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Prevention of β-Glucosidase Inhibition by High Molecular Mass Compounds During Enzymatic Wine Aroma Enhancement Using a Hollow Fiber Reactor

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Summary

Enzyme activity and stability in a membrane reactor for wine aroma enhancement can be higher than when the enzyme is present in a free state since the catalyst would only be in contact with the low molecular mass components of this beverage. To test this hypothesis, the activity and stability of two commercial β-glucosidases were measured in the presence of Tannat wine and of its low molecular mass fraction (<10 kDa) obtained by ultrafiltration. The relative activities of Endozym Rouge and Endozym β-split β-glucosidases were higher in this fraction (3.8 and 7.6 %, respectively) than in the whole wine (0.9 and 5.6 %, respectively). Both enzymes were also more stable in the low molecular mass fraction. Endozym β-split β-glucosidase retained about 75 % of its initial activity after 14 days in the low molecular mass fraction, as contrasted with only 37.5 % in the wine. The ability of Endozym Rouge β-glucosidase to hydrolyze the synthetic substrate *p*-nitrophenylglucoside was examined in a simple batch membrane reactor. A rate of hydrolysis comparable to that obtained with the free Endozym Rouge β-glucosidase was reached. Finally, Endozym β-split β-glucosidase was used to hydrolyze the synthetic substrate in a hollow fiber membrane reactor and a substrate conversion near 58 % was achieved.

Key words: glycosidases, β-glucosidase inhibition and stability in wine, wine aroma, membrane reactor

Introduction

Among the many applications reported for glycosidases, their use to increase wine aroma has been widely studied (*1–5*). This is because grape musts contain glycosidic aroma precursors, which remain unchanged after vinification, giving to the wine an aromatic potential that can be exploited by using exogenous glycosidases (*6*). The most important enzymes involved in the hydrolysis of the glycosidic precursors are α-L-rhamnosidase (EC 3.2.1.40), α-L-arabinosidase (EC 3.2.1.55), β-D-apiosidase and β-D-glucosidase (EC 3.2.1.21). When the glycosidic precursors are *O*-β-D-glucosides, the hydrolysis occurs in one step. A two-step reaction usually takes place when the precursors are *O*-diglycosides, where the α-L-rhamnosidase, the α-L-arabinosidase or the β-L-apiosidase acts first on the inter-sugar linkage to liberate the corresponding sugar and a β-L-glucoside, which in turn is subsequently hydrolyzed by the β-L-glucosidase to release the aromatic aglycone (*6*).

Commercial enzymes employed in winemaking (*3,7–10*) or other glycosidases produced by bacteria (*11*), yeast (*12*) or fungi (*13*) were tested for their glycosidase activity for aroma enhancement in white and red wines produced from several cultivars. It is important to take into account that wines usually contain a lot of compounds, with different molecular mass (MM), that have a nega-

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tive effect on the activity and stability of glycosidases, with the β-glucosidase being the most affected among the various glycosidase enzymes (*10,14*). The most studied inhibitors are usually low MM compounds, such as ethanol, glucose and gluconolactone. However, it should be noted that some high MM components of wine can also have an influence on glycosidase performance, for example tannins from grape skin and seeds and extracellular proteins produced by yeasts during fermentation, among others.

Immobilized enzymes usually have a greater stability than in a free state under catalysis conditions due to several causes, such as denaturation prevention, subunit dissociation prevention, or a more favorable environment. For this reason, different immobilization systems have been the subject of numerous studies (*15,16*). In addition, immobilization allows the reuse of enzymes without the need of separating them from the reaction medium, thus reducing the process costs. A proper immobilization technique may also permit the improvement of enzyme performance by improving some enzyme limitations: enzyme purity, stability (including the possibility of enzyme reactivation), activity, specificity, selectivity, or inhibitions. Among the diverse immobilization techniques, the use of pre-existing supports to immobilize enzymes (*via* covalent or physical coupling) and the immobilization without supports (crosslinked enzyme aggregates or crystals) are the most used or promising ones (*17*). The use of immobilized glycosidases for wine aroma enhancement has been extensively studied (*7,18–26*), but these enzymes have not yet found an application in the industry.

Among the different methods to improve enzyme performance, membrane reactors are being increasingly used for various applications (*27–29*). There are many studies on membrane reactors of the hollow fiber type, some of which include systems with the enzyme outside the fibers and the reaction medium including the substrate, which is able to diffuse through the membrane, inside them. This type of systems protects the enzyme, allowing its contact with a more favorable environment. Hollow fiber reactors have been used, for example, to study the kinetics of *p*-nitrophenylphosphate hydrolysis catalyzed by an alkaline phosphatase to produce *p*-nitrophenol (*30*), to hydrolyze galactooligosaccharides of soybean milk with an α-galactosidase (*31*), and to produce lactose-reduced skim milk (*32*). Hollow fiber reactors have also been used to treat wastewater, not only in pilot scale reactors but also in larger scale systems, reaching volumes of water of 375 m^3 treated per hour (*33*). However, to our knowledge there are no published data that address the use of hollow fiber reactors to confine glycosidases for wine aroma improvement.

Endozym Rouge and Endozym β-split are commercial enzyme preparations used in winemaking, either to obtain the highest extraction and stability of the coloring matter, or to enhance wine aroma. Both enzymes are also recommended by their supplier for the liberation of aroma from glycosidic precursors due to their glycosidase activities (*34*). Previous results indicated that these preparations were rich in β-glucosidase and α-arabinosidase activities, but they showed low levels of α-rhamnosidase. It was also observed that Endozym Rouge and Endozym β-split β-glucosidases were strongly inhibited by Tannat red wine, particularly the Endozym Rouge (*35*).

In this paper, the results of studies of the effect of the fraction of low molecular mass components (<10 kDa) from Tannat red wine on β-glucosidase activity and stability in two commercial preparations used in winemaking, Endozym Rouge and Endozym β-split are presented. These studies were performed to detect a possible beneficial effect of the use of membrane reactors, because in this type of system enzymes are confined in an environment of low molecular mass components and not the whole wine. A simple batch membrane reactor was also evaluated by comparison of the hydrolysis of the substrate *p*-nitrophenylglucoside in Tannat wine produced by the Endozym Rouge β-glucosidase contained in a dialysis tubing membrane with the results produced when the enzyme was added directly to the wine. Finally, a continuous hollow fiber reactor to hydrolyze the *p*-nitrophenylglucoside dissolved in Tannat wine, taken as a 'model' of wine glycoside precursor, was tested using Endozym β-split β-glucosidase.

Materials and Methods

Tannat red wine was produced by a local winery in Cafayate, Salta, Argentina. Dialysis tubing cellulose membrane with a cut-off of 12.4 kDa and *p*-nitrophenyl-β-D--glucopyranoside (*p*NPG) were from Sigma-Aldrich (St. Louis, MO, USA). Hollow fiber module (Microza SLP--0053) was from Pall (New York, NY, USA). *p*-Nitrophenol (*p*NP) was supplied by Merck (Darmstadt, Germany) and acetonitrile of HPLC grade by Carlo Erba (Rodano, Italy). Endozym β-split and Endozym Rouge are commercial enzymes used in the winemaking industry produced by *Aspergillus niger* from Pascal Biotech (Paris, France). All the other reagents were of analytical grade.

Enzyme assays

Endozym Rouge and Endozym β-split β-D-glucopyranosidase activities were determined with *p*NPG by removing samples from the reaction medium at different times and quantifying the *p*-nitrophenol produced by hydrolysis of the substrate.

In enzyme inhibition and stability assays, *p*NP was quantified by HPLC in an acidic medium as previously described (*10*). Activity units (U), expressed as μmol/min, were calculated from the slope of plots of *p*NP concentration *vs.* time.

International activity units (IU) were determined using 1.9 mM *p*NPG at optimum pH (previously determined to be 4.5) and 50 °C, measuring the absorbance of the produced *p*NP in basic medium at its absorbance maximum, 400 nm, and calculated from the slope of the time course plots by using a molar absorption coefficient, *ε*, of 16.44 mM (*10*).

Isolation of low molecular mass fraction from Tannat wine

The fraction of compounds with molecular mass (MM) lower than 10 kDa was obtained by the ultrafiltration of Tannat red wine in an AMICON cell (W.R. Grace & Co., Beverly, MA, USA) provided with a PM10 membrane. The ultrafiltrate was recovered for subsequent studies.

Inhibition of β-glucosidase activity

Endozym Rouge and Endozym β-split β-glucosidase activities were measured using 0.12 mM *p*NPG as substrate at 20 °C in a reaction medium with 96.6 % of Tannat wine or of the low MM fraction prepared from this wine. The volume of the reaction medium was completed with *p*NPG and enzyme solutions. Inhibition was calculated based on a 100 % value as the enzyme activity measured in 60 mM tartaric acid/Na tartrate buffer (pH=3.6) under the same conditions. Activity was determined by quantifying the hydrolysis product *p*NP by HPLC at different time intervals.

β-Glucosidase stability

In order to study Endozym Rouge and Endozym β-split β-glucosidase stability in Tannat wine and in the fraction of low MM, both enzyme preparations were separately added to these media to reach 0.16 IU/mL, the level of β-glucosidase recommended for aroma enhancement (*36*), and incubated at 20 °C. At selected times, samples of 100 μL were taken out and added to the same volume of 4 mM *p*NPG solution. Residual activity was determined at 50 °C by HPLC and it was calculated as the percentage of the activity at time zero.

Simple batch enzyme membrane reactor

A volume of 0.5 mL of Endozym Rouge solution (15.4 IU/mL) was added directly to 50 mL of Tannat wine in which *p*NPG was dissolved at a concentration of 2 mM. In another experiment, a closed dialysis tubing membrane with the same relative volume of enzyme (1 % of the wine volume) was introduced into the wine with the same *p*NPG concentration. These samples were incubated at 20 °C with mild stirring, and aliquots were taken from the wine at different times and analyzed by HPLC to quantify the *p*NP produced by *p*NPG hydrolysis. To examine the effect of different exchange areas between the dialysis membrane where the enzyme was confined and the wine, portions of the same quantity of enzyme were dissolved in different volumes of buffer (1, 2, 3 and 5 % of the wine volume), introduced in separated membrane tubing of the same diameter and added to 50 mL of wine portions. These samples were incubated at 20 °C for 3 days with mild stirring, and *p*NP was quantified at different times by HPLC to determine the initial rate of hydrolysis (v_i).

Hollow fiber enzymatic reactor

Fig. 1 shows the system employed to carry out the hydrolysis reaction. It consisted of a hollow fiber module with a polyethersulfone membrane of 10-kDa cut-off and 0.015 m^2 of working area, wine and enzyme reservoirs, two pumps and a fraction collector. The wine, in which the *p*NPG was dissolved, was pumped from a reservoir through the interior of the fibers, while the enzyme solution was recirculated through the exterior of the fibers. A fraction collector was coupled to the system to collect samples at the system output.

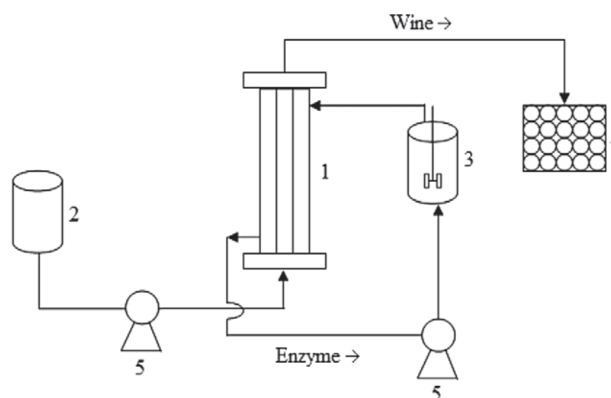

Fig. 1. Hollow fiber enzymatic reactor. 1=hollow fiber, 2=wine reservoir, 3=enzyme reservoir, 4=fraction collector, 5=pump

Tannat wine and 60 mM tartaric acid/Na tartrate buffer (pH=3.6) were circulated through the interior and the exterior of the fibers, respectively, until the recirculated buffer contained a constant amount of compounds with MM less than 10 kDa. After that, the wine inlet was replaced by 0.12 mM *p*NPG dissolved in Tannat wine, and 1 mL of Endozym β-split (174.1 IU/mL) was added to the enzyme reservoir (25 mL). The extent of hydrolysis was compared at 20 °C using two different flow rates for the recirculated enzyme solution, 0.11 and 2.9 mL/min, keeping the flow rate of the wine through the interior of the fibers at 0.11 mL/min in both cases. Fractions of 3 mL were collected at the end of the system and *p*NP concentration was measured by HPLC.

Statistical analysis

At least two replicate samples were measured in each determination. Graphs and statistical analyses (linear regressions) were carried out using PRISM software (Graph Pad, San Diego, CA, USA).

Results and Discussion

Inhibition of β-glucosidase activity by Tannat wine and its fraction with molecular mass lower than 10 kDa

We studied the possible advantage of the use of a membrane reactor for wine aroma enhancement by evaluating whether β-glucosidase activity and stability were less affected in the presence of a low MM fraction from the wine than by the whole wine. Such a low MM fraction would correspond to the phase in contact with the enzyme in this type of system.

The inhibition of hydrolysis by Endozym Rouge and Endozym β-split β-glucosidases caused by whole wine and by the low MM fraction prepared from the wine was measured using 0.12 mM *p*NPG. The percentage of inhibition was calculated considering 100 % to be

the initial rate of reaction determined in the buffer under the same conditions.

Table 1 shows that the β-glucosidase from Endozym β-split retained a higher activity, relative to the activity measured in the buffer, both in whole wine and in the fraction of low MM, than the β-glucosidase from Endozym Rouge. Both enzymes had a higher activity in the low MM fraction than in the Tannat wine.

These results showed that the initial rate of hydrolysis of *p*NPG, and therefore probably also of the glycosidic precursors, was higher in the low MM fraction than in wine, suggesting that at least some of the compounds causing inhibition were removed by ultrafiltration. From these results, it can be suggested that β-glucosidases confined in semipermeable membranes would be prevented from contact with some of the inhibitory compounds in wine and would therefore retain a higher activity on the precursors to increase wine aroma. However, any glucosides that remained in the low MM fraction are possible substrates for the β-glucosidase activity and could act as competitive inhibitors against the *p*NPG hydrolysis. Therefore, they would act as competitive inhibitors of the hydrolysis of glucosidic aromatic precursors. Furthermore, other β-glucosidase inhibitors, such as glucose and ethanol, will also be present in this low MM fraction (*10*).

β-glucosidase stability in Tannat wine and in the fraction with MM lower than 10 kDa

It is important that enzymes preserve at least part of their β-glucosidase activity to efficiently hydrolyze the aroma precursors after the rapid inhibition that occurs once they are added to wine. Thus, stability data are also of great importance when selecting exogenous enzymes to be used as immobilized catalysts, since such results allow us to know for how long we can expect that enzymes will maintain this remaining activity. If free enzymes are used, they must be active only during the period of aroma release, but when immobilized or confined enzymes are used, they must be as stable as possible to permit their prolonged use, thereby decreasing their effective cost.

For this reason, the β-glucosidase stability from both commercial preparations in wine was compared with the stability in the fraction of low MM, to predict if the use of membranes would improve enzyme stability. The residual activity of glycosidases during incubation at 20 °C was measured at 50 °C and the activity at time zero was considered 100 %.

The β-glucosidase activity from Endozym Rouge in Tannat wine was reduced to (9.8±3.7) % after 20 days of incubation, while the enzyme retained (34.8±4.7) % of its initial activity in the low MM fraction after 28 days (Fig. 2).

The Endozym β-split β-glucosidase showed a similar behavior, retaining more activity when it was incubated in the low MM fraction than when it was incubated in the whole wine, but this enzyme preparation was more stable than the one from Endozym Rouge. It retained (37.5±1.4) and (74.3±6.7) % of its initial activity after 14 days in whole wine and the low MM fraction, respectively (Fig. 3).

These results indicate that the ultrafiltration process removed from the ultrafiltrate at least some of the compounds affecting the enzyme stability. Therefore, we can conclude that the enzymes contained in semipermeable membranes will show higher stability than when they are in contact with the whole compounds present in the wine.

Table 1. Initial rate of hydrolysis catalyzed by Endozym Rouge and Endozym β-split β-glucosidases in Tannat wine and in Tannat wine with low MM fraction

Enzyme	Buffer		Tannat wine		Fraction (MM<10 kDa)	
	v_i/(μmol/(min·mL))	v_{iRel}/%	v_i/(μmol/(min·mL))	v_{iRel}/%	v_i/(μmol/(min·mL))	v_{iRel}/%
Endozym Rouge	$(7.9\pm0.8)\cdot10^{-4}$	100	$(0.70\pm0.06)\cdot10^{-5}$	0.9	$(3.0\pm0.1)\cdot10^{-5}$	3.8
Endozym β-split	$(2.5\pm0.1)\cdot10^{-3}$	100	$(0.14\pm0.01)\cdot10^{-3}$	5.6	$(0.19\pm0.01)\cdot10^{-3}$	7.6

v_{iRel}=percentage of the initial rate of hydrolysis relative to the rate observed in the buffer under the same conditions
Reaction conditions: 20 °C, 0.12 mM *p*-nitrophenyl-β-D-glucopyranoside in 60 mM tartrate buffer, pH=3.6

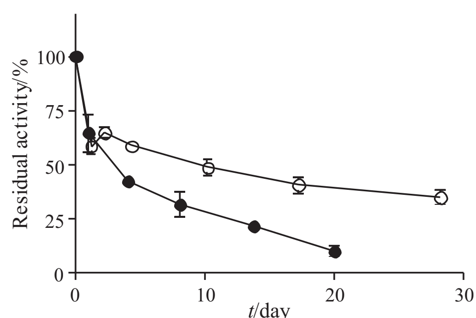

Fig. 2. Endozym Rouge β-glucosidase stability at 20 °C in Tannat wine and in the low molecular mass (MM) fraction. Enzyme added to samples: 0.16 IU/mL. ● Tannat wine, ○ low MM fraction

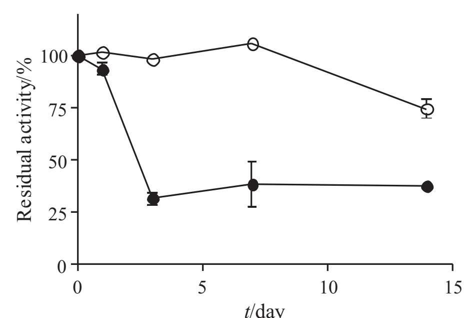

Fig. 3. Endozym β-split β-glucosidase stability at 20 °C in Tannat wine and in the low molecular mass (MM) fraction. Enzyme added to samples: 0.16 IU/mL. ● Tannat wine, ○ low MM fraction

Among the high MM components of wine there are proteins, polyphenols and polysaccharides (*37*). The confinement of the enzyme in the hollow fiber is precisely avoiding its contact with some of these compounds, which cannot pass through the membrane like low MM substances. Some of the endogenous proteins remaining in the wine are proteases, which are produced by *S. cerevisiae* metabolism for normal growth during fermentation (*38*). These enzymes have the ability to hydrolyze other proteins, such as the exogenous glycosidases, causing their denaturation. It should be interesting to study the effect of the addition of protease inhibitors to the wine to test if these enzymes are responsible for the loss of glucosidase activity or at least part of it. On the other hand, among the polyphenols, it was found that for example high MM tannins, the concentration of which is higher in red wines than in white ones, are capable of hydrophobically interacting with proteins (*39*), leading to aggregate formation and the subsequent loss of biological activity.

Rate of pNPG hydrolysis produced by the enzyme contained in a dialysis tubing membrane

Simple batch enzyme membrane reactor

To study the effect of the confinement of the enzyme on the hydrolysis rate of *p*NPG, chosen as a model of an aroma glycosidic precursor, the β-glucosidase from Endozym Rouge was added inside dialysis tubing into the Tannat wine containing dissolved *p*NPG. Thus, the enzyme was only in contact with the compounds of low molecular mass, which could diffuse through the dialysis membrane. The rate of substrate hydrolysis was compared with the rate of hydrolysis when the enzyme was added directly into the wine by taking samples from the wine at different time intervals and quantifying the released *p*NP by HPLC. β-Glucosidase from Endozym Rouge was chosen in this study since its activity and stability were the most affected by wine compounds. Because the differences in the measured values were expected to be higher, the changes were easier to detect.

In both cases, almost a 100 % conversion of *p*NPG was achieved (Fig. 4), but the reaction rate was slightly lower when the enzyme was contained in the membrane. Initial rates of reaction, calculated from the slope of the line at the beginning, were $(2.0\pm0.1)\cdot10^{-4}$ and $(3.4\pm0.1)\cdot10^{-4}$ μmol/(min·mL) when the enzyme was in the dialysis tubing and when added directly to the wine, respectively. This difference may be because in the case of the confined enzyme, both the substrate and the product of the reaction must cross through the dialysis membrane, and the rate of diffusion may possibly be a factor limiting the overall rate of reaction.

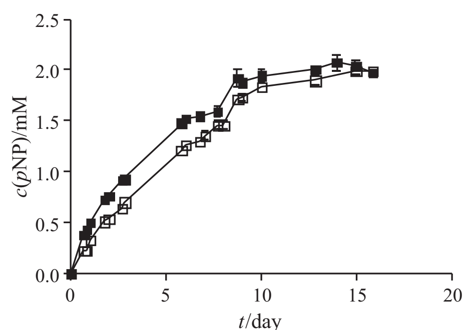

Fig. 4. *p*-Nitrophenyl-β-D-glucopyranoside (*c*(*p*NPG)=2 mM) hydrolysis by Endozym Rouge β-glucosidase in Tannat wine and in a simple enzyme membrane reactor. ■ enzyme added directly, □ enzyme contained in dialysis tubing; *p*NP=*p*-nitrophenol

In order to determine if the diffusion rate controlled the reaction, the transfer area was indirectly increased by changing the volume of the dialysis tubing in which the enzyme was contained, from 1 up to 5 % of the volume of wine to be treated, keeping the same quantity of added enzyme and the same diameter of membrane tubing. In this manner the contact surface of the membrane between the wine and the enzyme solution was increased. Even though the enzyme concentration decreased when the volume of the dialysis tubing was increased, the amount of catalyst in the total volume of substrate remained constant.

As can be observed in Table 2, when the volume of the dialysis tubing was increased, and thus the amount of exchange surface available for substrate and for compounds of low MM, the hydrolysis rate was appreciably increased. On an industrial scale, one of the available configurations of membrane reactors used in the confinement of enzyme is the hollow fiber membrane reactor.

Table 2. Initial rates of *p*-nitrophenyl-β-D-glucopyranoside hydrolysis by Endozym Rouge β-glucosidase added directly into the Tannat wine or contained in membranes of different volumes

Sample	v_i (μmol/(min·mL))·10^4
Enzyme added directly to the wine	3.4±0.1
Enzyme contained in dialysis tubing (1 %)*	2.0±0.1
Enzyme contained in dialysis tubing (2 %)*	3.1±0.2
Enzyme contained in dialysis tubing (3 %)*	3.2±0.2
Enzyme contained in dialysis tubing (5 %)*	3.3±0.1

*Volume of membrane where the enzyme was contained, expressed as the percentage of the total wine volume

pNPG hydrolysis in a hollow fiber enzymatic reactor

Although the latest reviews dealing with enzyme immobilization present the use of pre-existing supports (*via* covalent or physical coupling) and the immobilization without supports (crosslinked enzyme aggregates or crystals) as the most frequently used or promising techniques (*17*), there is also a tendency of looking for more than enzyme stabilization in the immobilization protocols, such as a reduction in inhibition problems. Since we observed that the β-glucosidase stability is higher and its inhibition is slightly lower when the high molecular mass compounds present in wine are removed, the hydrolysis of *p*NPG, chosen again as a model glycosidic precursor, was studied in a hollow fiber membrane reactor to check the usefulness of the strategy to prevent inhibition by large size compounds. Enzyme trapping is a technique that may keep the enzyme properties unaltered, but it can hardly improve them. However, inhibitory effects can be

reduced since enzyme is protected inside the fibers from interaction with inhibitors; in a completely different environment. Moreover, an additional benefit in membrane trapping systems could be obtained in the case of multimeric enzymes, since this type of arrangements prevents subunit dissociation, leading to an improvement in enzyme stability (*40*). This is due to a concentration effect, since dilution favors subunit dissociation (*17*).

During immobilization processes consisting of enzyme trapping, the adsorption of the enzyme in the membrane may occur, leading to negative or, sometimes, positive effects (*40*). However, in this particular case, it is very unlikely that this phenomenon take place since the membrane of the hollow fiber is constructed with polyethersulfone, which is the material more commonly used in the design of protein purification systems worldwide.

During the operation of the hollow fiber system, the substances with molecular mass lower than 10 kDa, including the *p*NPG, passed through the membrane to take contact with the enzymatic solution circulating outside the fibers, so the substrate could be hydrolyzed by the enzyme to release *p*NP, which diffused back again through the fibers into the wine, being collected at the reactor outlet.

β-Glucosidase from Endozym β-split was selected for this experiment since it was the most stable and least inhibited the enzyme among the different β-glucosidases tested (*35*). The *p*NPG concentration was set at 0.12 mM, based on the values of the concentration of natural glycosides in wine (*12,41,42*). The *p*NP concentration at the reactor outlet was determined by HPLC.

The results of this experiment are shown in Fig. 5. When both the wine and the enzymatic solution were pumped at a flow rate of 0.11 mL/min, the steady state was reached after pumping approx. 500 mL of wine, with a conversion of about 27 %. However, a conversion of about 58 % was reached when the flow rate of the recirculated enzyme solution was increased to 2.9 mL/min.

The low conversion at the slower flow rate could be due to insufficient mixing in the exterior of the fibers, which is where the enzyme acts by hydrolyzing the *p*NPG that passes through the membrane. If rapid mixing did not occur, *p*NPG and *p*NP may have accumulated in a poorly stirred zone near the fibers, remaining a part of the recirculated enzyme with no contact with the substrate. When the flow rate was increased, a greater conversion, probably because of better mixing of the enzyme solution outside the fibers, and thus less 'dead space', was achieved.

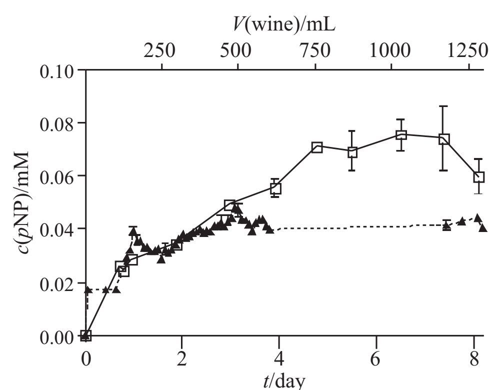

Fig. 5. *p*-Nitrophenyl-β-D-glucopyranoside (*c*(*p*NPG)=0.12 mM) hydrolysis by Endozym β-split β-glucosidase in the hollow fiber enzymatic reactor. Flow rate of recirculated enzyme solution: □ 2.9 mL/min, ▲ 0.11 mL/min; *p*NP=*p*-nitrophenol

Arévalo Villena *et al.* (*42*) found much lower conversions using a free commercial enzyme on the natural glycosides in three wine varieties; but, one year later, they reported hydrolysis between 7 and 32 % with a commercial enzyme on the natural substrates, and a conversion in the order of 60 % with a β-glucosidase from a yeast isolated from wine (*12*). Even though the conversion reached in this study was comparable with the results obtained by these authors, we must consider possible differences between the natural and the synthetic substrates, since glycosidases usually show higher activities on the synthetic substrates. However, it is reasonable to suppose that the natural substrates will also pass through the membrane to make contact with the enzyme, so they will be hydrolyzed and release the aromatic compounds, which can diffuse across the membrane to the wine. It is reasonable to make this assumption since polyethersulfone membrane selectivity is governed by molecular sieving, and this material has low physicochemical interactions and binding characteristics.

The use of hollow fiber reactors in the winemaking industry would involve high investment costs to acquire the modules. However, a corresponding decrease in total costs due to the availability of the enzyme for reutilization also has to be considered of economic importance, as well as the reduction of inhibition and the increase of the stability of the enzyme. All of these factors suggest that a smaller amount of the catalyst would be necessary to treat a certain amount of wine. A thorough economic study should be carried out to evaluate the potential benefits of these enzyme confinement systems.

Moreover, further research should include and evaluate other immobilization protocols, since the improvements may be even larger, because in any case, a better designed immobilization protocol may solve the stability problem and even the inhibition problems (*17*). An improved immobilization protocol (*e.g.* multipoint covalent attachment) could drive to an improved reactor performance.

Conclusions

Some of the inhibitors and other compounds affecting β-glucosidase stability are absent from the low molecular mass fraction of Tannat wine obtained by ultrafiltration. Thus, the enzyme shows less inhibition and greater stability in this fraction than in the whole wine.

Endozym Rouge β-glucosidase contained in dialysis tubing hydrolyzes efficiently the substrate *p*NPG dissolved in the wine in a simple batch reactor, and the initial rate of reaction is appreciably increased in proportion to the transfer area between the two phases.

A hollow fiber membrane reactor allowed efficient hydrolysis of the model substrate *p*NPG with a commercial β-glucosidase, yielding a high conversion. This result suggests that it could be possible to use this type of

reactor to hydrolyze the natural glycosides in the wine. However, this system should be further studied at the pilot scale, evaluating the hydrolysis of the natural substrates produced by all the glycosidases necessary to produce the complete release of the aromatic compounds. This could be accomplished either by using methods to quantify the nonhydrolyzed glycosides or through sensory analysis to get the data necessary to perform a detailed economic study.

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