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Immobilisation of glycosidases from commercial preparation on magnetic beads. Part 2: Aroma enhancement in wine using immobilised glycosidases

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Summary

Most of the terpenes in wines are conjugated to various sugars, representing a significant reservoir of aromatic precursors. To promote the release of these terpenes, certain enzymes, such as β-glucosidase, α-arabinosidase and α-rhamnosidase, are necessary. A simple and cost-effective procedure for the immobilisation of multiple glycosidase activities (β-D-glucopyranosidase, α-L-arabinofuranosidase, α-L-rhamnopyranosidase and β-D-xylopyranosidase) from commercial *Aspergillus niger* preparation onto magnetic beads as carriers was developed as reported in Part 1 (Ferner *et al.* 2016).

The aim of this work was to analyse a possible application of this immobilised biocatalyst due to its well-known advantages over soluble enzyme preparations – that is, control of the reaction process and preparation of enzyme-free products. Volatile compounds were analysed by gas chromatography (mass spectrometric detection). After the treatment of the model wine with different glycosides and white wine with immobilised glycosidases, the amount of free terpenes was significantly increased with respect to that of the control wine.

The results of this study are of considerable interest for possible future applications of immobilised enzymes in the wine-making industry.

K e y w o r d s : immobilisation; magnetic beads; glycosidases; magnetic separation; wine; aroma enhancement; enzyme assay; enzyme stability; sensory analysis.

Introduction

Wine is an ancient, manifold beverage that is a part of many cultures. In particular, the flavour components are among the major factors that define the nature and quality of wines. There are many factors that influence the flavour composition, including the grape variety, culture practices, soil type and climate (Rapp 1992, Styger *et al.* 2011). The sensory perception of wine is very complex and is formed by a variety of taste and odour-active compounds, such as terpenes, esters, aldehydes and methoxypyrazines (Styger *et al.* 2011). In particular, terpenes play a major role in wine aroma; they are responsible for the dominant flavours of the so-called bouquet varieties, such as Muscat, 'Gewürztraminer' and 'Morio Muskat' (Berger 2007, Meinl *et al.* 2009). These compounds can be present as either bound or free glycosides in grapes or wine. Glycosidically bound monoterpenes occur more frequently than the free ones (Günata *et al.* 1985a). In particular, for Muscat, monoterpene aroma occurs in up to 90 % of glycosidically bound monoterpenes (Park *et al.* 1991). Although glycosidic monoterpenes have no direct influence on the flavour of grapes and wines, they can be released by specific glycosidases or acid hydrolysis (Mateo and Jiménez 2000, Rapp 1992). Nevertheless, acid hydrolysis causes the rearrangement of aglyca; thus, selective enzymatic hydrolysis is much more interesting for the enhancement of wine flavour without modification or rearrangement of aglyca (González-Pombo *et al.* 2014, Günata *et al.* 1993).

In particular, β-glycosidases cause the release of naturally glycosidically bound aroma (Caldini *et al*. 1994, Günata *et al.* 1990, Hernández *et al.* 2003, Mendes Ferreira *et al.* 2001). Numerous published studies have reported that the use of enzyme preparations or exogenous β-glucosidases can increase the aroma components in wine (Caldini *et al.* 1994, González-Pombo *et al.* 2014, Martino *et al.* 2000).

The aroma compounds are usually bound to diglycosides and β-glucosidases can only act on monoglucosides. However, the release of aglyca is in most cases a sequential mechanism, meaning that other glycosidases, such as arabinosidase, rhamnosidase or xylosidase, are required to release the aroma (Günata *et al.* 1988). The first step is the cleavage of the inter-sugar linkage by a corresponding enzyme, such as α-arabinofuranosidase (Ara), α-rhamnosidase (Rha) or β-xylopyranosidase (Xyl). Only after this cleavage occurs, β-glucosidase (βG) can act and liberating the corresponding aglycone (Günata *et al.* 1988).

Natural enzyme activities originating from grapes or present in fermentation yeasts (e.g. *Saccharomyces cerevisiae*) can cleave sugars and release aroma. However, these enzymes are inhibited under typical wine conditions (acidic pH and glucose). Thus, enzymes from filamentous

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fungi have been the subject of research in recent decades to identify their possible use in the hydrolysis of glycosidically bound flavour precursors (Cabaroglu *et al.* 2003, Palmeri and Spagna 2007, Rosi *et al.* 1994). Since the 1970s, commercial enzyme preparations have been extensively used in fruit processing and wine making for maceration and/or clarification (González-Pombo *et al.* 2014). To date, winemakers use enzyme preparations, including numerous commercial enzymes, for various applications, such as improving or releasing flavour. The latter enzyme preparations are inactivated and precipitated by bentonite fining. In addition to the loss of active enzymes, this step incurs costs for winemakers because new enzyme preparations must be used for each new fermentation batch. Furthermore, residual enzyme activity is retained in the wine, which causes the flavour balance of treated wines to deteriorate after six months to one year. Therefore, complete removal of the enzymes is crucial. Enzyme immobilisation on magnetic particles could potentially be used to address these problems. In particular, the complete removal by magnetic separation and subsequent reuse of enzymes provide important benefits for winemakers. Thus, enzyme immobilisation on a magnetic carrier offers many advantages for fruit processing and wine making, including control of the reaction process and preparation of enzyme-free products. The control of the enzymatic reaction is an important factor for the treatment of wines for the rapid and controlled liberation of aroma precursors.

In the last few years, several purification procedures have been published for immobilising commercial enzyme preparations, including glycosidases [17-21]. However, purification of the enzymes prior to immobilisation is a complicated process that increases costs and limits their industrial applications. This process can also decrease enzyme stability [22]. In a previous paper, a simple method for the immobilisation of these enzymes from *Aspergillus niger* (including β-glucosidase, α-arabinosidase and α-rhamnosidase), which were present in a commercial enzyme preparation was described (Ferner *et al.* 2016). The goal of the present study was the application of obtained immobilised biocatalyst and to evaluate its potential use for flavour enrichment in wine with complete magnetic separation of the immobilised biocatalyst from wine after enzymatic reaction. We have examined the use of magnetic microparticles (M-PVA magnetic beads) to immobilise glycosidases from *A. niger*.

Material and Methods

Chemicals and reagents: All of the chemicals that were used were of analytical grade if not otherwise specified. Pentane, methanol (HPLC grade) and dichloromethane (analysis) were obtained from VWR (Darmstadt, Germany). Absolute ethanol, citric acid, sodium hydroxide and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Sodium carbonate was obtained from Th. Geyer (Renningen, Germany). The synthetic substrates p-nitrophenyl β-D-glucopyranoside (pNPG) and p-nitrophenyl β-D-xylopyranoside (pNPX) were purchased from Carl Roth (Karlsruhe, Germany), while p-nitrophenyl α-L-arabinofuranoside (pNPA) and p-nitrophenyl α-L-rhamnopyranoside (pNPR) were purchased from Carbosynth (Berkshire, UK) and used for activity assays. The carrier, M-PVA C22 magnetic beads, was obtained from PerkinElmer Chemagen Technologie (Baesweiler, Germany). 2-(N-Morpholino)ethane sulphonic acid monohydrate (MES) was purchased from Applichem (Darmstadt, Germany), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Merck (Darmstadt, Germany). Rapidase Revelation Aroma from Oenobrands (Montpellier, France) was used for commercial enzyme preparation. Membrane filters (0.2 μm, PET) were obtained from Macherey-Nagel (Düren, Germany).

The following chemical standards were used to identify and quantify the aroma compounds: linalool, linalooloxide, β-citronellol, geraniol and nerol, which were obtained from Sigma-Aldrich (Taufkirchen, Germany). α-Terpineol and the internal standard 4-nonanol were purchased from ABCR (Karlsruhe, Germany). The standards were dissolved in methanol with a concentration of 1 $g \cdot L^{-1}$ and stored at 26 °C until analysis. These solutions were stable for several months at -26 °C, and stock solutions were diluted just before measuring. The C18 reverse phase column Bond Elut C18 (500 mg) and Bond Elut ENV (200 mg) cartridge were purchased from Agilent (Waldbronn, Germany). Unless otherwise specified, twice distilled water was used for all of the experiments.

Enzyme assays: p-Nitrophenyl (pNP) glycosides were used to determine the activity of immobilised enzymes (Blondin *et al.* 1983). 0.1 mL of the enzyme suspension was added to 0.9 mL of a solution containing 5 mM pNPG or 1.5 mM pNPA, pNPR or pNPX dissolved in 0.1 M citrate/phosphate buffer. The assay was performed at pH 5.0 and 37 °C for 5 min. Then, 2 mL of 1 M carbonate buffer was added to stop the enzymatic reaction and to develop the yellow colour that was released by the p-nitrophenolate ions. Before measuring the absorbance of the released p-nitrophenolate ions, the insoluble beads were removed by membrane filtration and the absorbance of the obtained solution was measured. The liberated p-nitrophenol was measured against a blank at a wavelength λ of 400 nm and calculated using the molar extinction coefficient of *p*-nitrophenol (18,300 $M^{-1} \cdot cm^{-1}$). The activity was expressed in kat, where one kat is the amount of enzyme that releases one mole of p-nitrophenolate per second under the assay conditions. All of the experiments were performed in triplicate.

Immobilisation procedure: The commercial enzyme preparation Rapidase Revelation Aroma (free enzyme) was used for immobilisation onto magnetic microparticles. Superparamagnetic beads (M-PVA C22 magnetic beads) consisting of a polyvinyl alcohol matrix, which was subsequently carboxylated for protein binding, were used as magnetic carriers for immobilisation. The high magnetite content of the beads permits their rapid separation following their application. The beads had a polydisperse size distribution (1-3 μm). The immobilisation was performed according to the procedure in Part 1 (Ferner *et al.* 2016).

Aromatisation: Aromatisation was determined according to previous reports (Spagna *et al.* 2000 and 2002) with slight modifications. The glycosidic compounds from wine were isolated using

C18 reversed phase cartridges (Bond Elut C18, Agilent, 0.5 g of phase). The method that was proposed by (Williams *et al.* 1995), (Schneider *et al.* 2004) and (Arévalo Villena *et al.* 2006) was slightly modified. Each cartridge was pre-conditioned with 20 mL of methanol and 20 mL of high-purity water. Thereafter, 100 mL of the wine was loaded onto a cartridge. The cartridge was washed with 50 mL of water to eliminate sugar and with 10 mL of pentane/dichloromethane (2/1, v/v) to elute the free volatiles. The bound aroma fraction was finally eluted with 20 mL of methanol, and the extract was concentrated to dryness at 40 °C under vacuum. The obtained glycoside extract was stored at -26 °C until analysis.

For aromatisation, the glycoside extract of 500 mL of wine was redissolved in 100 mL of a model wine solution containing 9 $g \cdot L^{-1}$ tartaric acid in 10 % ethanol and adjusted to pH 3.5 with a sodium hydroxide solution (2 M). Twenty milligrams of the immobilised enzymes or fifteen milligrams of the soluble enzyme were added to 100 mL of the model wine solution containing the aromatic precursors, and the mixture was placed in a test tube. The tube was sealed and incubated at 25 °C for 24 h.

Enzyme stability and leakage test: A stability test for the immobilised enzymes was performed in wine adjusted to pH 3.5 with a sodium hydroxide solution (2 M) at room temperature. The stability assay was performed by incubating 210 mg of the beads in 30 mL of the solutions. Aliquots of the enzyme suspension with a concentration of 7 $mg \cdot mL^{-1}$ immobilised catalyst were taken at regular intervals and the activity was measured as described in the section "Enzyme assays". The activity of the immobilised glucosidase was determined over time as the percentage of change in the initial activity (at pH 5.0 and 37 °C). The leakage was determined by magnetically separating the beads and subsequently measuring the β-glucosidase activities of the supernatant as described in the section "Enzyme assays" (at pH 5.0 and 37 °C). All of the experiments were performed in triplicate.

Enzymatic treatment of wine: The soluble and immobilised enzymes were incubated with Morio Muskat from a 2014 vintage. To obtain comparable conditions, the pH of the wine was adjusted to pH 3.5 with 2 M NaOH. Fifteen milligrams of the immobilised enzymes or five milligrams of the soluble enzyme were added to 100 mL of both wines which corresponds to 24.5 nkat respectively 12.2 nkat per 100 mL β-glucosidase activity at pH 5.0 and 37 °C. The relatively higher proportion of β-glucosidase activity of the immobilised enzyme was to compensate the lower immobilisation yield of the arabinose, 0.9 nkat for the immobilised enzymes compared to 28.5 nkat in the commercial product per 100 mL wine.

The wine was incubated at 25 °C for 22 d. At the same time, a control wine without enzyme or magnetic support was performed to exclude a possible acid-catalytic release of glycosidically bound aroma compounds. For the sensory analysis, 1.5 g of the immobilised enzymes were added to 10 L of the wine and incubated accordingly.

The separation of the immobilised enzymes was performed using a high gradient magnetic separator (HGMS). The wine with the immobilised enzymes flows through the filter chamber, whereby theoretically only the magnetisable particles with the immobilised enzymes retard selectively within the HGMS filter, due to the magnetic forces acting through an applied magnetic field. The HGMS laboratory plant consisted of an electrical magnetic separator (Vulkan Technic Maschinen-Konstruktions GmbH, Wiesbaum, Germany; and PerkinElmer chemagen Technologie GmbH, Baesweiler, Germany), pump (IDEX Europe GmbH, Erlangen, Germany; and Masterflex SE, Gelsenkirchen, Germany), flow meter and a process control system. The high-gradient magnetic separator was equipped with a rotor-stator filter matrix. The wine suspension with the magnetic particles were pumped with a velocity of 400 $mL \cdot min^{-1}$ through the filter chamber. The retained magnetic particles form dense aggregations on the filter matrix. All other (non-magnetisable) constituents of the wine and solutes pass the filter unhindered. The quantification of the separation efficiency of the magnetic particles was performed through an analysis of the off-line samples by a flow cytometer (CyFlow Space from Sysmex Partec GmbH, Görlitz, Germany). The system was calibrated for suspensions with white wine. More detailed information of the separation process are described by Shaikh *et al.* (2016).

Isolation of volatiles. Solid-phase extraction (SPE): Volatiles were quantified after solid-phase extraction (SPE) from the Bond Elut-ENV cartridge (200 mg) as previously reported (López *et al.* 2002, Piñeiro *et al.* 2004). The cartridges were conditioned sequentially with dichloromethane (4 mL), methanol (4 mL), and finally 4 mL of a water-ethanol mixture (12 %, v/v). A volume of wine (50.0 mL) was spiked with the stock internal standard solution (50 µL) to produce a concentration of 100 $\mu L \cdot L^{-1}$ of the internal standard. The sample was passed through the preconditioned SPE column at 2-3 $mL \cdot min^{-1}$, after which the cartridge was washed with water (10 mL). Thereafter, the sorbent was dried by passing air through it (-0.6 bar, 10 min). The free volatile aroma compounds were eluted performed with 2 mL of dichloromethane. The extract was then hermetically capped and stored at -26 °C until GC-MS analysis. One microliter of extract was injected into the GC-MS in splitless mode.

Identification and quantification of the aroma compounds: Gas chromatography-mass spectrometry (GC-MS) analysis was performed using a Perkin Elmer gas chromatograph model Clarus 680 coupled to a mass selective detector (MSD) Clarus SQ 8 with a split/splitless injector (PerkinElmer, Rodgau, Germany). The injector was set at 180 °C. The flavour compounds were separated on ZB-WAX (cross-linked polyethylene glycol capillary; length: 30 m × 0.25 mm i.d.; 0.25 µm film thickness) from Phenomenex (Aschaffenburg, Germany). Helium was the carrier gas, and the flow rate was set to 1.0 $mL \cdot min^{-1}$ for liquid injection. The oven temperature was programmed as follows: 60 °C as the initial temperature and held for 5 min. Next, the temperature was increased to 120 °C at 1.5 $°C \cdot min^{-1}$, then to 180 °C at 5 $°C \cdot min^{-1}$ and finally to 220 °C at 20 $°C \cdot min^{-1}$ and held for 10 min. The source temperature was 220 °C, and the inlet line temperature was 200 °C. For the MS system, the temperatures of the transfer line and ionization source were 200 °C and 220 °C,

respectively: Electron impact mass spectra were recorded at 70 eV. The acquisitions were performed in the scan mode (from 50 to 350 m/z). The compounds were identified by comparing the retention times and mass spectra (NIST 11.0) with those of the standards. Quantitative data were obtained by calculating the peak area relative to that of the internal standard (4-nonanol).

Calibration: Six calibration solutions containing all of the analytes in the concentration range from 1 to 100 $mg \cdot L^{-1}$ and the internal standard at a fixed concentration (10 $mg \cdot L^{-1}$) - corresponding to 10-1000 $\mu g \cdot L^{-1}$ and 100 $\mu g \cdot L^{-1}$ in wine, respectively - were prepared in pentane by an appropriate dilution of the stock solution containing all of the analytes (1 $g \cdot L^{-1}$) and by the addition of the internal standard solution.

Wine sensory analysis: The sensory evaluation of the Morio Muskat was conducted with a panel of 10 expert judges from the DLR Mosel (Bernkastel-Kues, Germany). The sensory tests took place in a standard sensory analysis room. The wine was served at 12 °C (± 1) in glasses coded with three digit random numbers. During a duo-trio test, each panellist was offered three samples simultaneously. Two samples were equal, and one as different. The researcher indicated one of the equal samples as the reference sample. The panellist was asked which sample was equal to the reference sample.

The second session was a descriptive analysis aimed to evaluate differences in the following wine attributes: for flavour: flavour intensity, apple, passion fruit, lemon, floral, honey, fruity, and aftertaste; for odour: odour intensity, apple, passion fruit, lemon, floral, and fruity.

The Panellists were asked to first rate from 10 to 70 (10 = weak, 70 = strong) the odour attributes of the wine, followed by an oral evaluation of the sample. All of the sensory data were collected using EyeQuestion software (Version 3.8.13, Logic8 BV, Elst, Netherlands).

Statistical analysis: The statistical methods that were used for the sensory data analysis were two-way analysis of variance (ANOVA) and Least Significance Difference (LSD) test for the mean comparison of the aroma data. The EyeQuestion software was used for sensory data processing (Version 3.8.13, Logic8 BV, Elst, Netherlands).

The statistical method that was used for the other data analyses was one-way analysis of variance (ANOVA) and was carried out using the Microsoft Office Excel (2013) Add-In Analysis ToolPak.

Results and Discussion

Immobilisation: The commercial enzyme preparation was successfully immobilised on magnetic beads without any sample clean up. The detailed results are published in Part 1 (Ferner *et al.* 2016). βG showed an immobilisation yield and an immobilisation efficiency of 92.0 % and 67.9 %, respectively; for Ara, these values were 11.7 % and 6.8 %, respectively; for Rha, these values were 16.3 % and 72.7 %, respectively; and for Xyl, these values were 59.2 % and 57.3 %, respectively (Ferner *et al.* 2016).

Storage stability in wine and magnetic separation: The stability of the immobilised enzymes was studied under conditions that simulated their future application in wine. The storage stability of the commercial enzyme product was not interesting, because these kind of products are irreversibly deactivated after the enzymatic reaction and cannot be reused.

Storage stability in wine: Fig. 1 shows that there was almost no activity loss for the immobilised βG in wine at pH 3.5, which slightly decreased after approximately 10 d around 10 %. The immobilised Rha decreased to 74.0 % of its activity in wine after 70 d. Ara also showed a slight decrease in activity of 20.5 % and Xyl by 20.4 %.

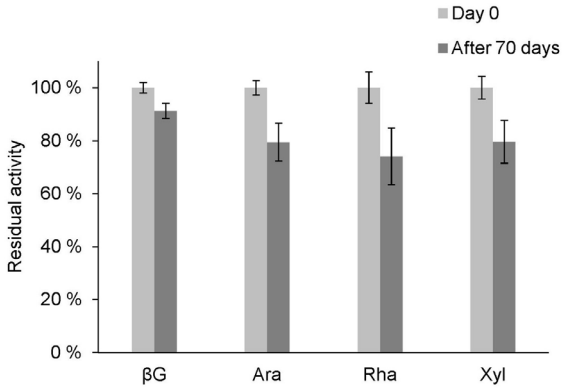

Fig. 1: Stability of the immobilised glycosidases in wine (pH 3.5) after 70 d of incubation at room temperature, the activity was measured at pH 5.0 and 37 °C.

In every case, no release of bound enzyme was observed. Furthermore, after the magnetic separation of the beads, the supernatants in every case showed no activity. Thus, the reduction in activity is by inactivation of enzymes and not due to loss of enzymes.

Compared to other studies, the immobilised glycosidases herein showed a significantly improved stability. Martino *et al.* (1996) referenced a residual activity of the immobilised β-glucosidase in wine (pH 3.2 and 25 °C) of 80 % after 55 d (Martino *et al.* 1996). Spagna *et al.* (1998a) found a stability of approximately 70 % in wine after 18 d for an arabinosidase and a residual activity of approximately 50 % for β-glucosidase (Spagna *et al.* 1998a). For rhamnosidase, Spagna *et al.* (2001) observed a decrease in activity in wine (25 °C) of approximately 50 % after 50 d (Spagna *et al.* 2001 and 2002) observed a residual activity in wine (25 °C) of 88 % for β-glucosidase and 48.8 % and 43.5 % for an arabinosidase and rhamnosidase, respectively, after 50 d (Spagna *et al.* 2002).

Magnetic separation: In the separation experiments it could be seen that there was no significant loss of activity of the coupled β-glucosidase by the separation with the HGMS and the associated shear forces. After 6 separations, the activity of β-glucosidase coupled to magnetite particles was still close to 100 % (Fig. 2). Pan *et al.* (2009) observed a similarly good reusability for a β-galactosidase coupled to magnetic chitosan particles. Thus, the magnetic separation did not affect the activity of the coupled β-glucosidase.

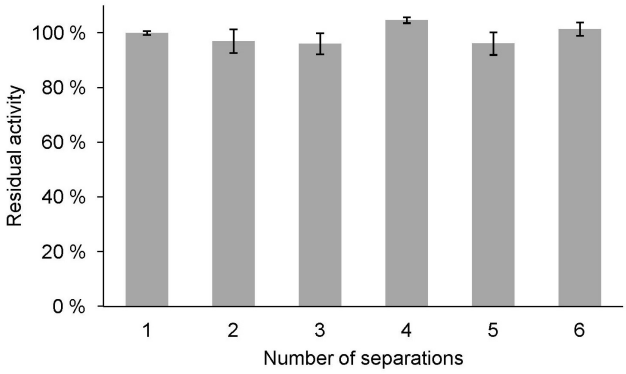

Fig. 2: Residual activity (%) of the immobilised βG after 6 separations with HGMS from wine (c particle) = 0.15 g·L^{-1}), the activity was measured at pH 5.0 and 37 °C.

The separation efficiency obtained was 99.57 % (± 0.098), it could be recovered almost 100 % of the magnetite particles used. Therefore, no negative influence on the immobilised enzymes by a magnetic separation is assumed and they can be reused much more than six times without having to expect a loss of activity. In addition, nearly 100 % of the added particles could be recovered from the wine. By another, usual in winemaking, filtration, shortly before bottling, any possible non-retained particles should be completely removed from the wine, so that the wine complies with the legal requirements.

Aromatisation: As previously mentioned, the activity of glycosidases on the aromatic precursors of wine is expected to release volatile compounds and therefore to increase the varietal aroma of wine. The glycosidases could release a considerable amount of terpenes in model wine and white wine ('Morio Muskat', 2014).

Hydrolysis of aromatic precursors in model wine: Both free and immobilised enzymes could release a high amount of total terpenes from the model wine solution (Tab. 1), although the action of the immobilised enzymes was lower (by approximately 50 %), at 1965.7 µg·L^{-1}, compared to 3912.9 µg·L^{-1} by the free enzymes. In particular, nerol and geraniol could be increased to a considerable extent. Nerol was increased up to 566.2 µg·L^{-1} by the immobilised glycosidases and to 1315.9 µg·L^{-1} by the free glycosidases. For geraniol, there were increases of 1322.8 µg·L^{-1} and 2217.6 µg·L^{-1}, respectively. The different rates of increase of the individual terpenes depended on the glycoside composition that was used. In this case, the glycoside extract that was used was obtained from a 'Morio Muskat', as this variety is particularly rich in glycosidically bound aroma substances.

Thus, both free and immobilised enzymes were capable of releasing a large amount of total terpenes from the model wine solution. However, the release of terpenes of the immobilised enzyme was lower by approximately 45 % compared to that of the free enzyme. Spagna *et al.* (1998a and 2002) also found a higher rate of increase in a model wine solution by free glycosidases compared to that by immobilised glycosidases (Spagna *et al.* 1998a and 2002). However, the difference was much lower, approximately 10 %. The lower release by the immobilised enzymes could be explained by the composition of the enzyme mixture of the glycosidases changing during immobilisation. The relative portion of β-glucosidases was higher for the immobilised biocatalyst. The release of the fragrant aglyca is a sequential mechanism; thus, the other glycosidases, such as Ara, Rha and Xyl, are important for the cleavage of the aromatic precursors.

Both the free and immobilised glycosidases act nearly similarly, the ratios of flavours with each other are approximately equal except of cis- and trans-linalooloxid. Other studies have reported a drastic reduction in the selectivity of the immobilised enzymes with respect to their free forms (Spagna *et al.* 1998a and 2001). This decrease may be attributed to diffusion problems and to the reduced accessibility of the tertiary alcohols (steric hindrance) to the active site of enzymes, which are immobilised on a support by the methods that were used in those studies.

Hydrolysis of aromatic precursors in white wine: As shown in Tab. 2, the treatment of white wine with glycosidases has a significant effect on the release

Table 1

Terpenols that were present in the model wine solution alone (control) and following treatment with the free and immobilised glycosidases

Aromatic compounds	No enzyme (µg·L^{-1})	Free enzyme (µg·L^{-1})	Immo. enzyme (µg·L^{-1})	Significance (*p* value)
cis-Linalooloxide	n.d.	85.7 ± 7.3	8.3 ± 2.5	< 0.001
trans-Linalooloxide	n.d.	149.0 ± 5.5	8.4 ± 3.3	< 0.001
Linalool	n.d.	32.4 ± 3.1	16.6 ± 1.2	< 0.05
α-Terpineol	n.d.	51.6 ± 4.6	13.1 ± 2.5	< 0.05
β-Citronellol	n.d.	58.9 ± 5.1	30.3 ± 3.7	< 0.05
Nerol	n.d.	1315.9 ± 13.6	566.2 ± 8.0	< 0.01
Geraniol	n.d.	2217.6 ± 29.1	1322.8 ± 15.3	< 0.05
Total terpenes		3912.9 ± 68.3	1965.7 ± 36.5	< 0.05

Mean ± standard deviation (S.D.).

Table 2

Effect of glycosidases on the concentration of monoterpenes in Morio Muskat wine. Mean concentration of the compounds (μg·L^{-1}) and standard deviation (n = 3)

Aromatic compounds	Control wine without glycosidases (μg·L^{-1})	Wine treated with free glycosidases (μg·L^{-1})	Significance (*p* value)	Wine treated with immobilised glycosidases (μg·L^{-1})	Significance (*p* value)
cis-Linalooloxide	55.8 ± 1.7	56.4 ± 1.3	n.s.	59.7 ± 6.6	n.s.
trans-Linalooloxide	26.0 ± 1.3	27.5 ± 2.1	n.s.	26.4 ± 1.3	n.s.
Linalool	97.4 ± 2.2	111.6 ± 5.7	< 0.01	110.7 ± 6.0	< 0.05
α-Terpineol	83.9 ± 3.8	93.9 ± 2.3	< 0.01	102.2 ± 15.9	n.s.
β-Citronellol	17.3 ± 0.6	20.8 ± 0.8	< 0.001	21.5 ± 4.5	n.s.
Nerol	14.7 ± 0.3	50.8 ± 2.4	< 0.001	42.8 ± 8.8	< 0.01
Geraniol	41.5 ± 1.3	198.7 ± 10.6	< 0.001	149.7 ± 32.3	< 0.01
Total terpenes	336.6 ± 11.1	559.9 ± 23.2	< 0.001	512.9 ± 75.4	< 0.01

n.s.: not significant; Mean ± standard deviation (S.D.);

of aglyca and therefore shows an increase in terpenes with respect to the control wine. The terpene level increased from 336.6 μg·L^{-1} to 512.9 μg·L^{-1} (152.4 %) after treating the wine with immobilised glycosidases and to 559.9 μg·L^{-1} (166.3 %) after treating the wine with free glycosidases. González-Pombo *et al.* (2014) observed a similar increase in the total terpenes by immobilised glycosidases, from 1119 μg·L^{-1} to 2132 μg·L^{-1} (190.5 %) (González-Pombo *et al.* 2014). The growth rate of linalool, 114.6 % by free glycosidases and 113.7 % by immobilised glycosidases, is comparable to that of González-Pombo *et al.* (2014), who observed a growth rate of 110.8 % (González-Pombo *et al.* 2014). In Particular, nerol and geraniol could be increased to a considerable extent. Nerol was increased from 14.7 μg·L^{-1} to 50.8 μg·L^{-1} by the free and to 42.8 μg·L^{-1} by the immobilised glycosidases. For geraniol, increases from 41.5 μg·L^{-1} to 198.7 μg·L^{-1} (478.8 %) and 149.7 μg·L^{-1} (360.7 %), respectively, were found. Again González-Pombo *et al.* (2014) detected a similar increase for geraniol from 98 μg·L^{-1} to 438 μg·L^{-1} (446.9 %) (González-Pombo *et al.* 2014). Although free and immobilised glycosidases act similarly, other studies have reported a drastic reduction in the selectivity of the immobilised enzymes with respect to their free forms (Spagna *et al.* 1998a and 2001). This difference may be attributed to the diffusion problems and reduced accessibility of the tertiary alcohols (steric hindrance) to the active site of enzymes, which are immobilised on a support by methods that were used in those studies.

The treatment of white wine with glycosidases had a significant effect on the release of flavours; terpenes were significantly increased compared to those in the control wine. Terpenes are very important aroma compounds for varietal wine aroma. There was a larger relative increase in primary terpene alcohols (nerol, geraniol) than in tertiary ones (linalool, α-terpineol). This behaviour may be due to the primary terpene alcohols that are present in Muscat grapes in an essentially bound form (Günata *et al.* 1985b) and because βG is less selective toward tertiary alcohols (Günata *et al.* 1990). This results can be explained by considering that tertiary alcohols have a larger steric hindrance than primary alcohols and are less accessible to the catalytic site of the enzyme (Spagna *et al.* 1998b).

Furthermore, the increase in aroma compounds by immobilised enzymes was slightly lower than that by free enzymes, but the difference was not significant. The difference was only significant for linalool, geraniol and nerol.

Nevertheless, the immobilisation of this commercial enzyme preparation onto magnetic beads fulfils the objective of obtaining an efficient biocatalyst to hydrolyse the conjugated aroma compounds of wine.

The difference in increase of the aroma compounds in the model wine compared to the wine could possibly be explained by the fact that in the used wine nearly all glycosidically bound aroma compounds were released by enzymatic treatment with free or immobilised glycosidases. In the model wine, on the other hand, an abundance of glycosidically bound flavors was present and the free or immobilised glycosidases could not release all glycosidically bound aroma compounds.

Sensory analysis: In almost all of the examined terpenes, an increase by enzyme treatment was observed. Because of the considerable rates of increase in the enzyme treated wines, a corresponding improvement in the sensory odour and flavour characteristics is expected. The impact of the released aroma compounds on the wine properties was evaluated by sensory analysis. For this, a 'Morio Muskat' treated with immobilised glycosidases was compared to untreated 'Morio Muskat' (control wine) by 10 judges. In the triangle test, 8 of 10 judges found significant differences between the control and enzyme-treated wine. The panellists found that the enzyme-treated wine was more intense in fruit and floral attributes than the control wine (Tab. 3). The treated wine showed a higher odour intensity of 64.5 compared to that of the control sample, at 40.3. The odour attributes apple, passion fruit, lemon and floral were significantly greater compared to those of the control wine. This results confirms that the increase in the concentration of the terpenes affects the wine aroma. In particular, the odour attributes

Table 3

Results of the significance tests of the descriptive sensory intensity test

	Control wine without glycosidases	Wine treated with immobilised glycosidases
Odour		
Odour intense	40.3	64.6
Apple	39.0	56.4[B]
Passion fruit	37.4	59.1[B]
Lemon	28.7	53.5[A]
Floral	38.9	68.3[A]
Fruity	40.6	58.9
Flavour		
Flavour intense	65.1	64.5
Apple	54.3	62.1
Passion fruit	45.4	59.2
Lemon	51.4	51.5
Floral	44.4	69.9[A]
Fruity	52.9	56.3
Aftertaste	46.4	35.2

Least Significance Difference (LSD):
A < 99.9 %, B < 99 %).

lemon and floral are associated with terpenes, which could be significantly increased by enzyme treatment compared to the control sample. Terpineol, nerol and geraniol occurred at higher concentrations in the treated wine. Geraniol and terpineol have a floral and nerol lemon/citrus flavour.

The differences in the flavour evaluation of the Morio Muskat were not as pronounced as in the odour. The flavour intensity of the treated and control wine was approximately identical. The taste attribute floral was again significantly greater, from 69.9 to 44.4, for the treated 'Morio Muskat'. Thus, treatment with enzymes also resulted in a more floral note of the wine in the flavour, but not so clear as in the odour.

Conclusion

The results showed that it is possible to successfully immobilise several glycosidases with different specificities from a commercial, oenological enzyme preparation onto magnetic particles and thereby influence the flavour characteristics of wine. The four glycosidases, βG, Ara, Rha and Xyl, present in a commercial enzyme mixture were immobilised onto magnetic microparticles and showed good stability in wine. A stability analysis of the immobilised glycosidases in the wine showed merely a slight loss of activity. The coupling was thus stable and represented no obstacle to its use in wine. The recovery of the magnetic particles from the wine was carried out using a high gradient magnetic field separator and the separation had no negative effect on the enzyme activity of the coupled enzymes.

The aromatization tests highlighted a sizeable increase in certain volatile compounds, such as terpene alcohols, obtaining nearly the same selectivity for both free and immobilised glycosidases. Further investigations showed that the coupled enzymes were capable of releasing aroma compounds from the wine. The terpene levels were significantly increased by the use of enzymes, free or immobilised. This results were particularly relevant for the monoterpenes nerol and geraniol. Here, a comparison between the coupled and the free enzyme preparation showed a slightly higher release of the compounds in the non-immobilised enzyme mixture. The lower release by the immobilised enzymes can be explained by the changed composition of the enzyme mixture due to immobilisation. The relative portion of the β-glucosidase was higher for the immobilised biocatalyst. The release of the fragrant aglyca is a sequential mechanism; thus, the other glycosidases, such as arabinosidase and rhamnosidase, are also important for the cleavage of the aromatic precursors. The sensory results showed that a significantly greater odorous aroma can be released in the wine using immobilised glycosidases.

The results are thus of considerable interest for the possible future application of immobilised enzymes in the wine-making industry.

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