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Titania/lignin hybrid materials as a novel support for α-amylase immobilization: A comprehensive study

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ABSTRACT

α-Amylase from *Aspergillus oryzae* was immobilized via covalent bonds and by physical interactions on a synthesized titania/lignin novel hybrid support. A temperature of 5 °C, a pH of 7.0, an initial enzyme solution concentration of 3.0 mg/mL and a 3 h process duration were found to be optimal for the highest activity of the immobilized enzyme. Moreover, the effect of temperature, pH, storage time and repeated catalytic cycles on the activity of free and immobilized enzyme was examined. Bound α–amylase showed enhanced thermal and chemical stability, and its reusability was also improved. Immobilized α-amylase retained over 80% of its initial activity when stored for 30 days at 4 °C. Kinetic parameters of the free and immobilized biocatalyst were calculated and compared. The maximum reaction rate (V_{max}) and turnover number (k_{cat}) were slightly lower for the immobilized enzyme than for the free enzyme. It should be clearly stated that this work presents a useful protocol to produce stable and active immobilized α–amylase onto titania/lignin hybrid which may also be applied to immobilization of other enzymes.

1. Introduction

Amylases are among the most significant enzymes used in many branches of industry. Their wide application is evidenced by the fact that they represent over 30% of the global enzyme market [1]. Amylases can be divided into three groups according to their mechanism of action on starch chains: α-amylases, β-amylases and γ-amylases. The most commonly used are α-amylases (1,4-α-D-glucan-glucanhydrolase, EC 3.2.1.1), which catalyze randomly the cleaving of the internal α-1,4 glycosidic bonds in starch molecules [2]. The main products of these reactions are various maltodextrins, usually with up to 6 glucose units in their structure. α-Amylases are used in a great variety of industrial applications, ranging from the paper and textile industries and detergent production to uses in medicine and clinical chemistry [3]. Nevertheless, due to their ability to catalyze starch hydrolysis, they are most commonly used in the food industry, for example in baking, brewing and alcohol production [4]. The widespread use of these enzymes is limited by their relatively low stability under reaction conditions and the creation of mass transfer limitations [5]. To overcome these problems and to facilitate separation of the enzyme from the reaction mixture, α-amylases are used in the form of immobilized, heterogeneous biocatalysts [6].

Immobilization is a process based on attachment of the enzyme to a solid matrix, which facilitates recovery and reusability of the enzyme. Moreover, immobilization improves stability and extends the lifetime of the bound peptides, compared with their native forms [7]. Enzymes are bound to the support by weak physical interactions or by much stronger covalent bonds. The interactions, by increasing the rigidity of the enzyme molecules, stabilize the tertiary and quaternary structures of the peptides, in consequence enabling their long-term reuse. Moreover, the negative effect of the harsh reaction conditions and inhibitors is strongly reduced [8].

Many types of materials may be used as matrices in enzyme immobilization. These may be broadly divided into both inorganic and organic supports, the latter including synthetic polymers and biopolymers of natural origin. An optimal matrix should offer high physical and mechanical stability, inertness, reusability, and good affinity to the peptides [9]. It should also positively affect enzyme activity and stability, and should protect the enzyme from microbial contamination.

Numerous supports of various origin have previously been used for immobilization of α-amylases, including polymers – polyaniline [10]; biopolymers [11]; inorganic materials – nano ZnO [12]; and composite materials – Fe_3O_4/polyaniline [13], chitosan/polyvinylpyrrolidone [14]. The last group of supports appears particularly promising, providing an opportunity to create materi-

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als with desirable properties, numerous reactive functional groups, improved biocompatibility and good mechanical stability. From this perspective, combining inorganic oxides with biopolymers results in the creation of a composite material suitable for the immobilization of α-amylases. However there is a lack of information about the immobilization of these enzymes on the surface of composite materials consisting of inorganic oxide and lignin, which is known as an activating biopolymer [15]. Lignin, as one of the most widespread biopolymers in the world, has many advantages. It is a waste material that is produced in large amounts in the pulp and paper industry. The biopolymer is harmless to living organisms and the renewable nature indicates that the lignin resources will never be depleted. Additionally, the presence in the structure of lignin variety of functional groups means that this material can easily be modified and combined with other substances, including inorganic compounds, or bigger molecules like enzymes or microorganisms.

Thus, in this study, for the first time (to the best of our knowledge), the α-amylase from *Aspergillus niger* was immobilized on a titanium dioxide/lignin hybrid matrix. FTIR spectroscopy confirmed the effective attachment of the enzyme to the solid support, and enabled identification of the type of interactions taking place. The effect of various initial process parameters, such as pH, temperature, initial concentration of enzyme solution and process duration, on the quantity of immobilized enzyme and its activity was evaluated to find the most suitable immobilization strategy. The storage stability and reusability of the bound enzyme, as well as the most suitable conditions for effective starch hydrolysis, were examined. Additionally, the thermal and pH stability of the resulting biocatalytic systems were studied. To further analyze changes in the affinity of the enzymes to the substrate molecules, sophisticated kinetic analysis of the free and immobilized α-amylase was also performed.

2. Materials and methods

2.1. Materials

Commercial titanium dioxide (product name Tytanpol® R-001) was supplied by Grupa Azoty SA (Poland). Kraft lignin, poly-*L*-lysine (PLL), dioxane and sodium (meta)periodate, used in the synthesis of the TiO_2/lignin hybrid, were obtained from Sigma-Aldrich (USA). Acetate buffer, phosphate buffer and tris-buffer were supplied by Amresco Company (USA). Immobilization was carried out using commercially available α-amylase from *Aspergillus oryzae* (EC 3.2.1.1, product number 10065) obtained from Sigma-Aldrich. Potato starch, maltose and 3,5-dinitrosalicylic acid (DNS) used for enzyme activity evaluation were purchased from Sigma-Aldrich, and 96% ethyl alcohol and 85% phosphoric acid from Chempur (Poland). Coomassie Brilliant Blue G-250 (CBB G-250) dye and bovine serum albumin (BSA), used in the Bradford method, were supplied by Sigma-Aldrich.

2.2. Synthesis of TiO_2/lignin support

The titania/lignin hybrid support was synthesized in three steps. In the first, titanium dioxide was modified using poly-*L*-lysine. This compound was used due to its compatibility with α-amylase (peptide) and presence of numerous reactive functional groups for the effective binding of lignin. For the purpose of TiO_2 functionalization, it was suspended in phosphate buffer at pH 7, and 10% (w/w) of PLL was added. The mixture was placed in a KS260 Basic shaker (IKA Werke GmbH, Germany) for 24 h at a temperature of 4 °C. After mixing, the product was centrifuged (Eppendorf Centrifuge 5810 R, Germany) and washed with water to elute unbound poly-*L*-lysine and eliminate phosphate buffer. The PLL-modified titanium dioxide was left to dry at ambient temperature for 24 h.

In the second step, the lignin was activated by dissolving it in a 9:1 (v/v) dioxane/water mixture and dropwise in an aqueous solution of sodium (meta)periodate (1.5 g in 30 mL of water). After addition of the oxidizing agent the mixture was mixed for 60 min (Eurostar Digital, IKA Werke GmbH, Germany). Finally, the activated biopolymer was evaporated on a Rotavarpor RII vacuum evaporator (Büchi, Germany) and dried for 6 h at 80 °C. The process has been described in detail in our previous work [16].

Finally, titanium dioxide and lignin were combined in a mass ratio of 1:1. For this purpose, modified TiO_2 was suspended in water and an appropriate amount of activated lignin was added. The mixture was placed in a KS260 Basic shaker (IKA Werke GmbH, Germany) for 6 h at a temperature of 4 °C. The product was then dried in an SF 75 dryer (Memmert, Germany) at 35 °C for 24 h and then used for immobilization of the α-amylase from *Aspergillus oryzae*.

2.3. Immobilization of α-amylase from Aspergillus oryzae

One of the main goals of the study was to optimize the parameters of the immobilization process, including time, pH, temperature, and the initial concentration of the enzyme solution. For immobilization of the α-amylase from *Aspergillus oryzae*, 0.5 g of the previously obtained TiO_2/lignin hybrid material was used, and 10 mL of the enzyme solution in buffer at appropriate pH (from 4 to 9), at a concentration in the range from 0.5 to 5 mg/mL, was added. The mixture was placed in a KS260 Basic shaker (IKA Werke GmbH, Germany), and was shaken for a specified period of time varying from 0.5 to 6 h at a temperature ranging from 5 to 30 °C. The immobilized enzyme was then centrifuged (Eppendorf Centrifuge 5810 R, Germany) and washed three times with the same buffer solution. The product and the filtrate were retained and subjected to further analysis.

2.4. Analysis of immobilized enzyme

The quantity of enzyme immobilized on the surface of the TiO_2/lignin material was determined based on the Bradford method by measuring the initial and final quantities of α-amylase in the immobilization medium [17]. A calibration curve based on BSA solutions at known concentration was used to calculate the quantity of the bound protein, expressed in milligrams of immobilized enzyme per gram of support.

The morphology of the titanium dioxide/lignin hybrid before immobilization was examined using SEM images recorded from an EVO40 scanning electron microscope (Zeiss, Germany). Before testing, the sample was coated with Au for a time of 5 s using a Balzers PV205P coater (Switzerland).

Analysis of the porous structure parameters was carried out to identify changes in the values of the surface area, mean pore size and pore volume for the titanium dioxide/lignin hybrids before and after immobilization. The N_2 adsorption-desorption isotherms under relative pressure (p/p_0) at 77 K were determined on an ASAP 2020 instrument (Micromeritics Instrument Co., USA).

To identify the functional groups present in the structure of the matrix and the product after immobilization, Fourier transform infrared spectroscopy (FTIR) was carried out using a Vertex 70 spectrometer (Bruker, Germany). Materials were analyzed in the form of potassium bromide tablets, made by mixing 250 mg of anhydrous KBr and 1 mg of the analyzed substance under a pressure of 10 MPa.