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IMMOBILISED LACCASE FOR PHENOLIC REMOVAL IN MUST AND WINE

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Summary

The possibility of the use of immobilised Laccase (EC 1.10.3.1.) for the enzymatic removal of phenolic compounds from must and wine has been attempted on laboratory scale. The immobilised enzyme was packed in column and model solutions of catechin or white grape must were eluted through. Oxygen saturation, flow-rate and immobilised enzyme amount were the main parameters used to study phenolic oxidation.

Introduction

Must and wine are complex mixtures of several different chemical compounds, such as ethanol, organic acids, salts and phenolic compounds. While ethyl alcohol and organic acids are responsible for the pleasant freshness of wine, colour and taste depend on the particular phenolic compounds present in different kinds of wine. Many groups of phenolic compounds are found in wine: cinnamic acid derivatives and catechins are present in different amount in all wines, while red and rosé wines are characterised by the presence of anthocyanins (Macheix *et al.*, 1991).

All phenolics are subjected to various fates during the shelf-life of wine and some problems can derive from their modification which are then involved in various chemical reactions (Singleton, 1987).

Different methods have been attempted to prevent must and wine phenol-dependent discoloration and/or off-flavour formation, such as phenolic removal with cross-linked polyvinylpyrrolidone, PVPP (Peri and Cantarelli, 1963), early

hyperoxidation (Guerzoni *et al*,1977) and enzymatic oxidation (Cantarelli *et al*,1989).

Our previous experience refers to this last method,in which polyphenol oxidation was obtained by means of **laccase.** This enzyme is an oxidase acting both on o-and p-diphenols (EC.1.10.3.1), present in must when infected by fungi, as for exemple ***Botrytis cinerea***. Since such an enzyme is not yet allowed as a food additive (JECFA, FAO/WHO Food Additives Data System), the use of immobilised laccase might be a suitable method to overcome such legal limits, since in this form it may be classified as technological aid.

Materials and Methods

Enzyme.
Laccase is an exocellular enzyme from a mutant strain of *Trametes versicolor* (Valenti,1975). The activity was assayed spectrophotometrically following the oxidation of 20 mM catechol in 50 mM citrate buffer pH 5, at 30°C. One unit is here defined as the amount of enzyme causing an increment of 1 mA/min at 420 nm in the above conditions.

Enzyme derivatives.
Agarose-based activated matrixes (Affi Gel-10 and 15, Bio-Rad) were coupled with the enzyme at different pH values. Best results in terms of immobilised active enzyme were obtained in both cases using as coupling buffer a 20 mM phosphate, pH 6.9, containing 0.1 M NaCl in the case of Affi Gel-10. A Silica-based matrix (Supelcosil Epoxy 540, Supelchem) was activated by reaction with 2 % , w/v , polyethyleneimine (BDH) titrated to pH 7.5 with HCl , for 16 hours. After exhaustive washing with distilled water, the gel was suspended in 20 mM phosphate buffer, pH 6.8, the enzyme solution added and adsorbed onto the cationic gel , then treated with 3 % glutharaldehyde in 20 mM phosphate buffer, pH 6.0, for 2 hours at room temperature. Finally, the gel was washed until the washings were negative to Schiff's reagent for aldehyde. The gel was further treated with 0.1 M $NaBH_4$, in order to stabilise the derivative.

Reactor performance.
Immobilised enzyme was packed into chromatographic columns of various size and a model solution (100 mg/L of catechin in 0.4 % tartaric acid pH 3.0, 10% in ethanol) or must (from Riesling grapes), equilibrated with N_2, air or O_2. The solutions were passed once through the column and collected at different flow-rates using the same reactor volume or at different reactor volumes and constant flow-rate. The eluted samples were treated with 1g/L PVPP.

Analyses
Total polyphenols (TPP: Scalbert *et al.*,1989), catechins (Nagel and Glories,1991) and colour (as mA at 420 nm) were measured

in all samples before and after laccase treatment and following PVPP treatment.

Results and Discussion

Immobilisation of laccase on the various supports resulted in different amounts of bound activity, ranging between 1000 to 1600 U/mL gel for Affi Gel 10 and 15, respectively, and 3000 U/mL in the case of the silica derivative.

With catechin, the immobilised enzyme showed a pH-activity shape similar to that of the free enzyme (Fig. 1)

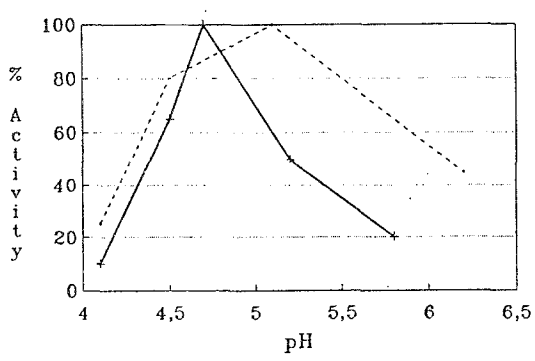

Fig. 1: pH-activity shape of laccase on catechin: free in solution (---) and immobilised (—).

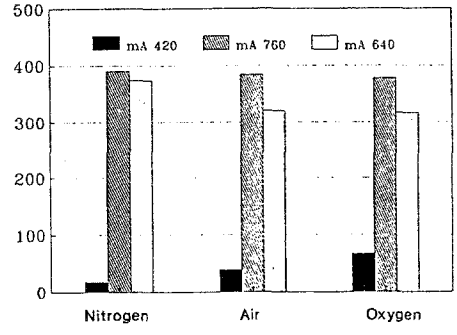

Fig. 2: influence of oxygen level on catechin oxidation by immobilised laccase. For details see text.

Fig. 2 shows how the presence of increasing levels of oxygen supply affected the oxidation of catechin by immobilised laccase. Residual catechin, as measured by mA 640 nm (Nagel and Glories,1991) or by mA 760 nm as total polyphenols (Scalbert *et al*,1989), was roughly the same with air or oxygen saturation, while browning at 420 nm was practically doubled with oxygen with respect to air saturation. This may reflect the formation of different products from catechin in those two conditions.

The polymeric compounds that formed under the enzymatic action (Singleton, 1987) ,in which total phenolic residues were maintained,as measured by A 760 nm (Scalbert *et al.*, 1989), can further oxidise in the presence of excess oxygen. Owing to their highly conjugated double bond system, a small increase in oxidised products resulted in a rather relevant colour formation at 420 nm.

Oxidation of phenolics depended mainly on the flow rate in the reactor (Fig. 3). Both the increase in colour and the decrease of TPP were maximal at low flow-rates (samples saturated with O_2)

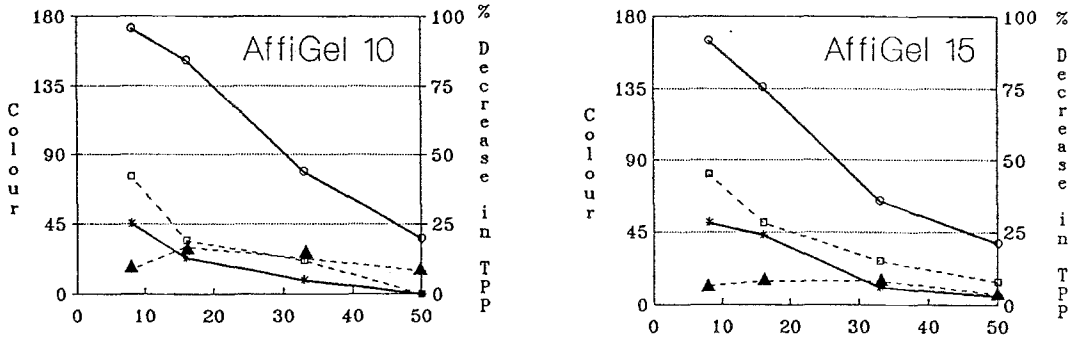

Fig.3: Dependence of catechin oxidation with immobilised laccase on flow-rate. Colour before(○) and after (▲) PVPP treatment; total polyphenol before (□) and after (✱) PVPP treatment

The immobilised enzyme could be reused at least eight times in successive experiments with neglegible loss in activity during a 6 months period, if the immobilised laccase was maintained at 4°C in 0.4 % tartrate buffer, pH 3, in the presence of 0.05% Thimerosal when not in use.

Since the enzymatic reactor turned progressively dark-brown in successive experiments, we studied the influence of contact time between the substrate and the reactor on such browning by maintaining a constant time-contact of about 15 sec between the substrate and the immobilised enzyme while increasing the volume of the reactor.

Obviously, the efficiency of phenolic oxidation increased with an increase in volume of the reactor bed (Fig. 4); furthermore, the reactor resulted in less colouration.

A probable cause can be attributed to a prolonged contact with the enzyme, as occurs at low flow-rates, which allows for the formation of highly polymerised polyphenolic compounds that are preferentially adsorbed to the matrix.

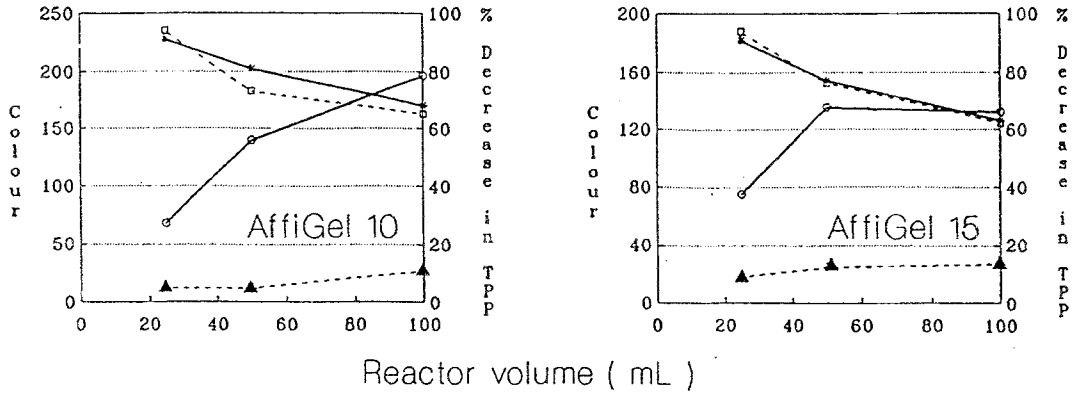

Fig.4 : Dependence of catechin oxidation with immobilised laccase on the volume of reactor at the same flow-rate. Colour before (○) and after (▲) PVPP treatment; total polyphenol before (□) and after (✱) PVPP treatment.

This can be supported by the fact that the efficiency of PVPP (1 g/L) in the removal of oxidised catechin, expressed as:

$$(A_B - A_A / A_B) * 100$$

(A_B and A_A being the values of the absorbance at 420 nm of the oxidised catechin before and after the addition of PVPP respectively) decreases with increasing flow rate (Tab.1).

Table I

% efficiency of colour removal by PVPP (1g/L)

Flow-rate (L/h)	Enzyme reactor AffiGel-10	AffiGel-15
8	91	93
16	80	87
33	70	76
50	58	71

In any case, on washing the browned reactor with 0.4 % tartrate buffer, pH 3, containing 10 % ethanol (v/v) and 0.5 M NaCl, some but not all the brown pigments were removed, without affecting the immobilised activity.

In conclusion, from the data obtained, it is reasonable to propose a laccase reactor for phenolic oxidation in white grape must, where oxidative discoloration can be a technological problem.

Few cases of routine use of immobilised enzymes in food industries are reported (Cheetham,1987), however new

applications might be of interest mainly in the field of liquid foodstuffs, such as fruit juices, wine and beer.

Aknowledgements

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