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ORIGINAL PAPER

Elucidating the binding efficacy of β-galactosidase on graphene by docking approach and its potential application in galacto-oligosaccharide production

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Abstract Herein, we propose the synthesis and characterization of graphene for the immobilization of β-galactosidase for improved galacto-oligosaccharide (GOS) production. The size of synthesized graphene was observed to be 25 nm by TEM analysis while interaction of enzyme with the nanosupport was observed by FTIR spectroscopy. Docking was obtained using molecular docking program Dock v.6.5 while the visual analyses and illustration of protein–ligand complex were investigated by utilizing chimera v.1.6.2 and PyMOL v.1.3 softwares. Immobilized β-galactosidase (IβG) showed improved stability against various physical and chemical denaturants. K_m of IβG was increased to 6.41 mM as compared to 2.38 mM of soluble enzyme without bringing significant change in V_{max} value. Maximum GOS content also registered an increase in lactose conversion. The maximum GOS production was achieved by immobilized enzyme at specific temperature and time. Hence, the developed nanosupport can be further exploited for developing a biosensor involving β-galactosidase or for immobilization of other industrially/therapeutically important enzymes.

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Keywords β-Galactosidase · Docking softwares · Galacto-oligosaccharide production · Graphene

Abbreviations

GO	Graphene oxide
GOS	Galacto-oligosaccharides
IβG	Immobilized β-galactosidase
SβG	Soluble β-galactosidase

Introduction

Enzyme immobilization is in use since long to impart long-term operational stability and reusability, besides increasing the shelf life of enzymes for a plethora of biotechnological and biomedical applications [1, 2]. However, this process can lead to partial loss of enzymatic activity in harsh immobilization procedures which could affect the native tertiary structure of enzymes irreversibly thereby leading to loss of its function and deactivation [3]. The nanoparticles-based enzyme immobilization provides solution for ideal characteristics in balancing the key factors. This ensures biocatalysts efficiency based on surface area and mass transfer resistance both vital for effective enzyme loading [4, 5]. There is an indication that enzyme bound to nanoparticles exhibits Brownian movement in aqueous solutions to facilitate better enzymatic activities as opposed to native enzyme. Beside, immobilization of enzymes by nanoparticles can reduce protein unfolding essential for their stability and improved performance [6].

Graphene as a candidate has attracted the attention of researchers for enzyme immobilization and in other biomedical fields including biomolecules detection,

bioimaging drug delivery, chemical sensors and phototherapy [7]. It is a carbon based nanoparticle possessing 2D crystalline structure of sp^2 bonded carbon atoms and exhibited thermal conductivity of $\sim$5000 W $m^{-1}K^{-1}$ and $\sim$2630 m^2 g^{-1} specific surface area, apart from having Young's modulus of $\sim$1.0 TPa, intrinsic mobility of $\sim 2 \times 10^5$ cm^2 v^{-1} s^{-1} and $\sim$97.7 % optical transmittance at wider wavelength range [8, 9]. These characteristics impart graphene tremendous in-plane thermal, structural, electrical and mechanical advantages. The presence of epoxy and hydroxyl groups on basal plane and carboxylic groups at the edges help further in surface modification and enhance efficiency in a controlled manner [10].

Structural biology provides information on the static structures of biomolecules. However, in reality, biomolecules are highly dynamic, and their motion is important to their function. Different experimental techniques are available to help study the dynamics of biomolecules [11]. Beside this, improved computational skills are on increase to encourage new theoretical methods. Together, all these give a renewed effort of solving the scientific problems at the molecular level. All the theoretical methods and computational techniques that have been used till date to model the behavior of molecules have been extensively reviewed [12]. The structure of enzyme as an important biomolecule has been tried by many techniques like crystallography, nuclear magnetic resonance and electron microscopy, but computational approach as by docking has proved to be very effective. Hence, more efforts are needed to peruse this line.

Docking is an important computational tool used to specifically predict protein–ligand interactions but two basic features are required for docking software; the accuracy and scoring reliability [13]. As for the former, it indicates how similar the prediction of ligand binding is to the ligand conformation that is determined experimentally whereas scoring reliability ranks ligands based on their affinities. As of now quite a few docking software are currently available. Among these, AutoDock is highly accessed and freely available [14]. Since protein-nanoparticle complexes are rather difficult to study using experimental tools, computational approaches show some promise. For example, structural models for carbon nanomaterials such as carbon nanotubes and fullerenes are available to substantiate this fact. This highlights that many protein-nanoparticle interactions can be studied by this method. Consequently, Yang et al. computationally studied the interactions in the fullerenol-lysozyme complex and effectively compared the outcome with experimental results [15]. Following these type of studies, a data repository can be created and utilized by researchers for the submission and retrieval of information on nanoparticles, including their physical and chemical composition, function and more so in vitro experimental characterizations. Such a lead also encourages data sharing and discussing the use of nanoparticles in biomedical and biotechnological applications globally.

β-Galactosidase (EC 3.2.1.23) is a hydrolase that catalyzes the *O*-glucosyl group of lactose into glucose and galactose, and is widely distributed in microorganisms, plants and animal tissues [16]. The general mechanism of lactose hydrolysis by β-galactosidase exhibited transgalactosylation which involves a multitude of sequential reactions with disaccharides (other than lactose) and higher saccharides, collectively termed as galacto-oligosaccharides as intermediate products [17].

Keeping these things in mind, an attempt is made to synthesize and modify graphene. Its application as a matrix was evaluated to immobilize β-galactosidase for producing GOS in biotechnology industries. Reusability study of immobilized enzyme was also investigated.

Materials and methods

Materials

Aspergillus oryzae based β-galactosidase and *o*-nitrophenyl β-D-galctopyranoside (ONPG) were procured from Sigma Chem. Co. (St. Louis, MO, USA). The reagents were prepared using chemicals of analytical grade in double distilled water.

Preparation of graphene

Graphene oxide (GO) was prepared by Zhao et al. method [18]. Important steps were: mixing 1.0 g of graphite with 25 mL of conc. H_2SO_4 in a flask, and the mixture was cooled in an ice bath by constant stirring. Then $KMnO_4$ (3.0 g) was added to it until a dark colored mixture was obtained. The resultant mixture was collected in distilled water, and to this 5 mL of H_2O_2 (30 %) was added to form bright yellow solution and then centrifuged. The isolated material was washed thoroughly by 6.0 N HCl and distilled water to obtain a neutral pH. The product was dried in vacuum at 50 °C for 12 h to get a dark brown solid matter. To obtain the modified matrix, ethanol was added in GO by constant stirring and by centrifuging (cooling centrifuge) at 10,000 rpm for 30 min. The precipitate was washed thrice with distilled water to remove traces of ethanol and later to be used for β-galactosidase immobilization.

Immobilization of β-galactosidase

β-Galactosidase was stirred slowly in modified GO (1.0 g) and kept for overnight. The product was then centrifuged to

remove unbound enzyme and washed it thrice with assay buffer. The enzyme activity was calculated by continuously shaking an assay volume of 2.0 mL (containing 1.79 mL of 100 mM assay buffer (pH 4.5), 100 µL β-galactosidase and 200 µL of 20 mM ONPG). The reaction was terminated by adding 2.0 mL of 1.0 M sodium carbonate solution. The product so formed was measured spectrophotometrically at 405 nm. The immobilization yield (Yi) of β-galactosidase was calculated as:

$$\mathrm{Yi} = \frac{\text{Activity of } \beta\text{-galactosidase obtained after immobilization per gram of GO}}{\text{Activity of } \beta\text{-galactosidase added per gram of GO}} \times 100.$$

Analysis of FTIR spectra of enzyme with nanosupport

FTIR analysis was assisted using Interspec 2020 model FTIR instrument (USA). The nondestructive analysis of interaction of GO was intended with β-galactosidase to monitor functional groups.

Molecular docking approach for computation analysis of graphene and β-galactosidase

Protein Data Bank (PDB) with PDB ID: 4IUG was used to analyze the crystal structure of β-galactosidase in complex with galactose. The 3-D structure of graphene was obtained from ChEBI (Chemical Entities of Biological Interest (https://www.ebi.ac.uk/chebi/) with ChEBI ID 36973. Docking was obtained using molecular docking program Dock v.6.5 [19–21]. The rigid body docking option was used. The bound galactose acted as probe and the region around 10 Å of galactose was employed for final docking of graphene. The visual analyses and illustration of protein–ligand complex utilized chimera v.1.6.2 and PyMOL v.1.3 softwares [22, 23].

Determination of kinetic parameters

Line-Weaver Burk plot was used to obtain kinetic parameters of soluble and immobilized enzyme. For this, the initial rates at varying concentrations of ONPG in 100 mM sodium acetate buffer at pH 4.5, 40 °C were measured.

Effect of galactose

Activity of soluble and ethanol activated graphene bound β-galactosidase (20 µL) was determined in the presence of increasing concentrations of galactose (1.0–5.0 %, w/v) in 100 mM sodium acetate buffer, pH 4.5 at 50 °C for 1 h. Activity of enzyme without added galactose was considered as control (100 %) for calculation of remaining percent activity.

Reusability of IβG

Activity of IβG (20 µL) was monitored for 12 consecutive days. It was analyzed in 100 mM sodium acetate buffer at pH 4.5 kept overnight at 4 °C. The activity measured on day 1 was considered as control and taken as 100 % in calculation of other percent activities.

Production of galacto-oligosaccharides

High performance liquid chromatography (Shimadzu, Japan) consisting of LC-10AT pump, SPD-10AVP, PDA detector, phenomenex C18 (250 mm × 4.6 mm, 5 µm) column, HPLC grade cartridge system (Phenomenex) and a class Nuchrom software was used to obtain oligosaccharides formation. Detector and column oven were maintained at 30 and 70 °C, respectively [24].

Statistical analysis

Each value represented the mean for three independent experiments performed in triplicates, with average standard deviations <5 %. The data expressed in various studies were plotted using Sigma Plot-9. The P values <0.05 were considered statistically significant.

Results and discussion

Keeping the presence of higher specific surface area and exceptional physicochemical properties of graphene and its potential in catalysis and environmental remediation in mind, we have tried to develop a simple and quick method for preparing and characterizing graphene by transmission electron microscopy (Fig. 1). The surface of graphene was activated by ethanol to maximize the availability of hydroxyl groups for easier binding of enzyme to graphene based nanosupport. The schematic representation of immobilization of enzyme on activated graphene is shown

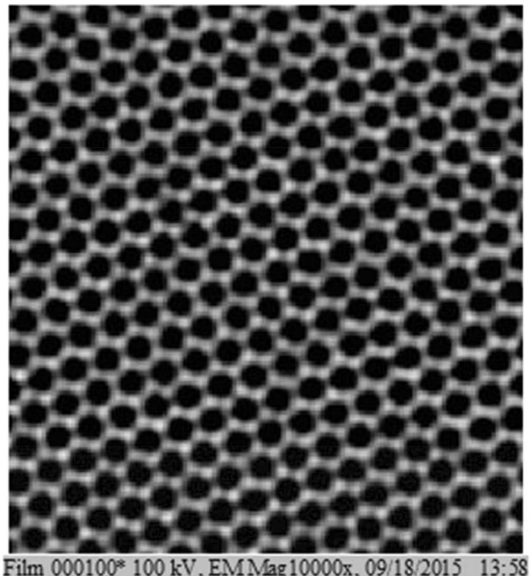

Fig. 1 Transmission electron microscopy. Transmission electron microscopy revealing the size of graphene

in Fig. 2. The covalent immobilization of β-galactosidase on ethanol activated graphene retained 93 % of original activity. FTIR spectroscopy performed for investigating various oxygen functionalities in modified nanomatrix have been displayed in Fig. 3. It was observed that O–H stretching vibrations were present at 3105 cm^{-1} while stretching vibrations from C=O to C–OH were obtained at 1735 and 1345 cm^{-1} respectively. The broadening of peaks from 1200 to 1700 cm^{-1} revealed attachment of β-galactosidase on modified nanomatrix. We found the computational automated docking as a powerful technique to identify transient structures involved in binding/interaction that are difficult to obtain by experimental structure determination. The present method definitely increases the understanding of enzyme engineering. The crystal structure of β-galactosidase from *Aspergillus oryzae* in complex with galactose was obtained from Protein Data Bank (PDB) with PDB ID: 4IUG, whereas the 3-D structure of graphene was obtained from ChEBI (Chemical Entities of Biological Interest (https://www.ebi.ac.uk/chebi/) with ChEBI ID, CHEBI: 36973. The docking between graphene and β-galactosidase using molecular docking program Dock v.6.5 [19] are shown in Fig. 4. The rigid body docking option of dock was used and the bound galactose was used as probe and the region around 10 Å of galactose was employed for final docking of graphene. Chimera v.1.6.2 [20] and PyMOL v.1.3 [20] gave visual analyses and provided insight of protein–ligand complex. The ligand interaction plots of protein–ligand complexes were illustrated by Ligplot +v.1.4.5 program [22, 23]. The dock/grid score (−74) was negative with high absolute value. Eight interacting residues revealed from docking analyses were Asn-140, Glu-142, Ala-237, Tyr-260, Phe-264, Phe-304, Glu-804, and Trp-806 which formed non-bonding interactions with graphene and made the complex stable. While observing the binding of graphene to natural substrate (galactose), four interacting residues Asn-140, Glu-142, Tyr-260 and Phe-304 were found overlapping (Fig. 5). Galactose, obtained as an end products of β-galactosidase catalyzed hydrolysis of lactose, acts as a competitive inhibitor for obtaining greater yield of GOS. Our results suggested that activated graphene bound β-galactosidase showed promising resistance to inhibition mediated by galactose as compared to its soluble counterpart (Fig. 6).

Immobilization protocols generally lead to alteration in kinetic parameters of the enzyme due to changes induced in their microenvironment [3]. The immobilized enzyme can therefore exhibit 20–30 % reduction in GOS yield from lactose due to the mass transfer resistance of particle carriers [25]. The enhanced stability was observed in our case also for immobilized β-galactosidase against various physical and chemical denaturants. Table 1 shows that K_m was increased to 6.41 mM as compared to 2.38 mM of soluble β-galactosidase as a result of immobilization without bringing significant change in V_{max}. This shows that the affinity of the immobilized enzyme decreased for its substrate. The velocity of enzymatic reaction decreased, thereby indicating a low accessibility of substrate to the active site and the lower transporting of the substrate and products into and out of the modified nanomatrix. These observations are in agreement with studies made in *A. oryzae* β-galactosidase and fenugreek β-amylase immobilized on magnetic polysiloxane–polyvinyl alcohol and functionalized graphene sheets [26, 27].

Reusability is an important criterion while selecting an enzymatic system for any application. These studies extended to this aspect and were investigated for β-galactosidase immobilized on activated graphene in the present work. It was observed that IβG retained 68 % of the initial activity even after its 10th repeated use (Fig. 7). The drawback generally encountered by soluble enzymes in industrial production of GOS can be overcome, even if partially, by the development of stable, robust and cheap nanomatrix. The glycosidic hydrolases that are commercially used for industrial production of GOS in food industry till date have been extensively reviewed [25]. There is continuous interest in exploring new immobilization methods for employing them in industrial uses to produce specific GOS mixtures with better yields. Hence, GOS production was obtained by β-galactosidase from *Kluyveromyces lactis*, *Bifidobacterium longum*, *Bacillus circulans* and *A. oryzae* in the recent past [28–30]. However, these systems suffered from drawbacks like they could not convert free galactose for GOS formation, enzymes were unstable and exhibited relatively lesser

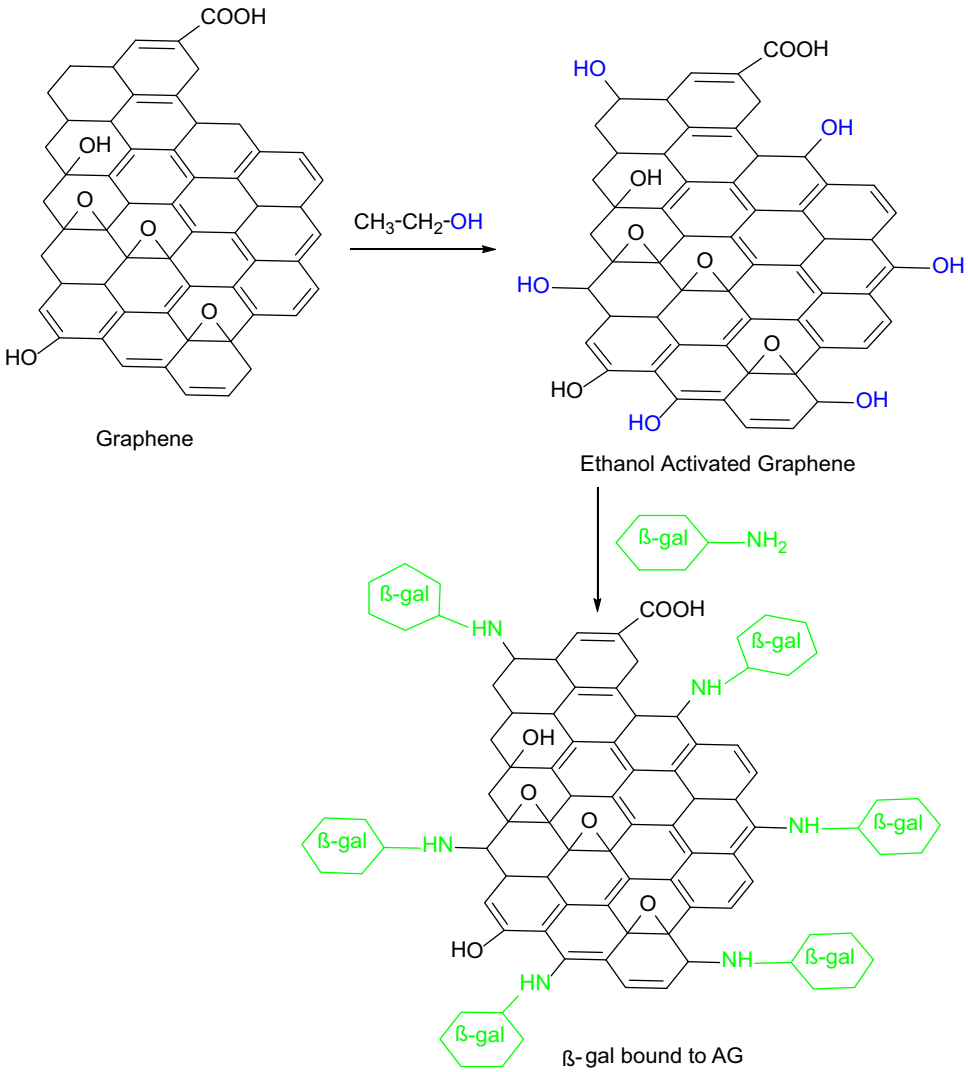

Fig. 2 Schematic representation for enzyme immobilization. Immobilization of β-galactosidase on activated graphene has been shown in *different colors*

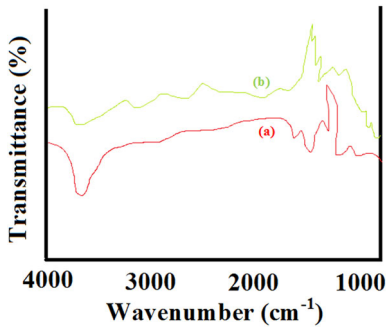

Fig. 3 FTIR spectra of β-galactosidase and activated graphene. FTIR analysis was done to monitor the functional groups **a** present in GO and **b** involved in binding β-galactosidase with GO

sensitivity toward glucose and galactose. The reusability of enzymatic system in this light assumes further importance.

This study depicted that GOS formation by modified graphene bound β-galactosidase in a batch reactor (at 50 °C) with an initial lactose concentration of 400 g/L (Fig. 8). Maximum GOS production achieved by immobilized enzyme was 40 % at 60 °C after 5 h. The probable reason for obtaining greater percent of GOS by IβG at higher temperature might be due to its increased stability as a result of immobilization. In a previous study, maximum GOS production achieved was 30 % using 241 U of immobilized β-galactosidase at pH 6, 40 °C, in comparison [31]. In another study, maximum formation of GOS

achieved by *A. oryzae* β-galactosidase was even less when immobilized on magnetic polysiloxane–polyvinyl alcohol being 26 % w/v of total sugars at 55 % lactose conversion at pH 4.5 and 40 °C [26]. In the present study, GOS formation was initially accompanied by a rapid decrease in lactose concentration, however, as GOS concentration decreased, the glucose and galactose concentration was increased. This indicates that transgalactosylation dominates in the initial reaction. In the long run, GOS production increases with increasing lactose conversion until it reaches a maximum conversion hovering at 50 %. Following that, GOS formation decreases and ultimately disappears at 100 % lactose conversion. Albayrak and Yang [32] suggested initial lactose concentration as the main factor for affecting GOS production. This explain the trend as the initial lactose concentration increased from 50 to 500 g/L, the maximum GOS content increased from 10.5 % (at 35 % lactose conversion) to 27 % (at 52 % lactose conversion). Pruksasri and co-workers earlier utilized nanofiltration membrane (NP030) at 45 bar for GOS purification at 5 °C and 60 °C and found that operating at 5 °C is more advantageous for obtaining high oligosaccharide yield. They obtained a product purity of 85 % (based on monosaccharide content) and an oligosaccharide recovery yield of 82 % [33]. Similarly, 2-D modeling of a packed bed bioreactor was exploited for obtaining GOS from milk whey. The effects of reactor to catalyst particle diameter ratio, feed flow rate and initial lactose concentration on substrate concentration distribution were investigated in detail [34]. They observed that the substrate conversion and product yield obtained experimentally showed an excellent agreement (97 ± 2 %) with the results predicted by 2-D model equation whereas the results

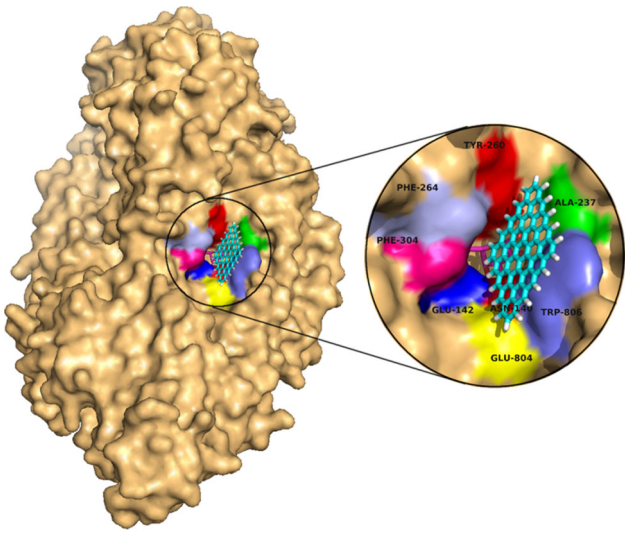

Fig. 4 Molecular docking. Enzyme is shown in surface representation in *light-orange color* with active site. The active site is zoomed in and the interacting residues are labeled and shown in *different colors* while the bound galactose and docked graphene are also shown in stick representation with carbon backbone in *magenta* and *cyan colors*, respectively

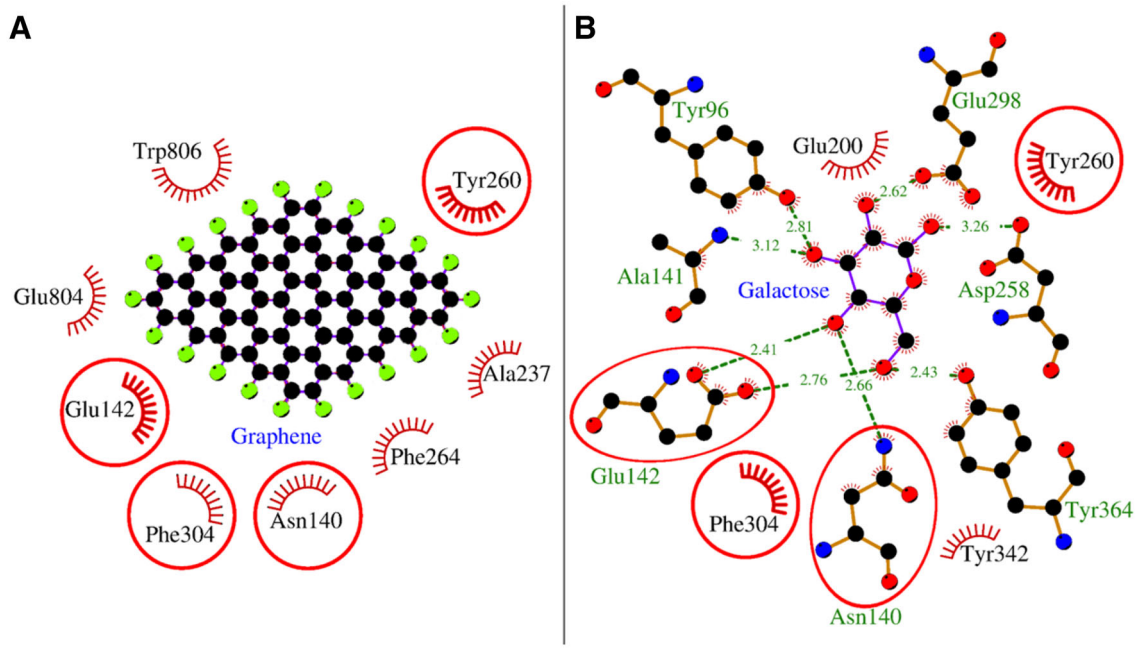

Fig. 5 The ligand interaction plots of graphene (**a**) and its comparison with that of natural substrate (galactose) (**b**). The residues forming hydrophobic interactions are shown as *red arcs* while the hydrogen bonds are shown as *green dashed lines* with indicated bond lengths. The interacting residues which are common for both the ligands are encircled

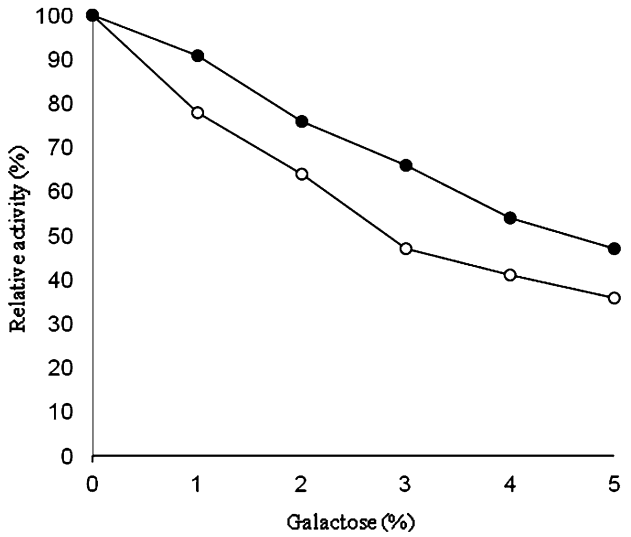

Fig. 6 Effect of galactose on soluble and ethanol activated graphene bound β galactosidase. Effect of galactose on soluble and immobilized β galactosidase (20 μL) was measured in the presence of increasing concentrations of galactose (1.0–5.0 %, w/v) in 100 mM sodium acetate buffer, pH 4.5 for 1 h at 50 °C. Activity of enzyme without added galactose was considered as control (100 %) for the calculation of remaining percent activity at other concentrations. *Symbols* show (*open circle*) soluble and (*filled circle*) immobilized β galactosidase

Table 1 Kinetic parameters for soluble β-galactosidase (SβG) and enzyme immobilized on activated GO (IβG)

Enzyme preparation	K_m (mM)	V_{max} (mM/min)
SβG	2.38 ± 0.14	499 ± 2.5
IβG	6.41 ± 0.22	508 ± 3.8

Each value represents the mean for three independent experiments performed in triplicates, with average standard deviations, <5 %

predicted by one-dimensional model equation did not lie within the desired confidence level (<90 %).

Conclusion

Immobilization of β-galactosidase is important for the industrial production of GOS where the use of the enzyme in free form is restricted by poor thermal stability and sensitivity to product inhibition apart from non-reusability drawback. The developed nanosystem showed greater activity of enzyme against various physical and chemical denaturants, and suitable for repeated use. The developed nanosupport can be utilized in future for development of biosensor involving β-galactosidase and for immobilization of other industrially/therapeutically important enzymes.

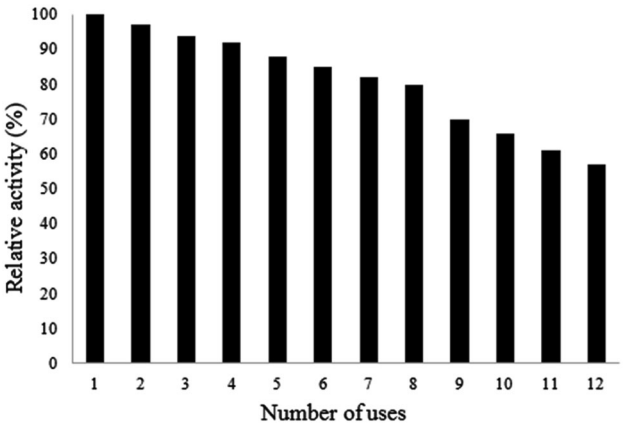

Fig. 7 Reusability of GO bound β galactosidase. Reusability of IβG was monitored for 12 successive days. The preparation was taken in triplicates and assayed for remaining percent activity. Activity determined on the first day was taken as control (100 %) for calculation of remaining activity after each use

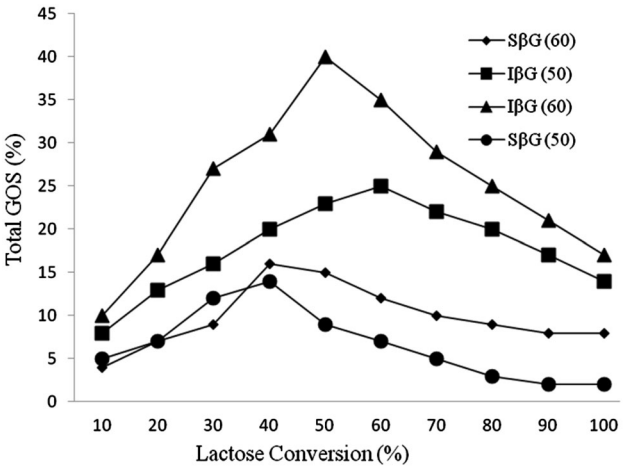

Fig. 8 GOS production by soluble and immobilized β galactosidase at various temperatures. Total GOS formation at 50 °C is shown as free (*filled circle*) and immobilized (*filled square*) *Aspergillus oryzae* β-galactosidase while at 60 °C, they are shown by symbols (*filled diamond*) and (*filled triangle*), respectively

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