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Encapsulation of glucose oxidase in alginate hollow beads to reduce the fermentable sugars in simulated musts

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ABSTRACT

The treatment of must with glucose oxidase (GOX) is a biotechnological alternative to the production of reduced-alcohol wines. Nevertheless, the low pH of must is a limiting factor for the enzyme's activity. This study reports on a simple immobilization method that uses enzyme encapsulation to overcome that limiting factor. The encapsulation of GOX was acceptably optimized using response surface methodology (RSM). An immobilization yield of 73% was obtained with 22 g L^{-1} Na-alginate, 20 g L^{-1} carboxymethylcellulose, and 12 g L^{-1} $CaCl_2$. The apparent K_m value of the immobilized GOX (4.55 mM) was slightly higher than that of the soluble enzyme (3.99 mM) and the activation energy was similar (~ 35 kJ mol^{-1}). The encapsulated enzyme maintained about 68% and 92% of its initial activity at pH 3.0 and 4.0, respectively. Moreover, GOX-loaded alginate hollow beads could be re-used with 7 reaction cycles in model must, with a final reaction efficacy of 37%. GOX was successfully encapsulated in Ca-alginate hollow beads, and their catalytic potential remained unaffected by the immobilization process. In view of these results and the activity of the immobilized enzyme at the acidic pH of the must, this encapsulated biocatalyst is a promising alternative for industrial use in the production of reduced-alcohol wines.

1. Introduction

In the wine sector, new strategies must be developed to solve the challenges arising from climate change and new market demands. Rising temperatures lead to higher and faster accumulation of sugars in ripened grapes. The accumulation of sugars implies a higher alcohol content of the wine following fermentation, which in some cases even surpasses ethanol concentrations of 15% (v/v) (Godden, 2000). These high concentrations of ethanol can cause unpalatable aromas and flavors (Gawel, van Sluyter, & Waters, 2007; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Compared to wines with a high alcohol content, reduced-alcohol wines have a number of social, health (Salomon, 2006) and economic benefits (Pickering, 2000) both for producers and for consumers.

The technologies available for the production of dealcoholized, low- and reduced-alcohol wines have been reviewed (Ozturk & Anli, 2014; Pickering, 2000; Pickering, Heatherbell, & Barnes, 1998; Schmidtke, Blackman, & Agboola, 2012). In general, these physical processes (Bui, Dick, Moulin, & Galzy, 1986; Mermelstein, 2000) are difficult to do, are expensive, and are liable to lose the aromatic compounds that affect the flavor balance (Heux, Sablayrolles, Cachon, & Dequin, 2006). One biotechnological alternative is to treat the grape juice from mature fruit with glucose oxidase (GOX) (Ozturk & Anli, 2014). GOX (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4), uses molecular oxygen, to catalyze the oxidation of β-D-glucose to δ-gluconolactone, which spontaneously hydrolyses to gluconic acid, and H_2O_2 (Zoldak, Zubrik, Musatov, Stupak, & Seldak, 2004). Enzymatic action results in a lower amount of glucose in the must, yielding wines with a reduced alcohol content (Heresztyn, 1987; Pickering, 1997; Villettaz, 1986, 1987).

The composition, sensory properties, and chemical stability of white wine made from GOX-treated juice have been described (Pickering, Heatherbell, & Barnes, 1999a, 1999b, 1999c). The low pH of the must was found to be a limiting factor in the catalytic capacity of the enzyme (Kakisz, Hendle, & Schmid, 1997; Pickering et al., 1998). Hence, a deacidification of the juice with $CaCO_3$ was beneficial, to obtain sufficiently low glucose concentrations to produce an acceptable reduction of alcohol in the wine (Pickering et al., 1998; Pickering, 2000).

The properties of immobilized enzymes suggested that they can differ from native enzymes (optimum pH and temperature, inhibitors, etc.). The hypothesis was that the immobilization of GOX in the must at such a highly acidic pH would improve the catalytic efficacy of the enzymes. Furthermore, the application of immobilized enzymes

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facilitates the separation of the final products and the enzymes can be reused or used in a continuous process.

As a low-cost, non-toxic, biocompatible substance that is resistant to microbial attack, and easily formulated, alginate is commonly used in immobilization processes, (Gombotz & Wee, 1998). The encapsulation in hollow Ca-alginate capsules is a commonly used method for the immobilization of biocatalysts (Chai, Mei, Wu, Lin, & Yao, 2004; Tanriseven & Doan, 2001).

This paper describes the encapsulation of GOX in hollow Ca-alginate beads to obtain a biocatalyst active at the pH of the must. The research is based on the fact that the hollow-beads, with a semipermeable layer containing a liquid phase, can enclose the enzyme at a suitable pH for its activity that differs from the pH of the must. The optimum conditions for enzymatic encapsulation were obtained using response surface methodology (RSM). The kinetic behavior, and the pH and temperature profiles of the GOX beads were studied. Finally, the operational stability of the encapsulated enzyme was evaluated in a simulated must system (Blouin & Peynaud, 2004).

2. Materials and methods

2.1. Materials

GOX (from *Aspergillus niger*, 150 U mg^{-1}), peroxidase (from horseradish, 250 U mg^{-1}), were purchased from Sigma Aldrich (St. Louis, Mo, USA). According to Sigma Aldrich, 1 unit (U) of GOX oxidizes 1.0 μmole of β-D-glucose to D-gluconolactone and H_2O_2 per min at pH 5.1 at 35 °C, and 1 unit (U) of peroxidase corresponds to the amount of enzyme that forms 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C.

Na-alginate and *o*-dianisidine were purchased from Sigma-Aldrich. CMC sodium salt low viscosity was obtained from BDH Laboratory Suppliers (Poole, England)·H_2O_2 35% (w/w) in aqueous solution stabilized (analytical grade) was purchased from Acros Organic (Geel, Belgium). The other reagents were of analytical grade (Acros Organic).

2.2. GOX assay

The activity of free and immobilized GOX was estimated by determining the amount of H_2O_2 that formed (Çil, Böyükbayram, Kiralpl, Toppare, & Yağci, 2007). A reaction mixture of 2.9 mL of glucose (15 mM) dissolved in a sodium acetate buffer (0.1 M, pH 5.1) and 0.1 mL of GOX solution (0.02 mg mL^{-1}), or the amount of immobilized enzyme corresponding to the encapsulation of 0.1 mL of GOX (0.02 mg mL^{-1}), was incubated for 10 min at 50 °C with constant shaking at 150 rpm. Then, 0.4 mL of acetate buffer, 0.1 mL of peroxidase (60 U mL^{-1}) and 2.4 mL of *o*-dianisidine (0.21 mM), as the coloring agent, were added to 0.1 mL of solution obtained from the enzymatic mixture. Finally, the enzymatic reaction was immediately stopped with the addition of 0.5 mL of H_2SO_4 (2.5 M). Absorbance was measured at 530 nm using a spectrophotometer Hitachi U-2000 (Ibaraki, Japan). An H_2O_2 standard curve was used to calculate enzyme activity. One unit of enzymatic activity (U) is expressed as the amount of enzyme required to produce 1 μmol of H_2O_2 min^{-1} using the aforementioned assay conditions. The values shown in both the figures and the tables represent the average of at least three triplicate assays.

2.3. Encapsulation of GOX in Ca-alginate

GOX was immobilized by encapsulation in Ca-alginate hollow beads using the method of Pilar, Ortega, Perez-Mateos, and Busto (2009), with minor modifications. Three mL of GOX solution (0.02 g L^{-1}) was mixed with 17 mL of a CMC solution (ranging from 11.9 to 22.0 g L^{-1}) containing $CaCl_2$ (11.5–28.4 g L^{-1}). The mixture was dropped into 600 mL of Na-alginate (11.5–28.4 g L^{-1}) and gently stirred in a 1 L beaker. After 10 min of gelation, about 300 mL of alginate solution was withdrawn, and the alginate solution containing the encapsulated enzyme was diluted with 500 mL of deionized water Milli-Q Integral 3 purification system (Merck-Millipore, Madrid, Spain). Then, the diluted solution was decanted and the immobilized enzyme was washed three times with deionized water (250 mL each time). The encapsulated enzyme was transferred into 20 g L^{-1} $CaCl_2$ solution, stirred and left to harden for 20 min. Finally, the $CaCl_2$ solution was decanted and the GOX-loaded alginate capsules were placed on filter paper to dry, about 15 min.

Table 1
Coded and real values for each variable of the central composite design.

Independent variables	Symbol	Coded variable levels				
		-α	− 1	0	+ 1	+ α
[Na-alginate] (g L^{-1})	X_1	11.5	15.0	20.0	25.0	28.4
[CMC] (g L^{-1})	X_2	11.9	14.0	17.0	20.0	22.0
[$CaCl_2$] (g L^{-1})	X_3	11.5	15.0	20.0	25.0	28.4

2.4. RSM

RSM was used to optimize the immobilization parameters. Preliminary experiments were done to determine the initial values of the variables influencing the immobilization process (data not shown). The range of the independent variables and their values are detailed in Table 1: concentration of alginate (X_1), CMC (X_2) and $CaCl_2$ (X_3). A central composite design, rotatable and orthogonal, was chosen (23 runs) (Ortega, Perez-Mateos, Pilar, & Busto, 2009). The dependent variable was the immobilization yield (IY, %) that is defined in Eq. (1):

$$IY\,(\%) = (U_e/U_o) \times 100 \quad (1)$$

Where, U_e= unit of GOX activity encapsulated, and, U_o = unit of GOX activity used initially for the encapsulation.

Optimized conditions were established using Statgraphics Centurion XVI (version 16.2.04) (Statpoint Technologies Inc., Warrenton, VA, USA). This software package was also used to fit the second-order model to the independent variables by using Eq. (2):

$$y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i}^{i<j} \sum_{j} b_{ij} X_i X_j + e \quad (2)$$

where, y is the dependent variable (response) to be modeled, X_i and X_j are the independent variables (factors), b_o, b_i, b_{ii}, and b_{ij} are regression coefficients, and e is the error. The model was simplified by dropping statistically insignificant terms ($p \geq 0.05$) by using analysis of variance (ANOVA) with the software Statgraphics Centurion XVI. The outcome of the ANOVA can be visualized in a Pareto Plot (Statgraphics Centurion XVI), where the absolute value of the standardized estimated effect (the estimate effect divided by the standard error) of each factor investigated on the IY is plotted. In this treatment a parameter is deemed to have a significant influence if the size of effect is > 2.

2.5. Determination of kinetic parameters

The kinetic parameters for free and immobilized GOX were calculated using different glucose concentrations ranging from 1 to 30 mM, in the standard assay mixture. The K_m and V_{max} values were calculated using a Hanes-Woolf plot. The Hanes-Woolf Eq. (3) is a modified form of the Michaelis-Menten equation according to which:

$$\frac{S}{V} = \frac{S}{V_{max}} + \frac{K_m}{V_{max}} \quad (3)$$

where S is the substrate concentration, V is the rate of the reaction, V_{max} is the maximum rate and K_m is the Michaelis constant. This plot was used because the distribution of errors is uniform in this case