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Aroma enhancement in wines using co-immobilized *Aspergillus niger* glycosidases

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ABSTRACT

A major fraction of monoterpenes and norisoprenoids in young wines is conjugated to sugars representing a significant reservoir of aromatic precursors. To promote their release, β-glucosidase, α-arabinosidase, and α-rhamnosidase from a commercial *Aspergillus niger* preparation, were immobilized onto acrylic beads. The aim of this work was the development and application of an immobilized biocatalyst, due to the well-known advantages over soluble enzyme preparations: control of the reaction progress and preparation of enzyme-free products. In addition, the obtained derivative showed increased stability in simile wine conditions. After the treatment of Muscat wine with the biocatalyst for 20 days, free monoterpenes increased significantly (from 1119 to 2132 μg/L, $p < 0.01$) with respect to the control wine. Geraniol was increased 3,4-fold over its flavor thresholds, and accordingly its impact on sensorial properties was very relevant: nine of ten judges considered treated wine more intense in fruit and floral notes.

1. Introduction

Monoterpenes and norisoprenoids from grapes are important aroma compounds, especially monoterpenes compounds in white wines. However, a major fraction of them is linked to sugars, as non-volatile compounds, representing a significant reservoir of aromatic precursors to be released. These conjugated compounds can be hydrolyzed by acid or enzymatic reactions. Due to the fact that acid hydrolysis causes rearrangement of the aglycones, enzymatic hydrolysis has attracted much interest for enhancement of wine flavor because it can efficiently release the glycosidically bound compounds without modifying the aglycones (Gunata, Dugelay, Sapis, Baumes, & Bayonove, 1993). Monoterpenes are linked to β-D-glucose units or to disaccharides containing glucose and a second sugar, such as α-arabinose, α-rhamnose, and β-apiose. The sequential mechanism of enzymatic hydrolysis is now well established: in the first step, the glycosidic linkage is cleaved by either an α-arabinofuranosidase (Ara), an α-rhamnosidase (Rha), or an β-apiofuranosidase (Apio), and then a β-glucosidase (BG) liberates the monoterpenols. Endogenous glycosidases from grapes and from *Saccharomyces cerevisiae* are severely inhibited in enological conditions, so enzymes from filamentous fungi and enological yeast species have been the subject of extensive research for their possible utility in the hydrolysis of glycosidic flavor precursors (Cabaroglu, Selli, Canbas, Lepoutre, & Günata, 2003; Palmeri & Spagna, 2007; Rosi, Vinella, & Domizio, 1994). Glycosidases from *Aspergillus niger* and some yeasts have been found to possess interesting properties for practical uses in winemaking (Gunata et al., 1993, Günata, Bayonove, Tapiero, & Cordonnier, 1990). The commercial enzyme preparations from *A. niger* have been extensively used in soluble form since the 1970s in fruit processing and winemaking to improve juice clarification, juice yield and colour extraction. However, the use of soluble preparations is troublesome mainly because the extent of the hydrolysis reaction is extremely difficult to control. Enzyme immobilization on an inert carrier offers many advantages for industrial processes including the development of continuous processes with a considerable cost saving as well as better control of the enzymatic reaction. The latter is a crucial factor for the treatment of young wines, to allow a rapid and controlled liberation of terpenes, while preserving a fraction of bound aromas as a reserve, to be released with time. Last but not least, the product obtained with an immobilized biocatalyst is enzyme free.

We have examined the use of epoxy-activated acrylic beads (Eupergit C) to immobilize glycosidases from *A. niger*. Eupergit C was selected because it has been identified as one of the most useful carriers for covalent immobilization of enzymes for industrial applications in the food industry, particularly due to its ability to stabilize protein conformation by multi-point attachment (Katchalski-Katzir & Kraemer, 2000; Mateo et al., 2007).

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It is well known that commercial enzyme preparations contain in addition to multiple glycosidase activities, collateral activities (e.g. esterases) which can produce unpredictable side effects (Palmeri & Spagna, 2007). Several purification procedures for glycosidases have been developed to overcome this obstacle (Martino, Pifferi, & Spagna, 1994; Spagna, Romagnoli, Martino, Bianchi, & Pifferi, 1998; Maicas & Mateo, 2005) and the soluble purified glycosidases have been used as an analytical tool for the study of glycosides present in wine (Martino et al., 2000). However, glycosidase purification prior to immobilization is an intricate procedure that increases cost, limiting its industrial application in wines and it usually results in preparations with decreased stability (Spagna et al., 1998).

Preliminary experiments in our laboratory have shown that Eupergit C can be a good support for the immobilization of glycosidases from a commercial *A. niger* preparation, while no esterase activity is bound. Therefore, we have focused on the development of an immobilized derivative with multiple glycosidase activities (β-glucosidase, α-arabinosidase and α-rhamnosidase) from a commercial *A. niger* preparation, widely used in the wine industry. The goal was to obtain an immobilized biocatalyst with adequate levels of bound glycosidase activities and to evaluate its potential use for flavour enrichment of a white Muscat wine.

2. Materials and methods

The glycosidases (β-D-glucopyranosidase, α-L-arabinofuranosidase, α-L-rhamnopyranosidase) from *A. niger*, were contained in the commercial preparation Cytolase PCL5 (DSM Food Specialties, MA Delft, Netherlands). The synthetic glycosides used as substrates [*p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA), *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR)] and the esterase substrate *p*-nitrophenyl butyrate were purchased from Sigma (St. Louis, MO). The bicinchoninic acid (BCA) reagent was purchased from Pierce (Rockford, IL). The glucose determination kit was from Spinreact S.A. (Spain). EUPERGIT C was kindly donated by Röhm Pharma (Darmstadt, Germany). The C 18 reverse phase column (Sep-Pak® cartridge) was supplied by Waters TM (USA) and the ISOLUTE® ENV+ was purchased from Biotage AB, Uppsala, Sweden. All other chemicals used were of reagent grade.

2.1. Enzyme assays

Glycosidases were assayed using *p*-nitrophenyl (*p*NP) glycosides. Depending on whether the enzyme was soluble or immobilized, 10 mL of a solution or suspension of enzyme were added to 1.25 mL of a solution of 5.5 mM *p*NPG, 1.5 mM *p*NPA or 1.5 mM *p*NPR dissolved in 0.1 M sodium acetate buffer at pH 4.5 (activity buffer) at 23 °C. The assay was performed for 5-10 min after which 1.25 mL of 0.2 M carbonate buffer, pH 10 was added to stop the reaction and the liberated *p*-nitrophenol was determined by measuring the absorbance at 400 nm. The molar extinction coefficient used was 18.300 mol^{-1} cm^{-1}.

We defined one unit of enzyme activity (EU) as that which releases 1 μmol *p*-nitrophenol/min under the specified enzyme assay conditions.

Esterase activity was assayed at 23 °C; 20 μl of 50 mM *p*NP-butyrate in acetonitrile was added to 2 mL 50 mM sodium phosphate buffer (pH 7.0). The enzyme reaction was started by the addition of 100 μl soluble or immobilized enzyme. The color of *p*NP produced was monitored at 400 nm. (Kilcawley, Wilkinson, & Fox, 2002).

2.2. Protein determination

The protein concentration in soluble samples was assayed according to the bicinchoninic acid (BCA) procedure (Smith et al., 1987).

2.3. Immobilization procedure

Immobilization of glycosidases was carried out by incubating 0.6 mL of Cytolase PCL5 (containing the following enzyme units/mL: 19 BG; 3,0 Rha; 0,8 Ara; 0,2 esterase) in 1 M sodium phosphate buffer pH 7.0 (5 mL total volume) with 1 g suction dried Eupergit C equilibrated in the same buffer with shaking for 96 h at 23 °C. The biocatalyst was then filtered, washed with 1 M sodium phosphate buffer pH 7.0 and distilled water. The excess epoxide groups on the derivative were blocked by incubation with 3 M glycine overnight at 6 °C. The immobilized biocatalyst was stored at 4 °C in 0.1 M sodium acetate buffer at pH 4.5.

The yield and the immobilization efficiency of the process for each glycosidase were determined. Immobilization yield was calculated by taking the difference between applied and recovered activity in the supernatant and washings as a percentage of the total activity applied of each glycosidase. Immobilization efficiency was calculated as the enzyme activity detected in the immobilized preparation as a percentage of the total activity bound.

2.4. Leakage test

The immobilized biocatalyst (100 mg filter-dry gel) was incubated with 1 mL of 25 mM sodium phosphate pH 7.0 at 23 °C under shaking for 2 h. The immobilized derivative was separated by filtration and the released activity was determined in the supernatant. Then, the immobilized derivative and the corresponding soluble enzyme controls were incubated with 1 mL of 30% ethylene glycol in 50 mM sodium phosphate pH 7.0 at 23 °C for 2 h. The released enzyme activities were determined in the supernatant.

2.5. Influence of immobilization conditions in the properties of the immobilized biocatalyst

The following key immobilization conditions were individually evaluated: incubation time (in the range 1–7 days); pH (5.0–9.0); buffer salt concentration (0.5–1.4 M sodium phosphate), and protein: support ratio (35–145 mg protein/g suction dried gel). The yield and the immobilization efficiency of the process for each glycosidase were determined. The results are the average of triplicate experiments and the error bars in figures represent the standard deviation.

2.6. Stability in model wine media

The stability assay was performed at pH 3.5 and pH 4.0 and 23 °C in model wine (ethanol 12% v/v, containing 3.5 g/L of tartaric acid, 2.5 g/L of malic acid and 60 mg/L of sodium metabisulfite). The stability assay was done by incubating 10 mg of protein (soluble or immobilized) in 2 mL of model wine solution. Experiments were performed in triplicate.

2.7. Enzymatic treatment of wine

The immobilized biocatalyst (1.5 EU of BG, 0.1 EU of Rha and 0.4 EU of Ara) was incubated with a Muscat white wine of the variety Early Muscat from the 2009 vintage (750 mL adjusted to pH 4.0 with 2 M NaOH) at 23° C with agitation. At the same time a control experiment without enzyme was performed by incubating the matrix (Eupergit C with the epoxy groups previously blocked with 3 M

glycine), in the same conditions (Control wine). Both mixtures were agitated and aliquots were taken over time to perform glycosyl-glucose assays. After use, the biocatalyst was washed with 0.1 M acetate buffer at pH 4.5 and the remaining activity was measured.

2.8. Glycosyl–glucose (G–G) assay

The G–G assay was used to determine the total concentration of glycosides in Muscat wine samples in order to monitor the effect of enzymatic treatment as a function of time.

Glycoside concentration was estimated by the glycosyl–glucose assay according to the method of Williams et al., 1995. In the first step, C 18 reverse phase was activated with 10 mL methanol followed by 10 mL distilled water. A volume of 10 mL of wine was loaded on the column and washed with 50 mL distilled water. Glycosides were eluted with 1.5 mL ethanol followed with distilled water to a final volume of 5 mL.

In the second step, glycosides were hydrolyzed in acidic conditions according to the method of William et al. (1995). In the final step, released D-glucose concentration was determined by an UV spectrophotometric method using a hexokinase/D-glucose-6-phosphate dehydrogenase enzyme assay kit. For each independent experiment, G–G analysis was performed in duplicate.

2.9. Isolation of volatiles

Volatiles were quantified after adsorption and separate elution from an isolute ENV+ cartridge packed with 1 g of highly cross-linked styrene-divinyl benzene (SDVB) polymer (40–140 mm, cod. no. 915-0100-C) as previously reported (Boido et al., 2003). The cartridges were sequentially equilibrated with methanol (15 mL) and distilled water (20 mL). A sample of 50 mL of wine diluted with 50 mL of distilled water and containing 0.1 mL of internal standard (1-heptanol at 230 μg/L in a 50% hydroalcoholic solution) was applied with an appropriate syringe (4–5 mL/min) and the residue was washed with 15 mL of distilled water. The aroma components were eluted with 30 mL of dichloromethane; the solution was dried with Na_2SO_4 and concentrated to 1.5 mL on a Vigreux column, stored at −10 °C, and, immediately prior to GC–MS analysis, further concentrated to 100 μL under a gentle nitrogen stream.

2.10. Identification and quantification of aroma compounds

GC–MS analyses were conducted using a Shimadzu QP 5050 mass spectrometer equipped with reference libraries (Adams, 2001; McLafferty & Stauffer, 1991) using a BP 20 (SGE, Ringwood, Australia) bonded fused silica capillary column (25 m × 0.25 mm i.d.), coated with poly (ethylene glycol) (0.25 μm phase thickness). The extract was analyzed by GC–MS using the conditions previously described (Fariña, Boido, Carrau, Versini, & Dellacassa, 2005). The identification of the compounds was confirmed by injection of pure standards and comparison of their retention index and relevant MS-spectra. Volatile compounds were quantified by GC in the same experimental conditions as previously described for GC–MS, by the internal standard method, using 1-heptanol as the internal standard.

2.11. Wine sensory analysis

The sensory evaluation of the wines was conducted with a panel of 10 judges experts from the Group of Science and Food Technology, lab of Sensory Evaluation, Faculty of Chemistry, Montevideo, Uruguay. The assessment took place in a standard sensory analysis chamber equipped with separate booths. The wines (40 mL) were served at room temperature (20 ± 1 °C) in glasses coded with three digit random numbers and placed in a random order. In the first session, triangle tests were performed to confirm the differentiation between the control and enzyme-treated wines. The second session was a descriptive analysis aimed to evaluate wine attributes. For each test, three samples were submitted to panelists, two samples were identical and the other was different. Panelists were asked to pick out which sample they felt was different from the other two.

2.12. Statistical analysis

One-way analysis of variance (ANOVA) was carried out by using a statistical program (STATISTICA 5.1).

3. Results and discussion

3.1. Influence of immobilization conditions in the properties of the immobilized biocatalyst

Eupergit C is an epoxy-activated acrylic support, able to chemically react with nucleophile groups on the protein surface. Due to its high density of oxyrane groups, enzymes can be immobilized by multipoint attachment on this support and thus can be stabilized for their use in industrial processes (Mateo et al., 2007). The immobilization mechanism involves a first step in which a rapid physical adsorption is favored by high ionic strength, followed by a covalent reaction between the enzyme and the activated support. The process is complex, so careful design of the immobilization conditions is required (Guisán, 2006). We have studied the influence of several important variables: coupling pH, reaction time, buffer salt concentration and protein load.

3.1.1. Coupling pH

In agreement with the higher reactivity of nucleophiles, immobilization yield increased with increasing pH in the range 5–9. As shown in Fig. 1A, the three glycosidases studied, namely β-D-glucopyranosidase (BG), α-L-arabinofuranosidase (Ara), and α-L-rhamnopyranosidase (Rha), showed good immobilization yields compared with previous reports (Tu et al., 2006; Spagna, Barbagallo, Greco, Manenti, & Pifferi, 2002). However, the immobilization efficiency decreased considerably at pH over 7.0, from about 95% for the three enzymes, to 50%, 75% and 58% for BG, Ara and Rha, respectively (Fig. 1B). For this reason pH 7.0 was selected for immobilization, as a compromise between immobilization yield and the efficiency of the process.

These results highlight the interesting feature of the epoxy groups on Eupergit C and show, in agreement with previous reports (Katchalski-Katzir & Kraemer, 2000 and Mateo et al., 2007) that they can efficiently react with proteins under very mild experimental conditions. At neutral pH, the covalent reaction involves initially the amino terminal group and is followed by the side chains of lysines (the most abundant nucleophiles on the protein surface), with formation of very stable secondary amine bonds.

In the pH range 5.0–9.0 the soluble enzyme preparation was very stable under the immobilization conditions, preserving 100% of the activity of the three enzymes (BG, Ara and Rha), so the decrease in the expressed activity of the immobilized biocatalyst at pH values over 7 probably indicates extensive multipoint cross-linking of the enzymes to the matrix, leading to conformational changes and enzyme inactivation.

3.1.2. Influence of incubation period

Although enzyme adsorption to the support may be a very fast process, the establishment of covalent bonding between them is a

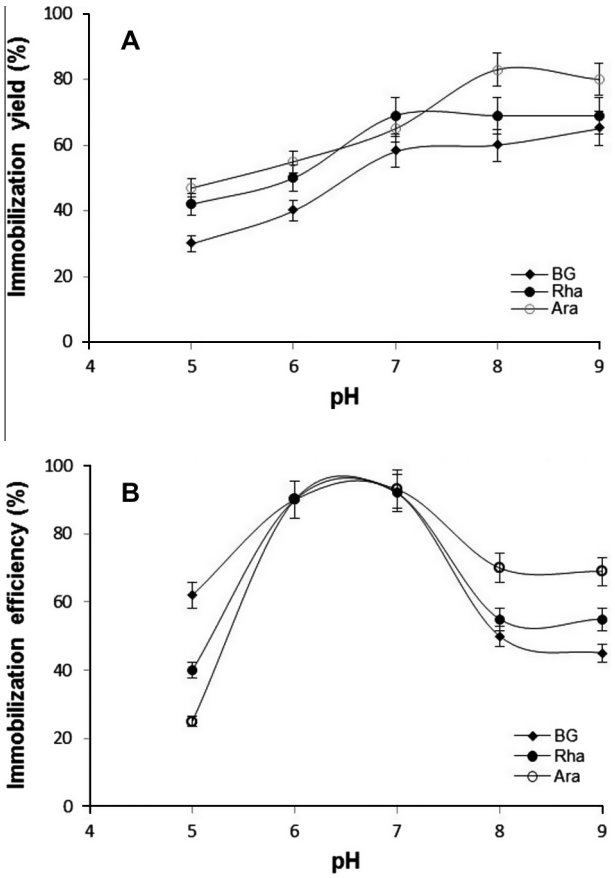

Fig. 1. Influence of coupling pH on immobilization yield (A) and immobilization efficiency (B) of the three glycosidases onto Eupergit C in 1 M sodium phosphate, after 96 h incubation time at 23 °C.

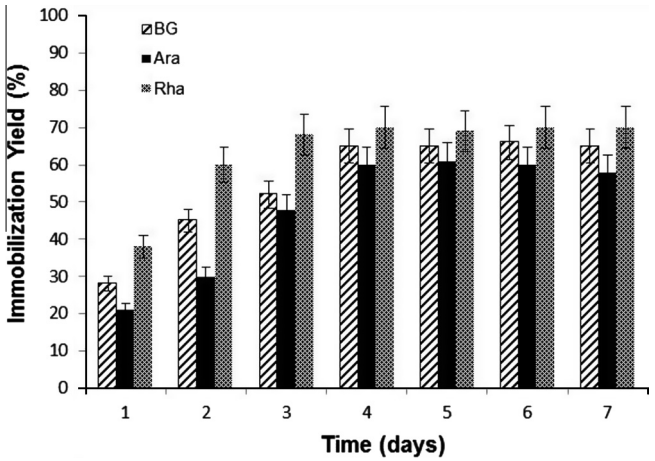

Fig. 2. Influence of coupling time on the immobilization yields of glycosidases onto Eupergit C in 1 M sodium phosphate pH 7.0 at 23 °C.

slow process, which requires the right positioning of the enzyme groups towards the rigid surface of the support (Tu et al., 2006; Mateo, Abian, & Fernandez-Lafuente, 2000). Under the conditions used, long term incubations were required to achieve high immobilization yields. As shown in Fig. 2, the immobilization yields of the glycosidase activities studied increased over the first three days reaching a maximum after 4 days of incubation, with yields of 65%, 63% and 70%, for BG, Ara and Rha, respectively. After this

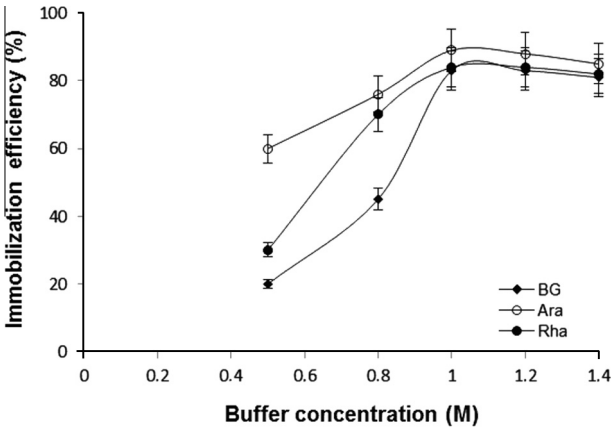

Fig. 3. Immobilization of glycosidases at different buffer salt concentrations (sodium phosphate pH 7.0) after 4 days of incubation at 23 °C.

incubation period the enzymes were covalently bound to the support, as confirmed by the absence of enzyme desorption upon treatment with low ionic strength buffer and with ethylene glycol, under the conditions reported in Methods (Leakage test).

3.1.3. Influence of buffer salt concentration

As expected considering the importance of the preliminary enzyme-support adsorption process, the immobilization yield increased with the increase of the buffer salt concentration for the three glycosidases studied. Maximum immobilization efficiency was achieved with 1 M sodium phosphate buffer pH 7.0 (Fig. 3) for all three glycosidases.

3.1.4. Influence of the protein load on the immobilization process

The effect of increasing protein loads was studied in the range 35–145 mg protein per gram of support (Table 1). At the highest protein load, the percentage of bound protein diminished, along with a slight decrease in immobilization efficiency. Therefore, for the three glycosidases, the optimum immobilization efficiency (around 90%) was achieved for the intermediate protein load (70 mg/g).

Overall, the optimum immobilization efficiency for the three enzymes studied was obtained incubating the enzyme preparation (0.15 mL, 70 mg protein/g support) at 23 °C for 4 days in 1 M sodium phosphate buffer pH 7.0. Under these conditions, the immobilized derivative contained 42 mg protein/g support expressing: 7 EU of BG, 0.3 EU of Ara, and 1.4 EU of Rha. The immobilization yields achieved (around 70%) are good compared with previous reports (Tu et al., 2006; Gallifuoco et al., 1998; Caldini, Bonomi, Pifferi, Lanzarini, & Galante, 1994).

3.1.5. Esterase activity

Esterase activity was determined in the applied soluble enzyme preparation from *A. niger* as well as in the supernatant fraction after immobilization. Interestingly, 100% of this collateral activity remained in the supernatant after the incubation period with Eupergit C, whereas no activity was detected in the immobilized derivative. A possible explanation of this experimental result is that under conditions of high protein load there would be unfavorable conditions to bind a minor enzyme component with lower affinity to the matrix.

This result is very important for applications in wine aromatization to avoid undesirable side flavors.

Table 1
Effect of protein load on the immobilization efficiency.

mg Applied protein/g support	Bound protein		Immobilization yield (%)			Immobilization efficiency (%)		
	mg/g	%	BG	Ara	Rha	BG	Ara	Rha
35	22 ± 2.1	63 ± 6.0	68 ± 7.0	85 ± 8.5	77 ± 7.5	83 ± 8.4	91 ± 9.0	100 ± 9.0
70	42 ± 4.6	60 ± 6.2	70 ± 7.1	65 ± 6.9	75 ± 7.5	89 ± 8.5	92 ± 9.0	88 ± 9.0
145	48 ± 5.1	33 ± 4.1	33 ± 3.8	43 ± 5.5	49 ± 5.2	76 ± 7.8	55 ± 6.3	80 ± 8.5

Mean ± standard deviation (S.D.).

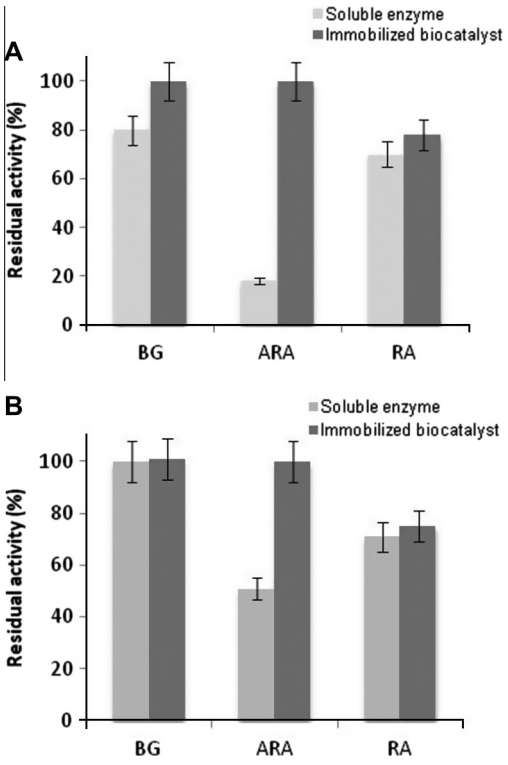

Fig. 4. Stability of glycosidases in model wine at pH 3.5 (A) and pH 4.0 (B), after 70 days of incubation at 23 °C. Model wine consisted of ethanol 12% v/v, containing 3.5 g/L tartaric acid, 2.5 g/L malic acid and 60 mg/L sodium metabisulfite.

3.2. Characterization of immobilized enzyme

A shift in pH optima following immobilization has been sometimes reported for several enzymes and is generally related to the different ionization of protein charged groups due to the carrier properties (Martino, Durante, Pifferi, Spagna, & Bianchi, 1996; Martino, Pifferi, & Spagna, 1996). However, in our work, optimum pH values for the three immobilized glycosidases (pH 4.0–4.5) were maintained with respect to the corresponding values of the soluble enzymes, and their respective pH-stability profiles were not significantly different in the pH range studied (pH 3–8). These results are in agreement with those obtained by other authors using Eupergit C as a support for the immobilization of glycosidases (Tu et al., 2006).

3.2.1. Stability in model wine

The stability of glycosidases was studied under conditions that simulate their future application in wine aromatization: model wine media at pH 3.5, at room temperature (Fig. 4A). Although the commercial soluble enzyme preparation is very stable, the immobilization process allowed further stabilization, particularly for arabinosidase at pH 3.5, in which case the residual activity

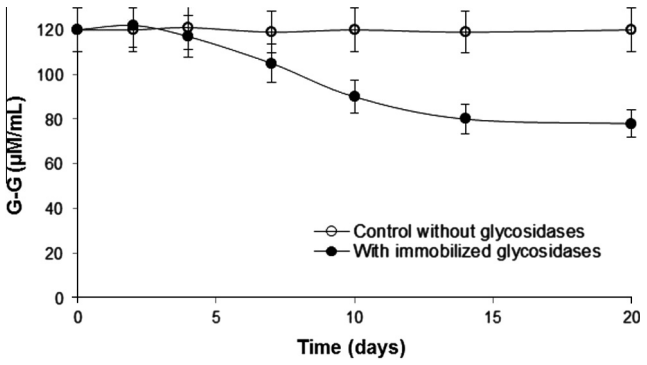

Fig. 5. Variation of glycoside content (G–G values) of Muscat wine at pH 4.0 and 23 °C, incubated with immobilized glycosidases or without glycosidases (control).

increased from 18% to 100% (stabilization factor of 5) after 70 days of incubation. The stabilization observed under stringent conditions could be an indication that the enzyme and the support are bound by multipoint attachment taking place during the long incubation period selected for immobilization.

3.3. Aromatization

3.3.1. The glycosyl–glucose (G–G) value decreases after incubation with the biocatalyst

In only twenty days, the immobilized biocatalyst produced a substantial decrease in the G–G value of Muscat wine, from 120 to 80 μM/mL (Fig. 5). During this period, the G–G value of control wine (treated with blocked matrix without enzymes) remained practically constant. After this time, the derivative was separated from the wine and the released aromas analyzed. Notably, the immobilized biocatalyst was extremely stable in Muscat wine, preserving 95% of its initial activity after the incubation period of 20 days.

3.3.2. Hydrolysis of aromatic precursors

The activity of glycosidases on the aromatic precursors of wine is expected to release volatile compounds and therefore to increase the varietal aroma of wines. As shown in Table 2, treatment of Muscat wine with the immobilized glycosidases achieved the release of monoterpenes (α-terpineol and geraniol, linalool oxides A–C) as well as norisoprenoids (vomifoliol and 3-oxo-α-ionol). ANOVA analysis demonstrates that treating wine with the immobilized glycosidases has a significant effect on the release of glycosides, showing an increase in monoterpenes (from 1143 to 2131 mg/L, $p < 0.0172$) and norisoprenoids (from 0 to 27 mg/L, $p < 0.001$) with respect to control wine. Except for the case of terpenes and norisoprenoids, the concentrations of other compounds including important aroma compounds such as esters did not change with respect to those present in the control wine.

Monoterpenes and norisoprenoids are both very important aroma compounds. After treatment with the immobilized

Table 2
Effect of immobilized glycosidases on the concentration of monoterpens and norisoprenoids in Muscat wine. Mean concentrations of compounds (μg/L) and relative standard deviations ($n = 3$).

Aromatic compounds	Descriptor	Odor threshold (μg/L)	Control wine without glycosidases (μg/L)	Wine treated with immobilized glycosidases (μg/L)	Significance (p value)
Linalool	Rose	50[a]	555 ± 86	615 ± 25	n.s.
α-Terpineol	Floral, pine	400[a]	182 ± 20	246 ± 17	<0.05
Geraniol	Fruit, floral	130[a]	98 ± 11	438 ± 26	<0.001
Oxide A (trans-furanic of linalool)	Leafy, sweet, floral, creamy, earthy	>6000[b]	47 ± 15	213 ± 79	<0.05
Oxide B (cis-furanic of linalool)	Leafy, sweet, floral, creamy, earthy	>6000[b]	28 ± 9	100 ± 3	<0.001
Oxide C (trans-piranic of linalool)	Leafy, sweet, floral, creamy, earthy	3000-5000[b]	151 ± 53	386 ± 28	<0.01
Oxide D (cis-piranic of linalool)	Leafy, sweet, floral, creamy, earthy	3000-5000[b]	59 ± 12	135 ± 56	n.s.
Total terpenes			1119 ± 182	2132 ± 211	<0.01
Vomifoliol	Dried fruit, raisins	_	nd	20 ± 3	<0.001
3-Oxo-α-ionol	Honey, apricots	_	nd	7 ± 1	<0.001
Total norisoprenoids			nd	27 ± 3	<0.001

nd: Below the limit of detection, ns: not significant.
[a] Riberau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, & D. (1998). Handbook of Enology, vol. 2, The chemistry of wine. Stabilization and treatments (2nd ed.), Wiley.
[b] Ribérau-Gayon. P., Boidron, J. N., Terrier, A. (1975) Aroma of muscat grape varieties. J Agricultural Food Chem 23, 1042–1047.

glycosidases, the concentration of practically all terpenes increased significantly (Table 2). The case of geraniol is of special relevance since it reached its odor threshold after treatment surpassing it by a factor of 3.4.

The impact of these compounds on wine properties was evaluated by sensory analysis, carried out by a panel of expert wine tasters. In the triangle test performed, all judges (10/10) found significant differences between the control and enzyme-treated wine ($p < 0.000$). Nine panelists (9/10) found that the enzyme-treated sample was more intense in fruit and floral attributes than control wines. This confirms that the increase in the concentration of terpenes had an impact in the wine aroma.

So, immobilization of this commercial enzyme preparation onto Eupergit C under the selected conditions fulfills the objective of obtaining an efficient biocatalyst to hydrolyze conjugated aroma compounds, without unwanted collateral activities. Since it is widely known that immobilized biocatalysts have inherent advantages over soluble enzyme preparations, this derivative is promising for technological applications. Notably, one of the most well-known drawbacks of the use of soluble commercial preparations is the impossibility to control the hydrolysis process, as well as the generation in wine of undesirable compounds; both problems were overcome by using this immobilized biocatalyst.

4. Conclusions

We have developed a simple and cost-effective procedure for the co-immobilization of multiple glycosidase activities (β-D-glucopyranosidase, α-L-arabinofuranosidase, and α-L-rhamnopyranosidase). The three glycosidases were efficiently immobilized and showed excellent stability in wine conditions.

This work highlights the importance of exogenous glycosidases for the enhancement of wine flavour. Thus, after a very short period (20 days) the amount of free monoterpenes in treated Muscat wine increased significantly and its impact on sensorial properties was very relevant: nine of ten judges considered treated wine more intense in fruit and floral aroma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.07.107.

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