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β-glucosidase immobilization on ion exchange resins for using as aromatic enhancement of wines

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The aim of this work is to study the process of β-glucosidase immobilization on particles of different ion exchange resins, and the stability of the catalytic beds obtained. The final target is to apply these catalysts in several operations of the winemaking industry directed towards the aromatic enhancement of wines.

1. Introduction

The aromatic profile of a wine is one of the fundamental characteristics that are analyzed when defining its quality. Nowadays a growing demand of young wines, with high characters fruity and variety, is been introduced in markets. Some of the major components of the aroma of the wines that contribute to the variety character are terpenes. The kind and quantity of these compounds that can be found in musts differentiates between varieties of grape. Terpenes are usually presents into two fractions: a free one that contributes directly to the aroma of the musts, and another bound one, forming non aromatic glycosides, in principle, although later they can liberate their aromatic load. Usually, the second fraction is quantitatively superior to the first one, and so it supposes a potentially profitable source to increase the aroma of the products. To obtain these aromas it is necessary to hydrolysate glycosides and consequently to release the bound compounds (Günata et al., 1985).

The hydrolysis of glycosides can be carried out by means of the use of commercial enzymatic reagents that contain high β-glucosidase activity. Enzyme must be added to wine after the alcohol fermentation is finished, in order to avoid interferences with the complex fermentative process that must be carried out. Moreover, in some cases, commercial reagents have not high purity grade and they contain different enzymes that causes other effects (Genovés et al., 2005; Yanai and Sato, 1999). Once enough glycosides have been hydrolyzed and the wanted aromatic unfolding has been reached, it is necessary to separate the enzyme from the medium to stop the hydrolysation process. To get this, it is usually carried out a typical bentonite treatment, followed by a decantation step. As much the first operation as the second one they are normally carried out in

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discontinuous mode, which implies difficult control of effectiveness and other disadvantages.

The enzyme immobilization on a solid bed would be an alternative proposal to the above mentioned treatment. This way, the wine can be percolated through a column of catalytic particles, allowing the operation in continuous mode and, therefore, leading to bigger control of the obtained hydrolysis degree and other advantages of this operation mode. Moreover, it would allow to avoid the reagent addition to the products, and so the later clarification stage. The intensity of the treatment is regulated here by the length of the bed and the percolation flow of the product.

Ion exchange resins are one of the most interesting possibilities in enzyme immobilization. These compounds are insoluble polymers and granular substances, able to exchange the ions fixed on the functional groups of the polymer with other ions in solution (Dorfner, 1990; Thompson, 1850). The main characteristic of the ion exchange resins is their reversibility. This way, in theory, a solid bed of this type loaded with enzymes can be regenerated easily when lose its activity, so you can reuse the catalyst indefinitely. The columns regeneration is carried out by means of exchanging the inactive enzyme molecules by the ones of the corresponding counterion. This is done by percolation through the bed of a counterion solution at the adequate pH. Later on, the counterion molecules must be exchanged by new molecules of the active enzyme, by means of percolation of an enzyme solution at different pH (Dechow, 1975).

An additional advantage of the proposed system is that ion exchange resins are already of generalized use in diverse alimentary processes, i.e., softening of drinking water (Ahmed et al., 1998) or conditioning of fruit juices (Coca et al., 2008; Pohl and Prusisz, 2006; Vera et al., 2003). Even, the use of ion exchange techniques in the own winemaking industry dates from 1945. Their first application was tartaric stabilization of wines (Gómez, et al., 2002). Recently it has been proven that it is also an effective technique in demetalization of wines (Lasanta, et al., 2005; Palacios, et al., 2001). In consequence, we can hope that enough sanitary guarantees exist for its use at industrial level in the alimentary sector.

2. Materials and methods

To carry out the above mentioned study, four different commercial ion exchange resins were used. Two of them are cationic resins, one of the strong acid type (Lewatit Monoplus SP 112, Bayer Chemicals AG) and the other of the weak acid type (Lewatit CNP 80, Bayer Chemicals AG). The third resin is of adsorption type (Lewatit VP OC 1600, Bayer Chemicals AG). The last resin is of covalent type (Amberzyme Oxirane, Rohm and Haas Company).

Lyophilized β-glucosidase with minimum activity of 2 IU/mg (Sigma-Aldrich) was used for the immobilization tests. The β-glucosidase activity was determined according to the method developed by Martino et al. (1994), modified in function of the special requirements of each experiment. The method is based on the hydrolytic activity that

presents a standard of 50 µL of the enzyme solution in a citrate-phosphate tampon (pH 3.3). The sample (or the standard) is added to 450 µL of a solution of *p*-nitrophenyl β-D-glucopyranoside 5.5 mM (Sigma-Aldrich), in the same tampon. The enzymatic reaction of glycoside hydrolysis is allowed to lapse during 10 minutes at temperature of 25 ºC and, later on, it is stopped by adding 1 mL of a solution of Na_2CO_3 1 M. This salt allows the evolution of the color of the *p*-nitrophenolate ion, which has been released in the reaction medium, and later is measured by means of a spectrophotometer (Hitachi 200, Perkin Elmer) at 400 nm (extinction coefficient $\varepsilon = 18.3 \cdot 10^3\ M^{-1}cm^{-1}$). In this case, an international unit of enzymatic activity (IU) is defined as the quantity of enzyme that is capable of catalyzing the hydrolysis of 1 µmol of glycoside per minute.

On the other hand, samples of 0.1 g of resin were used in the determination of the immobilized enzymatic activity, to which the *p*-nitrophenyl β-D-glucopyranoside solution (*p*-NPG) was added. Once lapsed the adequate reaction time, the solution was separated from the resin, and so the reaction stopped, in order to coloration measuring at 400 nm.

In this work, the equivalent capacity of the different resins was determined, in relation with the β-glucosidase immobilization, so we can know the maximum load capacity of the enzymatic activity of each type of resin. For these determinations an enzyme solution of 500 IU/L was used, to which 1 g of the resin was added. After the mixture, the fall of the enzymatic activity of the solution along the time was determined. This fall is mainly due to the immobilization, in front of the fall that presents the enzyme solution by spontaneous deactivation. This last phenomenon was measured by following the fall of the enzymatic activity of the solution in absence of resin. The differences of the enzyme activity between both solutions correspond to the quantity of enzyme that is immobilized at each time. When the difference of activities is stabilized it is considered that the immobilization process has concluded and the resins are enzymatically active.

To determine if active resins can present enzyme lixiviation during their operation at industrial level, specific lixiviation test were carried out. The experiments consist on submerging the active resin in a solution under the work conditions (pH 3.5) and shake it during a reference time previously established (1 h). Later on, the possible enzymatic activity in the solution, caused by the contact with the resin, is measured.

Finally, to stablish the deactivation kinetics of the immobilized enzyme, active particles submerged in the work solution were stored at room temperature for several days. During the storage, the activity in resin was determined at intervals.

3. Results and Discussion

The curve corresponding to the spontaneous deactivation of β-glucosidase in the work conditions is presented in Figure 1. If a first order kinetic is assumed (equation 1) we can obtain the deactivation kinetic constant (k_d) from the experimental data by linear regression. In this case k_d reaches a value of $1.61 \cdot 10^{-5}\ s^{-1}$.

$$\underset{\left(\begin{array}{c}\text{Fresh}\\ \text{Enzime}\end{array}\right)}{E} \xrightarrow{k_d} \underset{\left(\begin{array}{c}\text{Inactive}\\ \text{Enzime}\end{array}\right)}{D} \qquad E = E_o\, e^{-k_d t} \qquad (1)$$

The immobilization kinetics obtained for the different studied resins are presented in Figure 2. These curves correspond to the difference between the fall of enzymatic activity in presence of resin and in absence of it. Again we assume first order kinetics (equation 2) and we can obtain the immobilization kinetic constant (k_i) from the experimental data by linear regression.

Figure 1.- Spontaneous deactivation of β-glucosidase. Enzymatic activity in solution along time

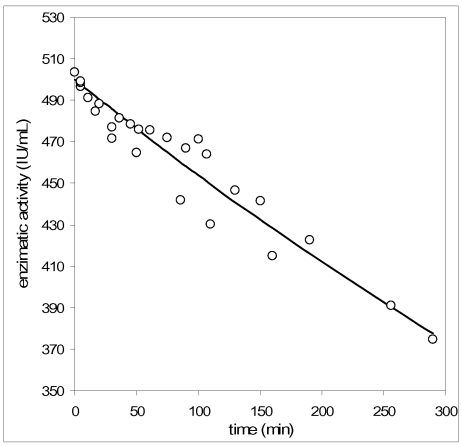
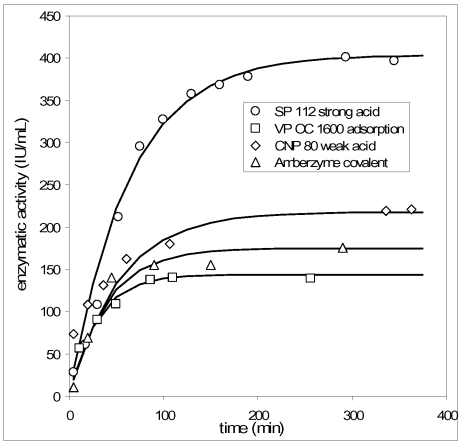


Figure 2.- Immobilization kinetics of β-glucosidase on different ion exchange resins.



$$\underset{\left(\begin{array}{c}\text{Free}\\ \text{Enzime}\end{array}\right)}{E} \xrightarrow{k_i} \underset{\left(\begin{array}{c}\text{Immobilized}\\ \text{Enzime}\end{array}\right)}{ER} \qquad ER = ER_{max}\left(1 - e^{-k_i t}\right) \qquad (2)$$

The kinetic data obtained from Figure 2 are shown in Table 1. In the same Table are presented the enzymatic activities observed in the resins at the end of the immobilization process (ER^*_{max}). Finally, it is calculated the percentage of activity that the immobilized enzyme molecules retain with regard to the free enzyme molecules.

Table 1.- Kinetic data for β-glucosidase immobilization on different ion exchange resins.

Resin	ER_{max} (IU/mL)	k_i (10^{-5} s^{-1})	ER^*_{max} (IU/kg)	Retention (%)
SP 112 strong acid	404	26.6	6.8	0.02
CNP 80 weak acid	218	31.5	11.1	0.12
Amberzyme covalent	175	42.2	29.6	0.21
VP OC 1600 adsorption	144	56.0	3.9	0.20

ER_{max} = maximum immobilized enzyme from solution; k_i = immobilization kinetic constant; ER^*_{max} = maximum immobilized enzyme in resin.

As it can be observed, in all the studied resins it is possible to immobilize the enzyme, although with different degree of effectiveness. Those that presented better results were the resin of weak acid type and the resin of covalent type, from the point of view of specific activity on resin. However, in the case of the weak acid type, the immobilized enzyme molecules retain half of the percentage of activity that in the other case.

In relation with the stability of immobilization, in none of resins was observed enzyme lixiviation once immobilized, not being detected appreciable enzymatic activity in solution after an exhaustive washing of the active particles.

Finally, in Table 2 it is presented the enzymatic activity in resin after storage for several days. Assuming also first order kinetics in the immobilized deactivation, as in the free deactivation (equation 1), we can obtain the kinetic constant of immobilized deactivation by linear regression. The values of this parameter are also shown in Table 2. According to data in Table 2, deactivation of the immobilized enzyme is from two to ten times slower than deactivation of the free one.

Table 2.- Kinetic data for the immobilized β-glucosidase deactivation on different ion exchange resins.

Resin	ER^* after 2 days (IU/kg)	ER^* after 4 days (IU/kg)	k^*_d (10^{-5} s^{-1})
SP 112 strong acid	6.4	6.0	0.036
CNP 80 weak acid	10.3	9.4	0.047
Amberzyme covalent	17.3	6.8	0.403
VP OC 1600 adsorption	1.3	0.7	0.512

ER^* = immobilized enzyme activity in resin; k^*_d = kinetic constant of immobilized deactivation.

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