanol and carbon dioxide; additionally, a myriad of by-products are formed. Malolactic fermentation is a secondary fermentation that reduces the acidity and brings biological stability to the wines; moreover, it improves the organoleptic characteristics of the product [1].

In traditional winemaking the fermentation processes are conducted in discontinuous mode, i.e. in batch. The seasonality of the raw-material, the grapes, defines largely the organization of this sector of activity and also the structure of the wine cellars. Nevertheless, continuous processes are known to be advantageous over batch processes. The continuous process is simpler to operate with low energy requirements, allowing almost complete utilization of the substrates and lowering the operating costs. Moreover, capital costs are reduced, with the possibility to obtain higher rates of production by using small bioreactors in the process. Superior productivities may be achieved by employing high concentrations of yeast or bacterial cells within the bioreactor. However, a conventional continuous process has limitations in the maintenance of high cell concentrations in the bioreactor [2]. To overcome this difficulty, immobilized yeast or bacteria cell systems provide high cell density with high flow rates that results in short residence times [3]. Reactors with immobilized cells have shorter fermentation times, higher productivity and operational stability of the cells, as well as easier downstream processing.

When dealing with immobilized cell systems it is of a big importance to choose the proper reactor type. This decision depends on the type of immobilization and type of support used, as well as on mass transfer requirements and conditions of the process. For continuous AF in wine production, multiphase reactors are used, including packed bed reactor, fluidized bed reactor, bubble column and air-lift reactor [3,4]. Packed bed reactor is among the most used for wine production with immobilized cells in continuous mode of operation [5,6]. In this type of reactor the immobilized cells are packed inside the reactor and a current of fermentation media is passed upflow (flooded bed reactor) or downflow (trickle-bed reactor) [7].

Most of the available data published about immobilized cell systems used in winemaking concerns batch processes, and in a less extent continuous alcoholic fermentation. Natural materials such as fruit pieces of apple, quince, pear, guava and watermelon [5,6,8–10], whole grains of corn, wheat and barley [11–13] or residues of the wine industry [14,15] are reported as supports for cell immobilization and further implied in batch winemaking. Most of these immobilized cell systems were found to be of good operational stability. In continuous alcoholic fermentation,

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1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.10.005

for winemaking, the used yeast cells are frequently immobilized on natural organic and inorganic materials. Immobilized cell systems using natural organic materials such as gluten pellets resulted in wines with improved quality [16]. Inorganic materials like kissiris and γ-alumina, are cheap, abundant and can be regenerated and reused, however this materials were considered undesirable as they leave mineral residues in the final product [17,18].

The immobilization methods mostly used for bacteria cell immobilization in malolactic fermentations are entrapment [19] and attachment to natural materials [20,21]. There are few available articles on the continuous malolactic fermentation of wines conducted with immobilized cells [22]. Moreover, according to what we know so far no works were published about an integrated continuous process of winemaking.

The main objective of this study was the integration of both alcoholic fermentation (AF) and malolactic fermentation (MLF) in a sequential continuous winemaking process. To achieve this global goal, both AF and MLF were implemented in distinct packed bed reactors operating with immobilized *Saccharomyces cerevisiae* and *Oenococcus oeni*, respectively.

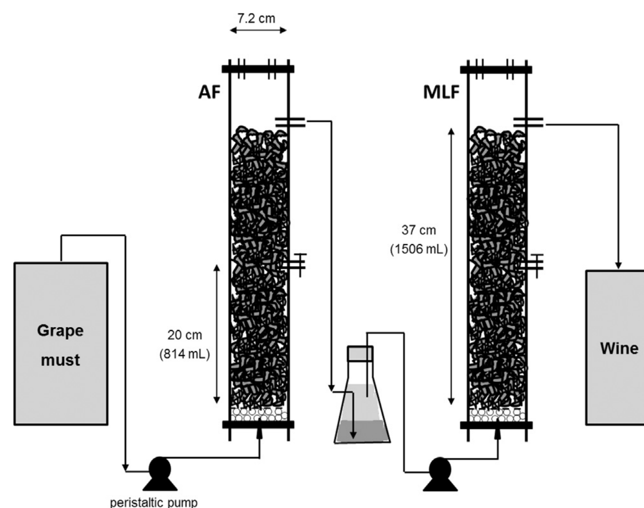

Fig. 1. Schematic representation of the integrated process of continuous winemaking.

2. Materials and methods

2.1. Inocula preparation

A commercial *S. cerevisiae* strain (Lalvin QA23®, Lallemand) was used in the alcoholic fermentation 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: yeast extract (10 g/L), peptone (20 g/L) and glucose (20 g/L). Cells were cultivated under static conditions, at 30 °C for 24 h, being subsequently recovered by centrifugation (*RCF* = 7000, 20 min), washed with distilled water and resuspended in the fermentation medium to obtain an initial concentration of 1 g/L (dry weight).

A commercial strain of *O. oeni* (Uvaferm® ALPHA, Lallemand) was the bacterial strain used in the malolactic fermentation experiments. The inoculum was prepared by cultivation of the bacteria in 500 mL Erlenmeyer flasks containing 200 mL of MRS Broth medium (Cultimed, Panreac, Barcelona). Cells were cultivated under static conditions, at 28 °C for 48 h, being subsequently recovered by centrifugation (*RCF* = 7000, 10 min), washed with distilled water and resuspended in the fermentation medium to obtain an initial concentration of 1 g/L (dry weight).

2.2. Support materials for cell immobilization

Grape skins and grape stems (from white grape varieties), separately, were used as support materials for cell immobilization. These supports were supplied by a local winemaking company, being washed with distilled water and dried at 60 °C until constant weight. Then, supports were sterilized for 20 min at 121 °C, before use.

2.3. Media composition for fermentation assays

Complex culture medium used in the alcoholic fermentation assays was composed by glucose (120 g/L), yeast extract (4 g/L), $(NH_4)_2SO_4$ (1 g/L), KH_2PO_4 (1 g/L) and $MgSO_4$ (5 g/L). Complex culture medium used in the malolactic fermentation had the following composition: glucose (15 g/L), yeast extract (4.0 g/L), meat extract (8.0 g/L), bacteriological peptone (10.0 g/L), $MgSO_4$ (0.2 g/L), $MnSO_4$ (0.05 g/L), sodium acetate (5.0 g/L), tween 80 (1.0 g/L), dipotassium hydrogen phosphate (2.0 g/L), di-ammonium hydrogen citrate (2.0 g/L) and malic acid (4.0 g/L).

The grape must used for alcoholic fermentations was obtained from a mixture of white grape varieties from the Appellation of Origin *Vinhos Verdes* region, with a total sugar content of ≈200 g/L, quantified by areometry [26]. The used wines for malolactic fermentation were produced in laboratory conditions and had an initial concentration of malic acid around 4 g/L. The grape must and wine were kept at 4 °C, before use. Initially, the studies were conducted with complex medium and later grape must and wine were used for alcoholic and malolactic fermentations, respectively. The choice of complex medium in the initial fermentations avoided difficulties with the supply and storage of grape must. Initially, the glucose content of the complex media (used in the alcoholic fermentation) was 120 g/L for a better and faster immobilization of the yeast cells.

2.4. Reactors preparation

Continuous alcoholic and malolactic fermentation assays were performed in distinct cylindrical tower packed bed reactors (7.2 cm inside diameter) with a total volume of about 1750 mL. Two sampling ports were available at 20 cm and 37 cm height, corresponding to working volumes (volume of the empty bed), of 814 mL and 1506 mL, respectively (Fig. 1). Both reactors were operated in upward flow mode.

Before use, the reactors were sterilized with sodium hypochlorite solution (1.5% active chlorine) during at least 4 d prior to fermentations [23]. Then, the reactors were washed with five volumes of sterilized water before filling with the sterilized support. At the bottom of each reactor, 1 cm height of glass beads (6 mm diameter) was placed to allow a regular repartition of the feeding medium in the whole section of the tower. Then, the reactor for continuous alcoholic fermentation was packed with grape stems (60 g or 90 g) or with grape skins (125 g or 260 g) in sterile conditions in the flow chamber. Similarly, the packed bed reactor for continuous malolactic fermentation was filled in with 260 g of grape skins. The assays with 60 g, 90 g and 125 g of material were carried out using the first sampling port, i.e. at 20 cm height; the assays with 260 g of support were performed using the total available volume at 37 cm height. The supports were restricted with an iron nets placed above the glass beads and above the support itself.

2.5. Fermentation assays

A schematic representation of the assays carried out in the present study is depicted in Fig. 2. Initially, for cells immobilization, the reactors were operated in batch mode. The reactors were

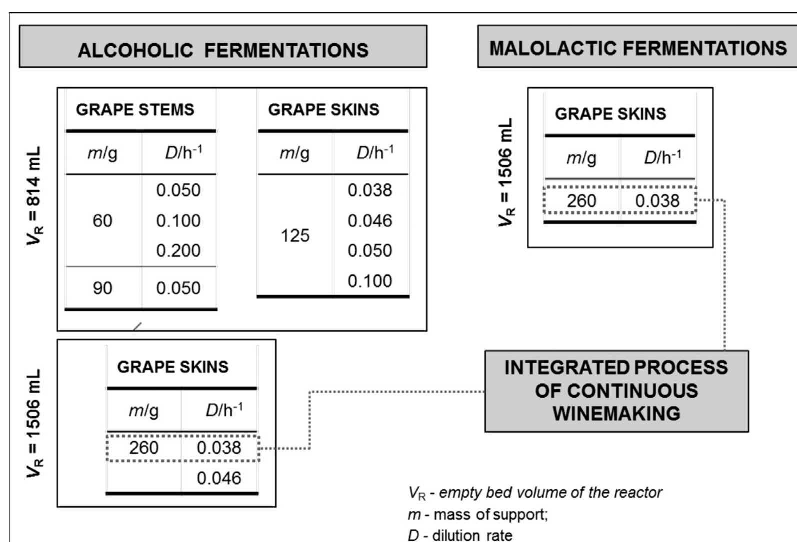

Fig. 2. Experimental design of the continuous fermentation assays conducted with immobilized cells.

charged with 1 L of complex medium and about 50 mL of yeast or bacterial cell suspension prepared as described in the inocula preparation subsection. Then, after 48 h of immobilization in batch mode, the reactors were switched to a continuous mode, and operated at different dilution rates (Fig. 2). After all the operational conditions of the reactors were established and the processes were stabilized, the complex medium was replaced by grape must for alcoholic fermentation, and wine for malolactic fermentation. Continuous AF and MLF were conducted initially in separate. After optimization of the processes conditions the two reactors were linked together.

All analyses were made in duplicate after the stationary state of the continuous process was attained.

2.6. Free and immobilized cells determination

Immobilized yeast cells concentration was determined by counting the yeast cells on a Neubauer chamber at the fermentations' end after washing the biocatalyst by agitation (120 min^{-1}) with 30 g/L NaOH solution, for 24 h at 30 °C, according to Genisheva et al. [24].

Death/live cells were determined after detachment of the cells by vigorous agitation of 0.5 g of the support with 30 g/L solution of NaCl, for 30 min. Then, the liberated cells were further stained with methylene blue and the dead/live cells were counted on a Neubauer chamber.

The concentration of immobilized bacterial cells was determined at the end of fermentation assays. About 3.5 g of material (wet weight) were placed in a 200 mL Erlenmeyer flask containing 20 mL of distilled water. Subsequently, it was autoclaved for 20 min at 121 °C. The autoclaved support was separated from the liquid using a strainer and left to dry at 60 °C till constant weight. The total volatile suspended solids, remained in water, were determined by gravimetry according to Clesceri et al. [25]. A blank experiment using support without immobilized cells was used to correct eventual losses of material during the autoclaving procedure.

Free yeast and bacterial cells concentrations, present in the fermentation medium, were estimated by measuring the absorbance at 600 nm, which was correlated to a calibration curve (dry weight × absorbance).

2.7. Fermentation parameters

The concentration of immobilized cells (X_{im}) was calculated as the ratio of the dry weight of cells to the dry weight mass of the support. The mass concentrations of malic acid ($C_{mal.ac}$), lactic acid ($C_{lac.ac}$) and acetic acid ($C_{acet.ac}$) were calculated respectively as the ratio of the mass of malic acid, lactic acid and acetic acid, per liter of fermentation medium. The conversion of malic acid ($\eta_{mal.ac}$) was determined as the ratio between the consumed malic acid and the initial malic acid. The mass concentrations of glucose (C_{gl}) and fructose (C_{fr}) were calculated as the mass of glucose and fructose per liter of fermentation medium. The alcohol strength (C_{et}) was calculated as the volume of ethanol present in 100 volumes of the fermentation product. The dilution rate (D) was defined as the ratio between the volumetric flow rate and the liquid phase volume of the packed reactor.

2.8. General physicochemical analysis

Total acidy (TA) and free SO_2 concentration were measured by titration according to the Methods OIV-MA-AS313-01 and OIV-MA-AS323-04A, respectively [26].

2.9. 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 detector (Jasco 830-RI), an ultraviolet detector (Jasco UV-2070) and a Varian Metacarb 67H column (300 mm × 6.5 mm) operated at 80 °C. A 5 mmol/L H_2SO_4 solution was used as eluent at a constant flow rate of 0.3 mL/min. These analyses were made in duplicate. The quantification of each compound was made by using a calibration curve (peak area vs. concentration).

2.10. Gas-chromatographic analysis

Major volatile compounds were directly analyzed after adding 410 μg of 4-nonanol (internal standard—IS) to 5 mL of wine. A Chrompack CP-9000 gas chromatograph equipped with a split/splitless injector, a flame ionization detector (FID) and

a capillary column, coated with CP-Wax 57CB (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 initially held at 60 °C, for 5 min, then programmed to rise from 60 °C to 220 °C, at 3 °C/min, and finally maintained at 220 °C for 10 min. The carrier gas was helium 4 × (Praxair) at an initial flow rate of 1 mL/min (125 kPa at the head of the column). The analyses were performed by injecting 1 μL of sample in the split mode (15 mL/min). The quantification of major volatile compounds, after the determination of the detector response factor for each analyte, was performed with the software Star-Chromatography Workstation version 6.41 (Varian) by comparing test compound retention times with those of pure standard compounds.

Minor volatile compounds were analyzed by GC–MS after extraction of 8 mL of wine with 400 μL of dichloromethane, spiked with 3.28 μg of 4-nonanol (IS), according to the methodology proposed by Oliveira et al. [27]. A gas chromatograph Varian 3800 with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000, was used. A 1 μL injection was made in splitless mode (30 s) in a Varian Factor Four VF-Wax ms (30 m × 0.15 mm; 0.15 μm film thickness) column. The carrier gas was helium 4 × (Praxair) at a constant flow rate of 1.3 mL/min. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range from 35 *m*/*z* to 260 *m*/*z* and an acquisition interval of 610 ms. The oven temperature was initially 60 °C for 2 min and then raised from 60 °C to 234 °C at a rate of 3 °C/min, raised from 234 °C to 250 °C at 10 °C/min and finally maintained at 250 °C for 10 min. The temperature of the injector was maintained at 250 °C during the analysis time and the split flow was maintained at 30 mL/min. The identification of compounds was performed using the software MS WorkStation version 6.9 (Varian) by comparing their mass spectra and retention indices with those of pure standard compounds. The minor compounds were quantified in terms of 4-nonanol equivalents only.

All the analyses of volatile compounds were carried out in triplicate.

2.11. Statistical analysis

The results were analyzed by ANOVA, using FAUANL software [28]. Fisher's Least Significance Difference (LSD) multiple comparison test was used to detect significant differences ($p < 0.05$) between samples.

3. Results and discussion

In previous studies, Genisheva et al. [14,21] showed the possibility of conducting, in batch mode of operation, alcoholic and malolactic fermentations with *S. cerevisiae* and *O. oeni* cells immobilized either on grape stems or on grape skins. The present study evaluated the possibility of conducting the same fermentations processes but in a continuous mode of operation. In the first part of this work, studies with immobilized yeast cells to carry out the AF for white wine production were made. Different amounts of two different supports (grape stems and grape skins) were used, and the system was operated at different dilution rates. Grape skins and grape stems were chosen as supports on the bases of two previous studies where different materials for the immobilization of yeast and bacterial cells were analyzed [21,24]. In the second part, assays with immobilized bacteria cells for malolactic fermentation of white wine, were conducted. Finally, the integration of the two continuous fermentative processes, in a whole winemaking procedure, was studied.

3.1. Continuous alcoholic fermentations

There are limited published data about the production of wines by continuous alcoholic fermentation with immobilized cells. Moreover, only a few works are concerning the use of natural materials, like pieces of fruits, for the immobilization of the yeast cells [5,6,8]. Even though the pieces of fruits are appropriate for winemaking, their cultivation, availability and cost are limited for industrialization [10]. In the present work were used residues from the wine industry (grape stems and grape skins), that are normally available in large amounts and have low cost. Continuous alcoholic fermentation assays were made with different concentrations of immobilized cells, as well as with different dilution rates. The obtained results are presented in Table 1.

The first two continuous fermentations were carried out with 60 g and 90 g of grape stems. The other two fermentation assays were carried out with 125 g and 260 g of grape skins, respectively. In all assays with grape skins these were hold down with small amount of grape stems, as well as with an iron net. Firstly the packed bed reactor worked in batch mode, with complex medium, for better cell immobilization. After 48 h, the reactor was switched to a continuous mode of operation.

The assays with 60 g of immobilized grape stems were conducted with three different dilution rates (0.05 h^{-1}, 0.10 h^{-1} and 0.20 h^{-1}), the most effective conditions being obtained at $D = 0.05\ h^{-1}$. At this dilution rate, the ethanol production (6 vol%) showed the best values compared to the two other dilution rates used. The residual glucose concentration was rather high, 42 g/L, and the amount of immobilized cells was low ($X_{im.T}$ = 5.1 mg/g; $X_{im.B}$ = 7.7 mg/g). Taking into account these results, the subsequent assay was carried out with 90 g of grape stems at $D = 0.05\ h^{-1}$. In this assay the initial sugar concentration was increased to 200 g/L, which theoretically would give an alcoholic strength to the final product of about 13 vol% However, the obtained results for alcoholic strength (4.3 vol%) were very low, which are in agreement with the high final glucose concentration observed (130 g/L).

To overcome this problem, grape skins were used as support material in a new series of continuous AF assays. In the first assay the total mass of the support was set to 125 g to obtain higher concentrations of immobilized cells. The initial concentration of glucose was 200 g/L and 5 different dilution rates were studied (Table 1). The best results for ethanol production (11.7 vol%) were registered for $D = 0.038\ h^{-1}$. Good results were obtained also when the reactor was operated at $D = 0.046\ h^{-1}$. Nevertheless, the final concentration of glucose, 21 g/L, was still high (Table 1). The obtained quantities of immobilized cells (205.8 mg/g on the top of the packed bed and 537.2 mg/g at the bottom of the packed bed) were much higher than in the previous assay using 90 g of grape stems as support (8.3 mg/g and 19.4 mg/g, on top and bottom of the packed bed, respectively).

In attempt to reach higher immobilized cell load in the reactor and to decrease the final glucose concentration, the last continuous alcoholic fermentations were carried out with 260 g of grape skins, using the total bed reactor volume, i.e. 1506 mL. Initially, the two first assays with complex medium were conducted with low concentration of glucose, 120 g/L, as it was observed previously (data not shown) that cell adhesion to the support, i.e. immobilization, is faster. However, at initial sugar concentration of 120 g/L, the obtained results for ethanol production were low, 7.1 vol% for $D = 0.038\ h^{-1}$ and 8.6 vol% for $D = 0.046\ h^{-1}$. For to reach a higher load of immobilized yeast cells, the initial glucose concentration was finally increased to 200 g/L. In result, the ethanol production increased as follows: 11.2 vol% for $D = 0.046\ h^{-1}$ and 12.7 vol% for $D = 0.038\ h^{-1}$.

As the reactor operation remain stable for more than 1 month (since the beginning of the assay with 260 g of grape skins) the

Table 1
General characteristics of the alcoholic fermentation assays with immobilized *S. cerevisiae* and multiple comparison analysis (Fisher's test; $p < 0.05$), including standard deviation (*sd*).

Support	m (g)	Media	t (d)	D (h^{-1})	$C_{gl.in}$ (g/L)	$C_{gl.fin}$ (g/L)	C_{et} (vol%)	*sd*	$X_{im.T}$ (mg/g)	*sd*	$X_{im.B}$ (mg/g)	*sd*
Grape stems	60	C	5	0.050	120	42	6.0[fg]	0.3	5.1	2.8	7.7	3.1
		C	2	0.100	120	92	2.1[h]	0.1				
		C	1	0.200	120	110	1.0[h]	0.0				
	90	C	25	0.050	200	130	4.3[g]	0.0	8.3	2.4	19.4	0.5
Grape skins	125	C	7	0.038	200	24	11.7[ab]	0.3	205.8	12.2	537.2	54.6
		C	7	0.046	200	21	11.4[bc]	0.9				
		C	42	0.050	200	29	9.9[cd]	0.2				
		C	43	0.058	200	36	11.1[bc]	0.8				
		C	6	0.100	200	90	8.6[de]	0.6				
	260	C	3	0.038	120	0	7.1[ef]	0.2	1476.8	128.1	2256.9	18.9
		C	5	0.046	120	3	8.6[de]	0.6				
		C	5	0.038	200	2	12.7[a]	0.3				
		C	19	0.046	200	42	11.2[bc]	0.0				
		M	47	0.038	200*	2	13.1[a]	0.1				

a–h—for each column, values with the same letters mean no significant difference at 95% confidence level; C—complex media; M—grape must; m—mass of the support; t—total fermentation time of the assay; $C_{gl.in}$—initial glucose concentration (* for grape must $C_{gl.in}$ corresponds to glucose + fructose); $C_{gl.fin}$—final glucose concentration; $X_{im.T}$—concentration of immobilized cells at the top of the reactor; $X_{im.B}$—concentration of immobilized cells at the bottom of the reactor.

Table 2
General characteristics of the wines obtained after continuous malolactic fermentation and multiple comparison analysis (Fisher's test; $p < 0.05$), including standard deviation (*sd*).

Fermentation	Medium	$\eta_{mal.ac}$ (%)	*sd*	$C_{mal.ac}$ (g/L)	*sd*	$C_{lac.ac}$ (g/L)	*sd*
1	Complex	91[a]	2.6	0.4[b]	0.1	8.6[a]	0.0
	wine (pH 2.9)	21[b]	1.7	3.2[a]	0.1	8.4[a]	0.3
2	Complex	93[a]	1.7	0.3[b]	0.1	5.8[b]	0.6
	wine (pH 3.1)	85[a]	0.0	0.4[b]	0.0	5.7[b]	0.0

a–c—for each column, values with the same letters mean no significant difference at 95% confidence level.

complex medium was changed to grape must. At these conditions and after achieved the steady state, the obtained results showed improved ethanol production, 13.1 vol% The reactor operated with grape must for 47 d. At a dilution rate of 0.038 h^{-1}, the final glucose concentration reached low values of 2 g/L, i.e. a dry white wine was produced. Moreover, the concentration of immobilized cells calculated at the end of the fermentation demonstrated high cell load in the entire reactor (1476.8 mg/g on the top and 2256.9 mg/g on the bottom).

When comparing the results between assays one can observed the differences in ethanol production. The differences in ethanol production between the assays with yeast cells immobilized on grape stems or on grape skins are dependent on the used support [24]. While in the assays where the same support is used the differences in ethanol production are mainly due to the dilution rate used.

During the continuous alcoholic fermentation with immobilized cells in the packed bed reactor working at $D = 0.038\ h^{-1}$, the flow rate of wine production was ≈960 mL/d, being the AF completed in 26.3 h. In our previous study using immobilized yeast cells on grape skins for wine production, in batch mode, at least 4 d were needed to complete the alcoholic fermentation in a 1 L container [29]. When compared to the results obtained in this study it is clear that continuous alcoholic fermentation with immobilized yeast cells are about 4 times more productive than fermentation in the batch mode.

3.2. Continuous malolactic fermentations

As far as we know there are only few studies of continuous malolactic fermentations with immobilized bacteria cells [22]. Based on the results obtained for the continuous alcoholic fermentation, continuous malolactic fermentations were also carried out with 260 g of support containing immobilized bacterial cells, operating at a dilution rate of 0.038 h^{-1}. Firstly, *O. oeni* bacterial cells were allowed to immobilize in the previously sterilized grape skins, using complex medium. For screening the bacteria development and growth, samples were taken at different time periods. After 48 h, the reactor was switched to the continuous mode of operation. Two continuous malolactic fermentations were carried out. For each fermentation assay, firstly the system was supplied with complex medium and then it was replaced by dry white wine (Table 2).

In the first fermentation, the malic acid conversion in continuous fermentation with complex medium was 91% (Table 2). However, when the wine was supplied a gradual decrease of the malolactic conversion was observed, possibly due to the low pH (2.9). At the 5th day of fermentation, the malic acid conversion was 21% and after 17 d the malolactic fermentation stopped completely. These results are in agreement with those referred by Ribéreau-Gayon et al. [1], which stated a pH of 2.9 as the limit for the growth of lactic acid bacteria.

In the second continuous malolactic fermentation assay, the conversion of malic acid using complex medium reached 93%. The higher pH value of the wine (3.1) in this assay facilitated the malolactic conversion (85%). According to Ribéreau-Gayon et al. [1], at pH 3.2 the bacterial growth is still very limited and malolactic fermentation only becomes possible at a pH of 3.3 or higher. However immobilized bacteria cells are more resistant against inhibitors [21,29] and the pH 3.1 was high enough for the immobilized bacteria cells to conduct successfully malolactic fermentation. In those conditions the used system gave high degradation of malic acid and long term operation stability (around 1 month).

3.3. Integrated continuous winemaking

This is a first attempt for developing an integrated continuous process of winemaking. Two assays of winemaking, integrating the two continuous processes were made (Fig. 1). The packed bed reactor with immobilized yeast cells was linked to the packed bed reactor with immobilized bacterial cells, using a *kitasato* flask as a

Table 3
General characteristics of the wine obtained after alcoholic fermentation (AF) and after malolactic fermentation (MLF), including standard deviation (*sd*), in the integrated process of winemaking.

	1st assay (pH 2.9)				2nd assay (pH 3.1)			
	AF	*sd*	MLF	*sd*	AF	*sd*	MLF	*sd*
$C_{mal.ac}$ (g/L)	2.57	0.06	2.55	0.05	2.44	0.12	0.81	0.03
$\eta_{mal.ac}$ (%)			0.8	0.4			66.7	1.0
$C_{acet.ac}$ (g/L)	0.60	0.00	0.62	0.00	0.35	0.04	0.60	0.01
$C_{lac.ac}$ (g/L)	1.64	0.01	1.43	0.01	1.63	0.18	4.54	0.02
C_{gl} (g/L)	0.40	0.01	0.34	0.00	0.33	0.01	0.00	0.00
C_{fr} (g/L)	0.88	0.02	0.44	0.00	0.75	0.04	0.00	0.00
C_{et} (vol%)	12.7	0.0	12.9	0.2	12.2	0.33	11.5	0.0
pH	2.9		nd		3.1		3.3	
TA (g/L)	nd		nd		2.4		0.2	

nd—not determined; *TA*–total acidity, expressed as tartaric acid.

clarifier between them. The outflow of the first reactor, after sedimentation of yeast cells, was indeed the inflow of the second reactor (Fig. 1). The whole system was operating in continuous mode at a dilution rate of 0.038 h^{-1}, using 260 g of grape skins as support material for cells immobilization. After reaching the steady state (fifth day of fermentation), the system was stable till the end of the study. The first assay was not successful as the wine produced in the first reactor had very low pH value of 2.9 (Table 3). However, in the second assay the wine produced by AF had higher pH (3.1) resulting in a final wine with expected good characteristics.

Table 3 presents some general characteristics of the wine obtained in this integrated process. In the first integrated assay, the degradation of malic acid was very poor, 0.8%. As mentioned before, the low pH value of the wine produced in the reactor with immobilized yeast cells could justify the results; additionally, the synergic effect of the high alcoholic strength might have influence. Here, the residual sugars, glucose and fructose, present after the alcoholic fermentation were further reduced during the MLF by 15% and 50%, respectively. No changes in ethanol concentration were found, as expected [30].

In the second assay of the integrated process, the wine produced in the first packed reactor had a pH 3.1. As a result, the degradation of malic acid in the second reactor was much higher 66.7%. In the total, 1.63 g/L of malic acid was consumed by the immobilized bacteria cells, and 2.91 g/L of lactic acid were produced. The system was able to metabolize malic acid, being the results comparable with previous studies [20]. The concentration of sugars, glucose and fructose, also decreased and may have been partially converted to lactic acid. According to Genisheva et al. [21] bacterial cells immobilized on grape skins were able to diminish the concentration of malic acid from 50% to 87% in 17 d of fermentation in batch mode of operation. In the present study, after the system reached the steady state, the continuous malolactic fermentation using immobilized bacterial cells converted about 67% of malic acid at a flow rate of 960 mL/d and a fermentation time of 26.3 h (i.e. $D = 0.038\ h^{-1}$). These results showed that MLF was about 17 times more efficient when conducting in the continuous mode of operation.

The low concentrations of residual sugars were completely exhausted during MLF, while the ethanol concentration did not change. Acetic acid is the most important volatile acid produced during MLF. Additionally, concentrations of this acid between 0.2 g/L and 0.6 g/L contributes to the complexity of the wine aroma [31,32]. In the present study, the concentration of acetic acid increased from 0.35 g/L to 0.60 g/L after MLF (Table 2). In fact it is well known that the concentration of acetic acid normally increased 0.1 g/L to 0.2 g/L after MLF [32], which corroborates the obtained results. The partial consumption of citric acid (results not shown) may also contribute to obtained results. The limit for volatile acidity in white wines, which comprises essentially acetic acid, is 1.2 g/L [26].

In order to obtain a better comparison between the wine produced in continuous alcoholic fermentation and the wine obtained after continuous malolactic fermentation, in the integrated system, a complete characterization of the products regarding aroma volatile compounds was made. The obtained results are presented at Tables 4 and 5. In total, 8 major volatile compounds and 19 minor volatile compounds were identified and quantified by GC–FID and by GC–MS, respectively.

3.4. Major volatile compounds

From the 8 major volatile compounds analyzed only acetaldehyde and four higher alcohols demonstrated significant difference before and after malolactic fermentation (MLF). Moreover, four

Table 4
Mean concentrations (*C*), confidence limits (*p* = 0.05) and aroma perception thresholds (*PT*) of the major volatile compounds at the end of alcoholic fermentation (AF) and at the end of the malolactic fermentation (MLF).

Compound	AF		MLF		*PT* (mg/L)
	C (mg/L)	±	*C* (mg/L)	±	
Acetaldehyde	20.9[b]	5.7	30.7[a]	7.4	10[Г]
Ethyl acetate	57.4[a]	16.9	66.0[a]	21.8	12.3[Д]
Methanol	24.6[a]	2.5	23.9[a]	10.8	668[Г]
Higher alcohols					
1-Propanol	85.3[a]	13.9	61.5[b]	16.6	830[Г]
2-Methyl-1-propanol	37.4[a]	7.6	30.0[b]	7.8	40[Г]
2-Methyl-1-butanol	14.9[a]	3.0	11.6[b]	3.1	
3-Methyl-1-butanol	102.9[a]	20.7	80.9[b]	24.2	30[Г]
2-Phenylethanol	6.6[a]	1.1	5.7[a]	3.3	14[Ж]
Total	**245.3**	31.8	**189.7**	39.9	

a, b, c, d—for each compound, values with the same letters mean no significant difference at 95% confidence level; Г—Moreno et al. [33]; Д—Escudero et al. [34]; Ж—Ferreira et al. [43].

Table 5
Mean concentrations (*C*), confidence limits ($p = 0.05$) and aroma perception threshold (*PT*) of the minor volatile compounds at the end of alcoholic fermentation (AF) and malolactic fermentation (MLF).

Compound	AF		MLF		*PT* (μg/L)
	C (μg/L)	±	*C* (μg/L)	±	
Fatty acid ethyl esters					
Ethyl butyrate	186.5[a]	65.9	148.7[a]	13.9	20[д]
Ethyl hexanoate	758.6[a]	78.6	545.3[a]	35.5	14[г]
Ethyl octanoate	257.7[b]	13.7	551.9[a]	47.9	5[г]
Ethyl decanoate	142.4[b]	43.9	222.0[a]	41.1	200[г]
Total	**1345.2**	112.4	**1467.9**	73.7	
Ethyl esters of organic acids					
Ethyl lactate	76.0[a]	22.1	32.3[b]	7.8	100,000[ж]
Diethyl succinate	nd		nd		100,000[ж]
Total	**76.0**	22.1	**32.3**	7.8	
Acetates of higher alcohols					
3-Methylbutyl acetate	2050.6[a]	309.1	1371.9[b]	161.6	30[ж]
Hexyl acetate	57.2[a]	15.1	37.1[b]	10.8	1000[м]
2-Phenylethyl acetate	170.4[a]	19.8	114.4[b]	3.1	250[ж]
Total	**2278.2**	310.1	**1523.4**	162.0	
Volatile phenols					
4-Vinylguaiacol	4.4[a]	2.9	1.7[b]	0.6	130[ф]
4-Vinylphenol	nd		nd		180[ф]
Total	**4.4**	2.9	**1.7**	0.6	
Volatile fatty acids					
Butanoic acid	36.5[a]	12.6	33.2[a]	5.2	173[г]
Hexanoic acid	688.4[a]	170.0	603.4[a]	45.0	420[г]
Octanoic acid	2617.0[a]	497.5	2382.3[a]	185.2	500[г]
Decanoic acid	420.9[a]	296.8	150.0[b]	54.4	1000[г]
Dodecanoic acid	64.9[a]	10.5	6.8[b]	2.4	10,000[ж]
2-Methylpropanoic acid	26.7[a]	7.5	22.1[a]	1.9	2300[г]
2 + 3-Methylbutanoic acids	35.8[a]	8.5	30.9[a]	8.9	33.4[г]
Total	**3890.2**	604.1	**3228.7**	198.5	

a–d—for each compound, values with the same letters mean no significant difference at 95% confidence level; nd—not detected; г—Oliveira et al. [42]; д—Guth [45]; ж—Moreno et al. [33]; м—Chaves et al. [46]; ф—Boidron et al. [47].

compounds were found in concentrations above their perception thresholds for both samples, after AF and after MLF. Acetaldehyde increased significantly ($p < 0.05$) after MLF, which is in agreement with other published data [20,30]. However, in our study, acetaldehyde was found in much lower concentrations compared to other published results with immobilized bacterial cells conducting MLF in batch mode [20]. Nevertheless acetaldehyde was always found above its orthonasal perception threshold of 10 mg/L [33]. Ethyl acetate was also found, in all the samples, in concentrations above its perception threshold of 12.3 mg/L [34] and similar to other published results [16]. Ethyl acetate is considered an important contributor to the wine aroma. At low concentrations (≤100 mg/L) this compound gives desirable “fruity” aromas to the wine; however, at higher concentrations it can impart “solvent” or “nail varnish-like” aromas [35]. Methanol is produced from the pectins of the skin of the grapes which undergo an enzymatic conversion [1]. Nevertheless the methanol concentrations found in wines produced with cells immobilized on grape skins were low (24.6 g/L after AF and 23.9 g/L after MLF). Sipsas et al. [16], using a packed bed reactor in continuous mode, reported methanol concentrations two times higher than those found in the present study. Also Tsakiris et al. [36], using yeasts immobilized on raisins to carry out AF (in batch mode), found methanol concentrations (93.2 g/L and 86.5 g/L) 4 times higher when compared to the results obtained in the present study (Table 4).

Alcohols having more than two carbons and only one alcohol function are called higher alcohols. As higher alcohols are produced during AF, they are absent in grape must, but are found in wines in relatively high concentrations, reaching values above 100 mg/L [37]. The present study shows that higher alcohols (except 2-phenylethanol) concentrations diminished significantly ($p < 0.05$) after MLF. Similar results were published by Agouridis et al. [30], using immobilized *Lactobacillus casei* cells on a delignified cellulosic material. The sum of the higher alcohols attained 245.3 mg/L after AF and 189.7 mg/L after MLF. These concentrations are in the normal range found in wines [37]. Excessive concentrations of higher alcohols may give “strong” and “pungent” notes to wines [38], while levels below 300 mg/L to 400 mg/L may impart “fruity” character [39,40].

In wines, 1-propanol is normally found in concentrations between 1 mg/L and 50 mg/L. However, in the present study 1-propanol was always found in concentrations higher than 50 mg/L, but it never reached its perception threshold of 830 mg/L [33]. After AF, the wine had much higher concentration of 1-propanol and was found statistically different ($p < 0.05$) from wine after MLF. Additionally, it was found in much higher concentrations than those reported by Sipsas et al. [16], 23 mg/L, in white wines produced continuously in a packed bed reactor with cells immobilized on gluten pellets.

The formation of higher alcohols is connected to the amino acids catabolism (Ehrlich pathway) and to the sugar metabolism of yeasts [37]. The higher alcohol 3-methyl-1-butanol was present, in both samples, in concentrations over its perception threshold of 30 mg/L [33]. The sum of 2-methyl-1-butanol and 3-methyl-1-butanol (117.8 mg/L) before MLF was higher compared to other published results (75 mg/L) for wine produced in continuous packed bed reactor [16]. According to Vilanova and Oliveira [37], 2-methyl-1-butanol and 3-methyl-1-butanol together with 2-phenylethanol are the higher alcohols that most contribute to the aroma of wine.

In general, factors that increase the fermentation rate, such as higher concentrations of yeast biomass, also increase the formation of higher alcohols. Moreover the content of higher alcohols

of wine varies according to the fermentation conditions, especially the species of yeast used in the fermentation process [1].

3.5. Minor volatile compounds

From the 19 minor volatile compounds identified and quantified, 9 were found to be statistically different ($p < 0.05$) in wines before and after MLF.

Fatty acid ethyl esters are formed enzymatically in a reaction between ethanol and fatty acids [37,41]. Esters have similar olfactory notes bringing "fruity" and "pleasant" characteristics to the overall aroma of wines [37]. However, changes in ester concentrations during MLF are strain specific [35]. All fatty acid ethyl esters, except ethyl decanoate were found in the wine samples in concentration above their perception thresholds, after AF (Table 5). Moreover, ethyl octanoate and ethyl decanoate increased their concentration after MLF, fact that is in agreement with Lerm et al. [32].

Ethyl esters of organic acids are formed during wine ageing by chemical esterification between ethanol and organic acids [1,35]. The most abundant ethyl esters of organic acids use to be ethyl lactate and diethyl succinate, which may attain higher levels in wines [37]. However in our samples these two compounds were not detected (diethyl succinate) or were found in low concentrations (ethyl lactate) once the analyzed wines were very young. The perception thresholds of ethyl lactate and diethyl succinate are very high (Table 5) and only ethyl lactate may occasionally contribute to the wine aroma [41].

Acetates of higher alcohols decreased slightly after MLF. Isoamyl acetate was found in all wine samples in concentrations above its perception threshold of 30 μg/L [33]. According to Oliveira et al. [42], isoamyl acetate and 2-phenylacetate, together with the ethyl esters ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate are the main contributors to the aroma of young wines. In our samples, these compounds, except 2-phenylacetate, were present in concentrations above their perception thresholds, indicating that the resulting wines may have "sweet" and "fruity" flavors.

Ethyl esters and acetates of higher alcohols, together, are very important for the flavor profile of fermented beverages, as the known synergic effect may reduce individual perception thresholds [35,41].

The volatile phenol 4-vinylguiacol was found statistically different for wines after AF and after MLF, showing less concentration after MLF. It is known that the concentrations of 4-vinilguiacol and 4-vinylphenol use to increase after MLF, as a result of the degradation of some phenolic acids by bacteria [32]. However, MLF may have contradictory effect on the sensory character of wine. It depends on the bacteria strain used, the presence and availability of precursors, the wine type and the vinification conditions [32].

The volatile fatty acids hexanoic acid, octanoic acid and 2 + 3-methylbutanoic acids (only for wine after AF) were also found in all samples in concentrations above its perception thresholds of 420 μg/L, 500 μg/L and 33.4 μg/L, respectively [43]. Although fatty acids are characterized by unpleasant notes (sweat, cheese), their flavor is essential to the aromatic equilibrium of wines [37,44].

4. Conclusions

Continuous fermentations are advantageous over batch fermentations as they have shorter fermentation times and higher productivities. Grape skins were found to be an appropriate support to be used in continuous alcoholic and malolactic fermentations. Immobilized cell systems on grape skins have an optimal mechanical stability for use in packed bed reactor in continuous mode of operation.

The integrated continuous process of winemaking gave good operational stability and promising results for further research. The obtained results on aroma compounds, suggested that the produced wines had "fruity" and "fresh" flavor. Immobilized bacteria cell system was strongly influenced by the pH value of the media and further studies in this aspect are needed. However malolactic fermentation was successful and well conducted.

The continuous mode of operation to carry out AF and/or MLF, in a winemaking process, opens the possibility to modulate the final characteristics of the wine by simply adjusting the dilution rate.

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