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Article

Activity and stability of bacterial cellulase immobilized on magnetic nanoparticles

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ABSTRACT

Magnetic nanoparticles (Fe_3O_4) were synthesized by co-precipitating Fe^{2+} and Fe^{3+} ions in an ammonia solution and treating under hydrothermal conditions. Cellulase was immobilized onto Fe_3O_4 magnetic nanoparticles via glutaraldehyde activation. Using response surface methodology and Box-Behnken design, the variables such as magnetic nanoparticle concentration, glutaraldehyde concentration, enzyme concentration, and cross linking time were optimized. The Box-Behnken design analysis showed a reasonable adjustment of the quadratic model with the experimental data. Statistical contour plots were generated to evaluate the changes in the response surface and to understand the relationship between the nanoparticles and the enzyme activity. Scanning electron microscopy, X-ray diffraction analysis, and Fourier transform infrared spectroscopy were studied to characterize size, structure, morphology, and binding of enzyme onto the nanoparticles. The stability and activity of the bound cellulase was analyzed using various parameters including pH, temperature, reusability, and storage stability. The immobilized cellulase was compared with free cellulase and it shows enhanced stability and activity.

1. Introduction

Enzymes are used as biocatalysts in chemical, pharmaceutical, and food industries [1,2]. Because the recovery and the reusability of free enzyme reactions are limited [3], attention has been paid to enzyme immobilization, which offers advantages over free enzymes, such as the possibility of continuous process, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture, and adaptability to various engineering designs [4].

The immobilization of biomolecules onto insoluble supports is an important tool to fabricate a diverse range of functional materials or devices [5]. It provides many distinct advantages including enhanced stability, easy separation from reaction

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mixture, possible modulation of the catalytic properties, and easier prevention of microbial growth [6]. Using magnetic supports for immobilization is promising. Magnetic carrier particles fulfill two functions of a magnetic material that allows ensembles to form with the species to be separated and surface properties that enable a selective separation.

The immobilization of enzymes onto nanomaterials such as nanopolymers [7], nanofiber [8], and nanoparticles [9,10] is of high interest because the reduction in the size of the enzyme carrier materials improves the efficiency of immobilized enzymes. Moreover, for surface attachment, smaller particles can provide a larger surface area for the attachment of enzymes, leading to higher enzyme loading per unit mass of particles [9]. However, for industrial biotechnology applications, immobilized enzymes on nanoparticles are limited by problems in recovery, e.g., via centrifugation or filtration.

Magnetic nanoparticles (MNPs) are potentially useful supports for bioactive materials such as peptides, enzymes, antibodies, and nucleic acids, and are easily recovered for reuse [11–15]. They have low toxicity, and their distinct advantage is their separation from reaction mixtures using magnets. Improved enzyme activity, loading, and stability using enzyme immobilized on MNPs have been shown by Dyal et al. [16].

Many chemical procedures used to synthesize magnetic nanoparticles and microparticles are applicable for bioapplications, such as classical co-precipitation, reactions in constrained environments (e.g., microemulsions), sol-gel syntheses, sonochemical and microwave reactions, hydrothermal reactions, hydrolysis and thermolysis of precursors, flow injection syntheses, electrospray syntheses, and mechanochemical processes [17–19].

Cellulase (1,4-(1,3;1,4)-D-glucan 4-glucanohydrolase, EC 3.2.1.4) can be used as a biocatalyst for cellulose hydrolysis. The cost of cellulase technology can be reduced by increasing the enzyme reusability and its stability. These may be accomplished by enzyme immobilization on suitable carriers. The immobilization of cellulase enzyme complex on magnetic supports has also reported [20]. The purpose of this study was to characterize the cellulase complex after direct binding to magnetic nanoparticles via glutaraldehyde cross linking and determine optimum operating conditions. Size and structure of the resultant nanoparticles were characterized by scanning electron microscopy (SEM). Binding of magnetite nanoparticles to enzyme was confirmed using Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD). Operating parameters for immobilized cellulase were evaluated using varying pH and thermal conditions, in addition to the binding efficiency of enzyme to the support, to determine the conditions for optimum hydrolysis reactions.

2. Experimental

2.1. Materials

Cellulase (from *Acinetobacter* sp. TSK-MASC) [21], Glutaraldehyde (25 wt% solution in water), $FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$ carboxymethyl cellulose (CMC), 3,5-dinitrosalicylic acid, Folin's reagent (2N), and sodium phosphate were purchased from Hi-media, Mumbai, India. All other chemicals were obtained from local suppliers and were analytical grade or better.

2.2. Preparation of magnetic nanoparticles

Magnetic nanoparticles (Fe_3O_4) were prepared by co-precipitating Fe^{2+} and Fe^{3+} ions with ammonia solution and treating under hydrothermal conditions as previously reported, but with minor modifications [13,22]. A 2:1 molar ratio of ferric and ferrous chlorides was dissolved in water under inert conditions. Chemical precipitation was achieved at 25 °C under vigorous stirring by adding 28% NH_4OH solution. The precipitates were heated to 80 °C for 30 min, and washed three times with water and once with anhydrous ethanol. The particles were dried for 24 h.

2.3. Immobilization of cellulase on magnetic nanoparticles

The immobilization of cellulase on magnetic nanoparticles followed the procedure described by Verma et al. [23] with minor modifications. The magnetic nanoparticles were suspended in deionized water at a concentration of 5 mg/mL. This suspension was sonicated for 1 h, and suspended in 1 mol/L glutaraldehyde solution in deionized water [23]. The support was activated by incubating the magnetic nanoparticles for 1 h at 25 °C in a shaker at 250 r/min. The reaction mixture was stored at 4 °C and sonicated at 1-h intervals to ensure uniform dispersion. After 2 h, the mixture was sonicated a final time and heated to 25 °C. The cellulase-bound nanoparticles were recovered by placing the container on a strong permanent magnet. They were washed twice in water and the resultant supernatants were used for protein analysis. The remaining precipitates were analyzed for enzymatic activity and stability.

2.4. Determination of enzyme activity

The cellulase activity was measured using CMC, and the amount of released glucose equivalent during the hydrolysis of CMC solution [24]. A 1% (w/v) CMC solution in acetate buffer pH 5 was used as the substrate. First, 1 mL of substrate solution and 1 mL of the immobilized enzyme were incubated at 37 °C for 1 h. The reaction was stopped by putting the enzyme reaction tubes in a boiling water bath for 15 min. The amount of reducing sugar was measured using the 3,5-dinitrosalicylic acid method [25]. One unit of enzyme activity is defined as the amount of enzyme producing 1 mol/L of glucose equivalent per min at 37 °C and pH 5. All experiments were conducted in triplicate, and reported as mean ± standard deviation.

2.5. Statistical optimization of immobilization process

Response surface methodology combined with Box-Behnken design (BBD) was established using Design Expert software (9.0.0.7 trial version). Four factors, enzyme concentration, magnetic nanoparticle concentration, cross linking (glutaraldehyde %), and cross linking time were optimized for