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Immobilization of *Aspergillus niger* cellulase on multiwall carbon nanotubes for cellulose hydrolysis

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ABSTRACT

In present study, *Aspergillus niger* cellulase was immobilized onto functionalized multiwalled carbon nanotubes (MWCNTs) via carbodiimide coupling. MWCNTs offer unique advantages including enhanced electronics properties, a large edge to basal plane ratio, rapid electrode kinetics and it's possess higher tensile strength properties due to their structural arrangements. The immobilization was confirmed by FTIR (Fourier transform infrared spectroscopy) and SEM (scanning electron microscope). The bionanoconjugates prepared under optimized condition retained 85% activity with improved pH and thermal stability. The $t_{1/2}$ of immobilized cellulase at 70 °C was four fold higher than free enzyme. The Km value indicates that affinity of bionanoconjugates towards substrate has increased by two times. The preparation could be reused ten times without much loss in enzyme activity. The enhanced catalytic efficiency, stability and reusability makes it useful for efficient cellulose hydrolysis.

1. Introduction

Cellulase is a mixture of enzymes with endoglucanase, exoglucanase, and β-glucosidase activity, which catalyzes the multi-step hydrolysis of cellulose to glucose. Cellulases have unique biotechnological conversion potential so, it provide opportunities to develop novel bioprocesses and products (Sharada et al., 2014). Nowadays, cellulases has been usage in different industries such as agriculture, brewery and wine, bioethanol, textile, food processing, paper and pulp, olive oil extraction and also used in pharmaceutical and medical sciences (Dincer and Telefoncu, 2007; Sharada et al., 2014). However the industrial application of free cellulase is limited because they are relatively unstable and cannot be efficiently reused, have low specific activity, as well as additional costs (Gokhale et al., 2013). In order to work out these problems, an immobilization of the cellulase enzyme on solid supports has been acknowledged as a viable solution (Chang et al., 2011; Gokhale et al., 2013). The immobilization of the enzyme can potentially improve stability, storage properties and enzyme reusability. Cellulase has already been immobilized on a number of various commercial materials which may be both insoluble or soluble carriers, as well as nano-scale materials (Ahmad and Sardar, 2014; Chang et al., 2011; Grewal et al., 2017; Ingle et al., 2017; Khoshnevisan et al., 2011; Perwez et al., 2017).

Nanomaterials are used as a matrix for enzyme immobilization, because its provide the upper limits in reconciliation the key factors that determine the efficiency of biocatalyst, including surface area, mass transfer resistance, and effective enzyme loading (Ahmad and Sardar, 2015; Jia et al., 2003; Wang et al., 2010; Zhang et al., 2009). The nanomaterials generally improves the efficiency of immobilized enzymes, because it provide a larger surface area for enzyme attachment, leading to higher enzyme loading per unit mass of particles (Grewal et al., 2017; Khoshnevisan et al., 2011; Perwez et al., 2017).

Recently, carbon nanotubes (CNTs) have been used as a matrix for immobilization of enzyme (Feng and Ji, 2011; Mubarak et al., 2014; Wang et al., 2010; Zhang et al., 2009). CNTs offer extraordinary properties such as high mechanical strength, electrical, and thermal properties that make them a very attractive material for a wide range of applications (Mubarak et al., 2014; Piccinino et al., 2015; Rivas et al., 2007). The bionanoconjugates (enzymes attached on nanotube) are stable and active at high temperature, providing a unique combination of useful attributes such as low mass transfer resistance, as well as recycling of the catalyst (Asuri et al., 2006; Mubarak et al., 2014; Zhang et al., 2009).

In this work, functionalized multi-walled carbon nanotubes (MWNTs) is prepared and used to immobilize cellulase enzyme. The bionanoconjugate can be further used in sacchrification of biomass and synthesis of plateform chemicals.

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2. Materials and methods

2.1. Materials

Multi-walled carbon nanotubes (MWCNTs), N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-(N-morpholino) ethanesulfonic acid (MES), N-hydroxysuccinimide (NHS), Cellulase from *Aspergillus niger* (Cat. No. C1184), Cellulase and Carboxy methyl Cellulose Sodium Salt (CMC) were purchased from Sigma Aldrich (USA). All other reagents used were of analytical grade and used without any further purification.

2.2. Determination of cellulase activity

Cellulase activity in native enzyme and bionanoconjugates were measured by the method described earlier (Ghose, 1987). The bionanoconjugates was continuously shaken at 50 rpm in water bath shaker for the entire duration of assay. One unit of enzyme is defined as the amount of enzyme required to produce one μmol of reducing sugar per min under assay condition. The amount of reducing sugar was estimated using the dinitrosalicyclic acid methods (Miller, 1959). Protein was estimated by the dye binding method, using bovine serum albumin as the standard (Bradford, 1976).

2.3. Oxidation of multi walled carbon nanotubes (MWCNTs)

Oxidation of MWCNTs was carried out as described by Mubarak et al. (2014). Pristine MWCNTs (1000 mg) were suspended in 100 ml of concentrated acid mixture of HNO_3:H_2SO_4 (1:3, v/v). The mixture was sonicated in an ultrasonic bath (53 kHz frequency) at 40 °C for 4.5 h. The resulting mixture was washed with milliQ water to remove the acid.

2.4. Modification of oxidize MWCNTs with EDC

Modification of oxidize MWCNTs was accomplished using carbodiimide chemistry (Jiang et al., 2004). A suspension (2 mg/ml) of oxidized nanotubes in MES (2-(N-Morpholino)ethanesulfonic acid) buffer (50 mM, pH 6.2) was added to an equal volume of 400 mM N-hydroxysuccinimide (NHS) in MES buffer. The mixture was sonicated for 30 min in an ultrasonication bath. N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (20 mM) was then added to initiate the coupling of NHS to the carboxylic groups on the oxidized nanotubes. The mixture was stirred at 400 rpm for 30 min. The activated nanotubes solution was rinsed thoroughly with MES buffer to remove excess EDC and NHS.

2.5. Conjugation of enzyme on EDC modified MWNTs

Cellulase enzyme was added to the activated nanotube and left overnight at 4 °C with constant shaking for conjugation. The nanotube-enzyme suspension was then washed with buffer to remove any unbound enzyme. The enzyme activity was calculated in the supernatant and washings. To calculate the immobilization efficiency the enzyme load was varied (0.5 U–50 U) and the immobilization was carried as above. A control experiment was done using an identical procedure, only without using EDC and NHS. Only 10% enzyme was bound on nanotube in control.

2.6. Kinetic properties of enzyme and bionanoconjugates

km and Vmax values of free cellulase and bionanoconjugates (cellulase immobilized on functionalized MWCNT) were determined by measurement of enzyme activities with various concentrations of substrates. The kinetic parameters were calculated using Lineweaver-Burk plot (Hashemifard et al., 2010).

2.7. Thermal inactivation kinetics

The thermal inactivation kinetics of enzyme in free and bionanoconjugates system was studied at 70 °C at different time interval (Nadar et al., 2016). Free and nanoconjugates cellulase (dissolved in assay buffers) will be incubated separately at 70 °C. After each interval of 15 min till 120 min, samples were withdrawn, cooled quickly and assayed for the remaining residual activity. A semi-log plot of residual activity vs. time was used to calculate the deactivation rate constant (k_d) as the slope. The half life ($t_{1/2}$) defined as time required for activity to decrease to half of its original and was calculated as 0.693/kd.

2.8. Temperature optima

Appropriate aliquots of free and nanoconjugates cellulase (dissolved in acetate buffer, pH 5.0) were incubated separately, at different temperatures and the activities were determined using CMC as the substrate. In another set of experiment, Cellulase and bionanoconjugates incubated with substrate at 70 °C for continuous hydrolysis. Aliquotes were withdrawn at various intervals of incubation and the amount of reducing sugar was estimated.

2.9. Effect of pH

Appropriate aliquotes of free and nanoconjugates cellulase were incubated separately, in buffers of different pH and the activities were determined using CMC as the substrate. In another set of experiment, free and nanoconjugates cellulase were incubated in the buffer of different pH values separately, for 90 min. The free and nanoconjugates cellulase was brought to optimum pH and then activity was determined.

2.10. Reusability of the bionanoconjugates

The immobilized enzyme preparations were made to 0.5 ml with the assay buffer and incubated with 0.5 ml of the substrate under shaking condition at 50 °C, separately. After 30 min the supernatants were removed and the enzyme activities were estimated in the supernatants. The immobilized enzyme preparations were washed three times with 1 ml of assay buffer. For second cycle the immobilized enzymes were incubated with 0.5 ml of fresh substrate and the reaction was carried out as described previously.

2.11. Fourier transform infrared spectroscopy

FTIR spectra was recorded on a FTIR Spectrometer (Cary 660, California, USA). The spectra were recorded from 4000 to 400 cm^{-1}.

2.12. Scanning electron microscopic

SEM images were examined under Scanning microscope (Zeiss EVO 18, New York, USA).

3. Results and discussion

Multiwalled carbon nanotubes (MWCNTs) were functionalized with N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in presence of N-hydroxysuccinimide (NHS) and used as a matrix for cellulase immobilization. Table 1 shows the Immobilization efficiency of bionanoconjugates (*Aspergillus niger* cellulase immobilized onto functionalized multiwalled carbon nanotubes). The immobilization efficiency (B/A) is defined as the ratio of the measurable enzyme activity (calculated by subtracting the unbound activity in the supernatant and wash) in the immobilize enzyme (B) to the total bound activity (A). A broad range of organic functional groups can be introduced onto the surface of particles using mild conditions (Ahmad and Sardar, 2014; Mahouche-Chergui et al., 2011; Mazumder et al., 2016). The