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Immobilization of *Aspergillus oryzae* β galactosidase on zinc oxide nanoparticles via simple adsorption mechanism

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ABSTRACT

The present study demonstrates the immobilization of *Aspergillus oryzae* β galactosidase on native zinc oxide (ZnO) and zinc oxide nanoparticles (ZnO-NP) by simple adsorption mechanism. The binding of enzyme on ZnO-NP was confirmed by Fourier transform-infrared spectroscopy and atomic force microscopy. Native ZnO and ZnO-NP showed 60% and 85% immobilization yield, respectively. Soluble and immobilized enzyme preparations exhibited similar pH-optima at pH 4.5. ZnO-NP bound β galactosidase retained 73% activity at pH 7.0 while soluble and ZnO adsorbed enzyme lost 68% and 53% activity under similar experimental conditions, respectively. There was a marked broadening in temperature–activity profile for ZnO-NP adsorbed β galactosidase; it showed no difference in temperature-optima between 50 °C and 60 °C. Moreover, ZnO-NP adsorbed β galactosidase retained 53% activity after 1 h incubation with 5% galactose while the native ZnO- and soluble β galactosidase exhibited 35% and 28% activity under similar exposure, respectively. Native ZnO and ZnO-NP adsorbed β galactosidase retained 61% and 75% of the initial activity after seventh repeated use, respectively. It was noticed that 54%, 63% and 71% milk lactose was hydrolyzed by soluble, ZnO adsorbed and ZnO-NP adsorbed β galactosidase in batch process after 9 h while whey lactose was hydrolyzed to 61%, 68% and 81% under similar experimental conditions, respectively. In view of its easy production, improved stability against various denaturants and excellent reusability, ZnO-NP bound β galactosidase may find its applications in constructing enzyme-based analytical devices for clinical, environmental and food technology.

1. Introduction

β Galactosidase (E.C.3.2.1.23) is a hydrolytic enzyme that catalyzes the breakdown of lactose into glucose and galactose. These enzymes are abundant among animals, plants and microbial sources. Its use has been suggested in various food, dairy and fermentation industries to hydrolyze milk and whey lactose [1–3]. β Galactosidases have been exploited as a drug to treat infants with a genetic deficiency of intestinal lactase. Thus, the technological importance of this enzyme arises mainly due to the problems associated with whey disposal, lactose crystallization in frozen concentrated desserts and milk consumption by lactose-intolerant populations [4–7].

In accordance with the present scientific knowledge, soluble enzymes cannot be used in industrial and environmental applications due to product inhibition, instability, non-reusability and difficult recovery. In order to overcome such limitations, enzyme immobilization has been considered as one of the best alternatives to use them at large scale [8–10]. Immobilized biocatalyst can be reused several times and provides several benefits like easy separation from reaction mixture, no contamination of product by the enzyme, continuous processing, multi-enzyme reaction systems, operational and long term stability [11]. Recently, immobilization of industrially important enzymes onto nanomaterials with improved performance has paved the way to myriad of application-based commercialization [12]. Nanostructure materials exhibited interesting properties such as large surface to volume ratio, high surface reaction activity, high catalytic efficiency and strong adsorption ability that makes them potential candidate materials to play an important role in enzyme immobilization [13]. The large surface area of nanomaterials provided better matrix for the immobilization of enzymes leading to increased enzyme loading per unit mass of particles. Moreover, the multipoint attachment of enzyme molecules to nanomaterial surface reduces protein unfolding resulting in the enhanced stability of the enzyme attached to the surface of nanoparticles [14].

In the present work, an attempt has been made to compare the immobilization and stabilization of *Aspergillus oryzae* β galactosi-

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dase by using native zinc oxide (ZnO) and zinc oxide nanoparticles (ZnO-NP). Immobilized enzyme preparations were characterized by Fourier transform-infrared spectroscopy (FT-IR) and atomic force microscopy (AFM) in order to determine the linkage between matrices and the enzyme. Effect of various physical and chemical denaturants on the activity of soluble β galactosidase (S-β gal), β galactosidase adsorbed on native ZnO (ZnO-β gal) and β galactosidase adsorbed on ZnO nanoparticles (ZnO-NP-β gal) were monitored. Reusability of immobilized β galactosidase was also evaluated. Moreover, soluble and immobilized β galactosidase preparations were exploited for the hydrolysis of lactose from milk and whey in stirred batch processes at 50 °C.

2. Experimental methods

2.1. Materials

A. oryzae β galactosidase was obtained from Sigma Chem. Co. (St. Louis, MO, USA). 2-Nitrophenyl β-D galactopyranoside (ONPG), zinc acetate dihydrate, ethylene glycol, 2-propyl alcohol, native ZnO, triethylamine and glycerol were obtained from SRL Chemicals (Mumbai, India). All reagents were prepared in double distilled water with chemicals of analytical grade.

2.2. Synthesis of ZnO nanoparticles and their characterization by XRD analysis

Zinc oxide nanoparticles (ZnO-NP) were synthesized by sol–gel method. The sol was prepared by using zinc acetate dihydrate (99.5%), ethylene glycol, 2-propyl alcohol and glycerol. $Zn(CH_3COO)_2{\cdot}2H_2O$ (100 g) was mixed with 25 mL ethylene glycol at 150 °C for 15 min to obtain a uniform transparent solution. The solution was solidified to a transparent brittle solid on cooling down to room temperature. This solid was then dissolved in 200 mL of 2-propyl alcohol. The resulting solution was highly hydrophobic and converted into gel upon addition of few drops of water. Triethylamine was added to facilitate the hydrolysis of zinc acetate. The solution gets transformed into light brown powder upon incubation at 200 °C for 6 h. The resulting powder was initially heated in a programmable furnace at 450 °C for 8 h and then at 700 °C for 5 h to remove all organic impurities yielding white pure ZnO powder. The characterization of ZnO-NP obtained was performed by X-ray diffraction according to the procedure described earlier [15].

2.3. Optimization of immobilization of β galactosidase on native ZnO and ZnO-NP

Soluble β galactosidase (600 U) was independently adsorbed on varying concentrations of native ZnO and ZnO-NP (25–150 mg) overnight in 0.1 M sodium acetate buffer, pH 4.5 at 30 °C.

2.4. Adsorption of β galactosidase on native ZnO and ZnO-NP

β Galactosidase (4800 U) was independently mixed with 1.0 g native ZnO and ZnO-NP and was continuously stirred overnight in sodium acetate buffer, pH 4.5 at 30 °C. Immobilized enzyme preparations were collected by centrifugation at 3000 × *g* for 20 min. The resulting complexes were washed thrice with 0.1 M sodium acetate buffer, pH 4.5 and finally suspended in the same buffer and stored at 4 °C for further use [16].

2.5. Assay of β galactosidase

The hydrolytic activity of soluble and immobilized β galactosidase was determined by measuring the release of *o*-nitrophenol from ONPG at 405 nm. The reaction was performed by continuous shaking in an assay volume of 2.0 mL containing 1.7 mL of 0.1 M sodium acetate buffer, pH 4.5, 2.0 U β galactosidase and 0.2 mL of 20 mM ONPG. The reaction was stopped by adding 2.0 mL of 1.0 M sodium carbonate solution and product (*o*-nitrophenol) formation was measured spectrophotometrically at 405 nm. In case of immobilized enzyme, the reaction mixture was centrifuged at 3000 × *g* for 10 min to remove enzyme and the collected supernatant was analyzed for the formation of *o*-nitrophenol at 405 nm [17].

One unit (1.0 U) of β galactosidase activity is defined as the amount of enzyme that catalyzes the formation of 1.0 μmol of *o*-nitrophenol (ε_m = 4500 L/mol/cm/min) under standard assay conditions.

2.6. Atomic force microscopy (AFM)

Tapping mode AFM experiments of ZnO-NP, β galactosidase and ZnO-NP adsorbed β galactosidase were performed using commercial etched silicon tips as AFM probes by exposing the nanomatrix with the same protein-free buffer as the enzyme-contacted surfaces with typical resonance frequency of ca. 300 Hz (RTESP, Veeco, Japan).

2.7. FT-IR spectra

FT-IR spectra of ZnO-NP, β galactosidase and ZnO-NP adsorbed β galactosidase were monitored by using INTERSPEC 2020 model FT-IR instrument, USA. The calibration was done by polystyrene film. The samples were injected by Hamiet 100 μL syringe in ATR box. The syringe was first washed with acetone followed by distilled water. FT-IR analysis was done to monitor the functional groups of compounds.

2.8. Effect of pH

Enzyme activity of soluble and immobilized β galactosidase (2.0 U) was assayed in the buffers of different pH (3.0–9.0). The used buffers were glycine–HCl (pH 3.0), sodium acetate (pH 4.0, 4.5, 5.0), sodium phosphate (6.0, 7.0) and Tris–HCl (pH 8.0, 9.0). The molarity of each buffer was 0.1 M. The activity at pH 4.5 was taken as control (100%) for the calculation of remaining percent activity.

2.9. Effect of temperature

The activity of soluble and immobilized β galactosidase (2.0 U) was measured at various temperatures (30–80 °C) in 0.1 M sodium acetate buffer, pH 4.5 for 15 min. The enzyme activity at 50 °C was taken as control (100%) for the calculation of remaining percent activity for soluble and immobilized enzyme.

Moreover, soluble and immobilized enzyme preparations were incubated at 60 °C in 0.1 M sodium acetate buffer, pH 4.5 for varying times. Aliquots of each preparation (20 μL) were taken at indicated time intervals and chilled quickly in crushed ice for 5 min. The enzyme was brought to room temperature. The activity of enzyme without incubation at 60 °C was taken as control (100%) for the calculation of remaining percent activity.

2.10. Effect of galactose

The activity of soluble and immobilized β galactosidase (2.0 U) was determined in the presence of increasing concentrations of galactose (1.0–5.0%, w/v) in 0.1 M sodium acetate buffer, pH 4.5 for 1 h at 37 °C. The activity of enzyme without added galactose was considered as control (100%) for the calculation of remaining percent activity.