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Kinetic study of tyrosinase immobilized on polymeric membrane

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ABSTRACT

Kinetic properties of tyrosinase immobilized on a polymeric membrane for the production of L-3, 4-dihydroxyphenylalanine (L-DOPA) were investigated. A comparison with the properties shown by the free enzyme used in a stirred tank reactor was also carried out. The values of the Michaelis–Menten constant indicated that the immobilized tyrosinase exhibited better affinity for the substrate (K_m = 1.56 mM and 2.10 mM for the immobilized and free enzyme, respectively).

The stability (pH, thermal, storage and operational) of both free and immobilized tyrosinase was also evaluated. Results showed that the immobilization enhanced the enzyme stability. The optimum pH and temperature for the activity of both free and immobilized enzyme were found at pH 7.0 and 35 °C, respectively. However, the immobilized tyrosinase was more stable in the whole range of pH and temperature. These advantages of the immobilized enzyme make it a good candidate for its use in different industrial processes.

1. Introduction

Enzymes are used as catalysts to enhance the rate of the reactions and operate in milder conditions of pH, temperature and pressure compared to chemical catalysts that require severe conditions. They are also eco-friendly with respect to the chemical ones. Due to these advantages, use of enzymes reduce manufacturing costs and adverse impacts on the environment. However, considering their high costs and the difficulty of separating them from the reaction medium, different routes were followed to overcome these limitations. In this context, the possibility of immobilizing the enzymes on different supports represents one of the most effective methods in this research field. In fact, the immobilization process offers many advantages such as higher enzyme stability, easy enzyme recovery from the reaction medium and sometimes higher catalytic activity [1,2]. Nowadays, different methods are available for enzyme immobilization ranging from covalent bond [3,4] to physical entrapment or adsorption [5,6]. The main application areas of immobilized enzymes are the food and pharmaceutical industries [7,8]. A number of papers have reported the immobilization of several enzymes. For example, lipase, one of the most widely studied enzymes, was recently immobilized in different mesoporous organosilicates (PMOs) containing ethane and benzene groups with large cage-like pores [9]. The authors demonstrated that these supports are promising candidates for lipase immobilization. Sing and Kayastha reported the immobilization of *Cicer* α-galactosidase onto chitosan and amberlite beads [10]. Their studies evidenced that the immobilized enzyme exhibited better activity and stability with respect to the free form. Recently, invertase was immobilized on hydrophobic polyurethane to produce inverted sugar syrup from sugarcane juice using an enzymatic bioreactor [11].

An interesting biocatalyst is tyrosinase (polyphenol oxidase EC 1.14.18.1), a copper enzyme largely distributed in animals, plants and microorganisms [12,13]. Tyrosinase is used at the industrial level for the removal of phenolic compounds from wastewater [14], as biosensors to identify different pollutants [15] and also in the stereospecific synthesis of quinones, phenols and polymers [16].

In previous papers we successfully demonstrated the possibility of producing L-DOPA using tyrosinase immobilized on polymeric and inorganic membranes [3,5].

The aim of this work was to provide relevant information for potential application at the industrial level of the immobilized tyrosinase on tubular polyamide membranes for L-DOPA production. The effect of pH and temperature on enzyme activity and stability was investigated in order to assess the optimum operating conditions. The Michaelis–Menten kinetic parameters (K_m, V_{max} and k_{+2}) for free and immobilized tyrosinase were also determined.

2. Materials and methods

2.1. Materials

L-tyrosine (purity 98 wt%), ascorbic acid (purity 99 wt%), L-DOPA, Bradford's kit, Tyrosinase (EC 1.14.18.1 obtained from

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mushroom as lyophilized powder), sodium phosphate dibasic (98 wt%) and sodium phosphate monobasic (98 wt%) were purchased from Sigma-Aldrich (Milan, Italy). 1020 AE polyamide (PA) membranes having a nominal molecular weight cut-off of 20 kDa were supplied by Berghof Membrane Technology GmBH & Co KG (Germany) [5].

2.2. Analytical determinations

Tyrosinase concentration was determined by Bradford's method [17]. The L-DOPA concentration was measured by HPLC analysis. The column was Alltima C 18, 5 μm, 250 mm × 4.6 mm (Alltech, Italy). The mobile phase was 25 mM of KH_2PO_4 (pH=4). The operating conditions were flow rate of 0.9 mL min^{-1}, temperature of 25 °C, pressure of 118 bar and wavelength of 270 nm. The retention time of L-DOPA was 5.8 min. The analysis of samples was carried out after the collection process to avoid L-DOPA oxidation by air.

2.3. Free tyrosinase activity assay

The activity of free enzyme was determined by following the production of L-DOPA using a stirred tank reactor. The experiments were achieved using one liter of the reaction mixture constituted of 50 mM sodium phosphate buffer, 2.5 mM of L-tyrosine and 2.5 mM of L-ascorbic acid. The ascorbic acid was added to the reaction medium as an antioxidant agent to reduce the oxidation of L-DOPA in dopaquinone [5]. During the reaction 0.5 mL aliquots were collected each 15 min. A volume of 0.5 mL of hydrochloric acid (2 M) was added to the samples to stop the reaction. The concentration of L-DOPA in the collected samples was determined by HPLC analysis.

2.4. Immobilization of tyrosinase

The enzyme was immobilized using the procedure reported in a previous paper [5]. Briefly, tyrosinase solution, prepared dissolving an appropriate amount of the enzyme in 150 ml of phosphate buffer (50 mM; pH=7), was re-circulated along the shell side of the membrane module at an axial velocity of 0.06 m s^{-1} and a trans-membrane pressure of 0.2 bar. According to this method, due to its molecular weight (120 kDa) and the membrane cut-off (20 kDa), the biocatalyst was entrapped into the macrovoids of the sponge layer and thus did not pass through the thin layer. During the process, the permeate flux was measured in the time and the process was stopped at the steady state. Besides, the permeate was collected and filtered for other two times. Afterwards, the membrane was repeatedly washed with phosphate buffer until no protein was detected in washing solutions (by measuring the optical density at 280 nm).

The mass of the immobilized enzyme was calculated by mass balance between the initial enzyme amount (in the feed solution) and the one present in permeate and retentate streams. The enzyme concentration in the feed, permeate, retentate and washing solutions was determined by the colorimetric Bradford's method [5]. The enzyme activity was determined by following the production of L-DOPA in the time as already done for the free form.

2.5. Membrane reactor setup

All the reactions with the immobilized tyrosinase were carried out using the equipments described elsewhere [5].

The membrane was 18 cm long with an internal/external diameter of 11.5/12 mm and having an external surface area of 65 cm^2.

The effects of pH and temperature on the activity of free and immobilized tyrosinase were investigated working in a total recycle mode. In this configuration, both the permeate and retentate streams are recycled back to the feed tank. The kinetic parameters were determined under the same operation mode at the optima values of temperature and pH (35 °C and pH 7). The reactions were performed using 1 l of feed solution (2.5 mM of L-tyrosine and 2.5 mM of L-ascorbic acid). The feed solution was fed along the membrane module in laminar flow regime.

Experiments in a continuous operation mode were also carried out (T=35 °C and feed volume of 10 l) to investigate the possibility of enhancing the performance of the catalytic process.

During the continuous operation mode, the substrate was fed and recycled through the enzyme-loaded membrane and the permeate was collected in a graduated cylinder.

2.6. Effect of pH and temperature on the activity of free and immobilized tyrosinase

The influence of pH on the catalytic activity of soluble and immobilized enzyme was determined performing reactions at different pH values (5.0–9.0). The effect of temperature was investigated in the range 25–55 °C and maintaining constant the other parameters. The results for both pH and temperature were normalized, considering as 100% enzymatic activity the highest value obtained in each of the series of measurements made. All activity tests were conducted in triplicate.

2.7. Determination of kinetic parameters

The kinetic constants K_m (constant of Michaelis–Menten) and V_{max} (maximum rate of the reaction) of free and immobilized tyrosinase were determined by measuring initial rates (v_0) of the reaction obtained as a function of different substrate concentrations [S] (0.45 mM; 0.63 mM; 1.25 mM; 1.60 mM; 2.50 mM) in phosphate buffer (50 mM, pH 7) at 35 °C. The initial rate (v_0) is the slope of the initial linear portion of the curve obtained following the product formation as a function of time [18]. K_m and V_{max} values were calculated from the Lineweaver–Burk equation [19].

The k_{+2} value, defined as a turnover number, was calculated as follows:

$$k_{+2} = \frac{V_{max}}{[E]_t} \quad (1)$$

The turnover number represents the number of substrate molecules converted into product per enzyme molecules per unit time when the enzyme is saturated with substrate.

The catalytic efficiency (CE) of an enzyme is defined by the following equation:

$$CE = \frac{k_{+2}}{K_m} \quad (2)$$

A large value of this ratio indicates an easy formation of the reaction product.

The efficiency factor, η, was calculated from the maximum reaction rates of the immobilized tyrosinase over that of the free one [20]:

$$\eta = \frac{\nu_{immob}}{\nu_{free}} \quad (3)$$

where ν_{immob} and ν_{free} are the maximum reaction rate of the immobilized and free enzyme, respectively.

All the experimental tests of both free and immobilized enzyme were carried out at 35 °C which is the optimum value for both free and immobilized tyrosinase.