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Cicer α-galactosidase immobilization onto functionalized graphene nanosheets using response surface method and its applications

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ABSTRACT

Cicer α-galactosidase was immobilized onto functionalized graphene with immobilization efficiency of 84% using response surface methodology (Box–Behnken design). The immobilized enzyme had higher thermal stability than the soluble one, attractive for industrial applications. Immobilization of the enzyme lowered the K_m to 1/3rd compared to the soluble enzyme. Raffinose family oligosaccharides (RFOs) are mainly responsible for flatulence by taking soybean derived food products. The immobilized enzyme can be used effectively for the hydrolysis of RFOs. After ten successive runs, the immobilized enzyme still retained approximately 60% activity, with soybean RFOs. The easy availability of enzyme source, ease of its immobilization on matrices, non-toxicity, increased stability of immobilized enzyme and effective hydrolysis of RFOs increase the *Cicer* α-galactosidase application in food processing industries.

1. Introduction

Enzymes are biomacromolecules (usually proteins) that catalyze biochemical reactions without consuming themselves in the reaction. They play indispensable roles in regulating various reactions occurring in numerous biological processes such as signal transduction, gene expression, immune responses, etc. In addition to this, the enzyme demands mild experimental conditions (pressure, temperature, etc.) which make it more popular in comparison to chemical catalyst; reducing manufacturing cost as well. To improve the catalytic properties of enzyme; immobilization has been proven to be an efficient technique. In general, immobilized enzymes have certain advantages over the soluble enzyme such as high thermal stability, reusability, broad range of pH optima, increased storage stability and easy separation from reaction mixture.

Recently, several new types of carriers and technologies have been used to improve traditional enzyme immobilization to enhance enzyme loading capacity, enzyme activity, stability and to reduce biocatalyst cost for industrial biotechnology. These include Amberlite MB-150 (Dwevedi & Kayastha, 2009; Singh & Kayastha, 2012a); glass beads (Kishore & Kayastha, 2012) and most recently nanoparticles based immobilization of enzymes (Dwevedi, Singh, Singh, Srivastava, & Kayastha, 2009). Among them, nanoscaled materials due to their small size, high surface to volume ratio and desired aqueous suspending ability have exhibited advantages over traditional bulk materials and received a great deal of attention in recent years. Nanoparticle based immobilization served three important features in comparison to the conventional immobilization methods (Ansari & Husain, 2012):

(i) nano-enzyme particles are easy to synthesize in high solid content without using surfactants and toxic reagents,
(ii) homogeneous and well-defined core–shell nanoparticles with a thick enzyme shell can be obtained and
(iii) particle size can be conveniently tailored within utility limits.

In addition, with the increasing attention paid to cascade enzymatic reaction and *in vitro* synthetic biology, it is possible to achieve co-immobilization of multi-enzymes on these nanoparticles (Ansari & Husain, 2012).

The growing interest in carbon family materials has shown various ways for producing arrays of novel functional nanomaterial. Among them, graphene one atom thick planar sheet having hexagonal arrangement with sp^2 bonded carbon atoms has got significant attention of scientific society in recent years. Graphene holds a number of extraordinary electronic, optical, thermal and mechanical properties. Due to fast development of synthesis and functionalization approaches, graphene and its derivatives have shown extraordinary potentials in many fields such as energy

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technology (fuel cells), nanoelectronics and sensors. Biomedical application especially for drug/gene delivery is the recent emerging field for graphene/derivatives applications. In addition to the above properties graphene is suitably an ideal matrix for enzyme immobilization; it alters the aggregation/denaturation of enzymes on its surface and it maintains native state necessary for enzyme's activity.

Response surface methodology (RSM) is one of the popular statistical methods used for modelling and analyzing the relationships between several independent variables and response variable(s). In general use of RSM for optimization purpose makes the process more authentic and rapid; furthermore, it prevents the experimental repetition, enables the work to be carried out with less reagent and labour consumption, thus making the process more economical.

α-Galactosidase (α-D-galactoside galactohyrolase, E.C. 3.2.1.22) catalyzes the hydrolysis of terminal galactose residues from substrate and is widely distributed in microorganisms, plants and animals. Several industrial applications of α-galactosidase are known, mainly in sugar (Kobayashi & Suzuki, 1972) and paper industry (Ratto, Siika-aho, Buchert, Valkeajarvi, & Viikari, 1993). In addition to this, α-galactosidase is also used to improve the gelling properties of galactomannans (Bulpin, Gidley, Jeffcoat, & Underwood, 1990), Fabry's disease treatment (Desnick, Ioannou, & Eng, 2001), blood conversion (Liu et al., 2007) and transglycosylation (Eneyskaya, Golubev, Kachurin, Savel'ev, & Neustroev, 1998). Although, the soy-based foods are proteinaceous diet but the presence of RFOs deteriorate its popularity. These α-linked oligosaccharides are the major cause of flatulence (De Lumen, 1992). Since the humans and other monogastric animals lack the α-galactosidase necessary for the breakdown of stachyose and raffinose (Gitzelmann & Auricchio, 1965) due to which the oligosaccharides accumulate in the lower intestine and undergo fermentation by the anaerobic microorganisms, causing flatulence (Steggerda, 1968). α-Galactosidase can be used for improving the nutritional value of soy-based foods (Mulimani & Ramalingam, 1995).

Development of simple and reliable protocol for enzyme immobilization is always an important aspect of biotechnology. Therefore, in the present work we developed a simple immobilization protocol for *Cicer* α-galactosidase immobilization on functionalized graphene using RSM. Furthermore, the attachment was characterized by using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and fourier transform infrared spectroscopy (FTIR). The enzyme kinetics of immobilized α-galactosidase was studied and compared with the soluble enzyme. The application of immobilized enzyme in hydrolysis of RFOs was also investigated and compared with the soluble enzyme. To the best of our knowledge this is the first report of immobilization of *Cicer* α-galactosidase onto the functionalized graphene nanosheets.

2. Materials and methods

2.1. Plant materials and chemicals

Dry seeds of white chickpea (*Cicer arietinum*) (Pusa 1053) were purchased from local market. The chemicals for buffers preparation were of analytical or electrophoresis grade from Merck Eurolab GmbH, Darmstadt, Germany. All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Milli Q (Millipore, Bedford, MA, USA) water with a resistance of higher than 18 MΩ cm was used throughout the experiments.

2.2. Enzyme preparation

Cicer α-galactosidase was purified from white chickpea (Singh & Kayastha, 2012b).

2.3. Functionalized graphene sheets preparation and immobilization of *Cicer* α-galactosidase

Functionalized graphene was synthesized by thermal exfoliation of graphite oxide. Graphite powder (<50 μm, 1 g) was mixed with strong oxidizing solution of conc. H_2SO_4 (98%), fuming HNO_3 (90%) and potassium chlorate (98%) at room temperature and stirred for 24 h. The solution was then washed with distilled water and 10% HCl solution and dried at 80 °C under vacuum. Next, it was thermally exfoliated to synthesize graphene by rapid heating under an Ar atmosphere. The sample was flushed with Ar gas for 15 min, and then inserted into a tube furnace pre-heated to 1050 °C and kept in the furnace for 30 s. Further the functionalized graphene sample was cooled down to the room temperature under Ar gas flow. Functionalized graphene was suspended in Milli Q water followed by sonication at room temperature for 10 min and left undisturbed for 30 min so that the larger non-exfoliated flakes settled down. Suspended functionalized graphene was gently collected so that the larger flakes stay undisturbed.

The enzyme was immobilized onto the functionalized graphene using spacer arm (cysteamine) and a cross-linker (glutaraldehyde). The functionalized graphene was dissolved in Tris–HCl buffer (50 mM, pH 8.0) to prepare 1 mg/mL graphene solution and divided into 29 aliquots according to the design of the experiment (Table 1). These aliquots were equilibrated with same buffer (1 mL reaction volume) overnight, followed by cysteamine treatment (1 mL reaction volume) prepared in the same buffer and kept under dark for 4 h at room temperature. These were washed with same buffer and treated with glutaraldehyde (1 mL reaction volume) for 4 h. Washing was carried out with Tris–HCl buffer (50 mM, pH 8.0) and incubated overnight with enzyme at 4 °C. Thereafter, the unbound enzyme was washed off with the respective buffer. It is important to note that washing and centrifugation at 12,000 rpm for 5 min is an important step throughout the immobilization studies to settle down the graphene aliquots.

2.4. Characterization

Functionalized graphene sheets (both native and coupled) were characterized using TEM (Technai 20 G^2, 200 kV), SEM (Philips: XL 20) and FTIR (Perkin Elmer Spectrum 100 instrument). For TEM studies, a drop of sample was placed on electron microscope 200 mesh copper grid and allowed to evaporate the water to complete dryness of the sample, followed by loading into the machine. For SEM studies, samples were sprinkled on the stub having layer of silver glue for striking the particles. The fine structural details were obtained using secondary electron imaging mode. For FTIR studies, samples were made by mixing the material with KBr powder followed by vacuum drying. The spectra were recorded in the range of 400–4000 cm^{-1}.

2.5. Experimental design and statistical analysis

Preliminary experiments were performed to determine the initial values of various factors (amount of functionalized graphene, cysteamine, glutaraldehyde and enzyme) involved in enzyme immobilization. Based on these results Box–Behnken design was prepared for optimization of enzyme immobilization onto graphene sheets. The variables and their levels selected for obtaining immobilization were: amount of functionalized graphene (500, 1000 and 1500 μg), cysteamine concentration (5, 10 and 15 mM), % glutaraldehyde (1.5%, 2.25% and 3.0%) and amount of enzyme (100, 300 and 500 μg). The final volume of each used solution was 1 mL. The data were analyzed using Design Expert programme (version 8.0) and the coefficients were interpreted using *F*-test.