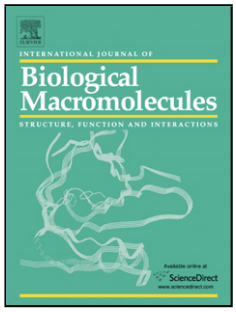

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PII: S0141-8130(17)31049-8
DOI: http://dx.doi.org/doi:10.1016/j.ijbiomac.2017.07.088
Reference: BIOMAC 7896

To appear in: *International Journal of Biological Macromolecules*

Received date: 22-3-2017
Revised date: 4-7-2017
Accepted date: 13-7-2017

Please cite this article as: {http://dx.doi.org/

Immobilization of *β*-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite~~s~~ improves enzyme stability and resistance to inhibitor

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1. Introduction

The enzyme, β-galactosidase (EC 3.2.1.23) has several advantages in the food industry as well as in analysis. It acts as a therapeutic agent to hydrolyze lactose from milk products, thereby alleviating the adverse effects caused by lactose malabsorption [1,2]. Apart from feeding the lactose intolerant patients, it is employed to enhance the flavour and sweetness of the dairy products as well as to prevent lactose crystallization in frozen concentrated deserts [3]. By the use of β-galactosidase, the problems associated with whey disposal can be eliminated [3]. This enzyme is also used in the synthesis of galacto-oligosaccharides [1]. However, native or soluble enzyme cannot be used directly in a biotechnology industry due to considerations like loss of enzymatic activity under harsh environmental and long storage conditions along with reusability impossibility [4].

All these circumstances can be overcome by the use of immobilized enzymes which provide operational and long term stability as well as reusability [1]. The main objective of enzyme immobilization is to improve enzyme features, like stability via multipoint attachment or multisubunit immobilization, activity, selectivity or specificity as well as resistance to inhibitors etc. Multipoint covalent immobilization increases the rigidity of immobilized enzyme, thereby making it less sensitive to conformational changes caused under drastic conditions and hence enhancing the activity as well as the stability of the immobilized enzyme over the free enzyme [5]. Lipases possess flexibility in their active site due to which their functional properties like activity, selectivity and specificity can be easily modulated with the help of diverse techniques and hence lipase immobilization becomes one of the most promising one [6]. There are various methods employed for the immobilization of β-galactosidase like physical adsorption, covalent binding, entrapment and cross linking etc.[2].

Among the various conducting polymers, polyaniline (PANI) is one of the most significant, due to their electrical conductivity, easy synthesis, and environmental stability [7]. It has gained much popularity in biosensor applications, since it acts as an efficient mediator for electron transfer in the redox or enzymatic reaction [7]. Nanomaterials have proved to be an excellent immobilization support due to exceptional properties they exhibit like small size, large surface area etc. [9,10]. These particles reduce diffusional limitations and thereby increase enzyme loading. But they possess certain disadvantages too, like they usually tend to agglomerate in native conditions because of which the surface area of the NMs needed for immobilization is compromised. However, this can be minimized by functionalization or surface modification of the nanomaterials by treating with different chemical agents to either form an electrostatic surface for adsorption or provide functional groups for covalent coupling [11]. Furthermore, the cost of the fabrication process, large scale applications and inactivation of enzyme by interfaces and suffer autolysis are also some of the setbacks in the use of nanoparticles in immobilization industry [12,13].

Recently, multiwalled carbon nanotubes (MWCNT) have attracted considerable attention due to their extra ordinary mechanical and electrical properties that make them an ideal reinforcing agents for high strength polymer composites [14]. Cobalt, because of its electrical and thermal properties is also extensively employed for the development of nanoparticle based biosensors [15]. Hence, the exceptional properties of the PANI, Cobalt and MWCNT can be combined and easily exploited in the electro-enzymatic reactions for the use of biosensors with immobilized biomolecules. Earlier, PANI has been reported as a coating for magnetite NP for the immobilization of β-galactosidase [16]. Furthermore, several enzymes have been reported to be immobilized on PANI grafted CNT by various workers [17-19].

In this manuscript, an attempt has been made to synthesize a non-porous support, polyaniline cobalt mutiwalled carbon nanotube nanocomposite (PANI/Co/MWCNTNC) by in situ

polymerization and subsequent attachment of *β*-galactosidase on this support by means of physical adsorption and covalent binding has been accomplished. **Fig. 1** illustrates the overall scheme of the methods and chemicals employed in this study. The binding was confirmed by transmission electron microscopy (TEM), scanning electron microscopy (SEM), and Fourier-transform infrared spectroscopy (FT-IR). The effect of temperature, pH and galactose on the activity of both soluble and immobilized enzyme preparation was investigated. The kinetic parameters were studied and reusability of the immobilized enzyme preparations was also assessed. The genotoxicity of the support in free and bound form was evaluated by the using plasmid nicking assay.

2. Materials and methods

2.1. Materials

β- D-galactosidase (E.C. 3.2.1.23) from *Aspergillus oryzae* was purchased from Sigma-Aldrich Chemicals Co. USA, o-Nitrophenyl *β*-D-galactopyranoside (ONPG) and sodium carbonate were obtained from Sisco Research Laboratories, Mumbai, India. Multiwalled carbon nanotubes (purity ≥95% wt %, 10 nm diameter), sodium dodecyl sulphate (SDS) and ammonium persulphate (APS) were obtained from Central Drug House Private Limited, New Delhi, India. All other chemicals were used without posterior purification and were of analytical grade.

2.2. Synthesis and characterization

The synthesis of PANI/Co/MWCNTNC was done according to the procedure described by Giri *et al* [20] with slight modifications. In a beaker 150 ml of 1 M HCl and 50 mL of 0.2 M SDS were taken followed by the addition of 60 mg of MWCNTs. This mixture was sonicated

for about 30 min at room temperature. Simultaneously, in another beaker, 1 mL of aniline monomer in 50 mL of 1 M HCl was taken and calculated amount of $CoCl_2$ (3% w/v) was added by stirring to achieve a homogenous suspension. Both the solutions prepared in the previous step were mixed and sonicated for another 10 min. Upon stirring, 50 ml of 1 M HCl containing 2.04 g of APS was added drop by drop to the solution for the complete polymerization of the monomer. The mixture was kept at 0-5^{o}C for 24 h. Finally, the resulting precipitate was filtered and washed with distilled water and ethanol and then dried at 60^{o}C for further studies.

The free and enzyme bound nanocomposite preparations were characterized by TEM (JEOL-2100, 200 κV), SEM (JSM-6510 LV, 15 κV) and FT-IR (Perkin Elmer instrument, USA, Spectrum- II, wave no. 4000-400). For TEM studies, samples were prepared by drop coating diluted solutions on carbon coated copper grids at normal atmospheric conditions. For SEM studies, small samples from PANI/Co/MWCNTNC and air dried immobilized enzyme were mounted on carbon tape on a copper stub, followed by 60 nm of gold deposition using a sputter coater. For FT-IR, samples were crushed and thoroughly mixed with KBr to form transparent pellet. UV-vis spectrophotometer (Shimadzu, UV-1800) was used to measure enzyme concentration.

2.3. Immobilization of β-galactosidase on PANI/Co/MWCNTNC

Free *β*-galactosidase was independently adsorbed by varying concentrations of PANI/Co/MWCNTNC from 2 - 12 mg by continuously shaking the mixture in sodium acetate buffer, pH 4.5 at 25^{o}C. The enzyme concentration was also varied from 1 - 5 mg mL^{-1} keeping the NC concentration (10 mg) fixed. Soluble *β*-galactosidase was then covalently immobilized on PANI/Co/MWCNTNC by using glutaraldehyde as cross-linking agent. During this procedure, firstly, the NC concentration was increased from 2 - 10 mg, the

concentration of glutaraldehyde being fixed as 2%. Secondly, the glutaraldehyde concentration was varied from 0.5% (v/v) to 3% (v/v) maintaining the NCs concentration at 6 mg.

In the case of simple adsorption method, PANI/Co/MWCNTNC (250 mg) were suspended and sonicated in 0.1 M sodium acetate buffer, pH 4.5. For covalent method, the NC (250 mg) was mixed with glutaraldehyde (2%, v/v) in continuous stirring condition for 2 h. The activated support was centrifuged and washed thrice with assay buffer, 0.1 M sodium acetate, pH 4.5 to remove traces of glutaraldehyde. β-galactosidase (24.67 U) was added individually in both types of colloidal suspensions. The mixture was stirred slowly for 5 h (simple adsorption) and 2 h (covalent) and then centrifuged at $3000\times g$ for 10 min at 4^{o}C. The unbound enzyme was removed by washing thrice with assay buffer and preserved at 4^{o}C for further studies. The supernatant and all the washes were also stored at 4^{o}C for the calculation of immobilization yield [21].

2.4. Stability and kinetic studies

2.4.1. pH-activity profile

The activity of free and immobilized β-galactosidase (0.04 U) was assayed in buffers of varying pH 2.0-9.0. The buffers used were glycine-HCl (pH 2.0 & 3.0), sodium acetate (pH 4.0 & 5.0), sodium phosphate (pH 6.0 & 7.0) and Tris-HCl (pH 8.0 & 9.0). The molarity of each used buffer was 0.1 M. The activity at pH 4.5 was taken as control (100%) for the calculation of residual percent activity [22].

2.4.2. Temperature-activity profile

The activity of free and immobilized β-galactosidase (0.04 U) was measured at different temperatures (20-80^{o}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 calculation of relative activity of the free and immobilized enzyme at other temperatures [22].

2.4.3. Thermal denaturation

Thermal stability of free and immobilized β-galactosidase (0.04 U) was calculated in terms of loss in enzyme activity when incubated at 60°C in 0.1 M sodium acetate buffer, pH 4.5 for varying times in the absence of substrate. Aliquots of each preparation (20 µL) were taken out at interval of 15 min and chilled quickly in crushed ice for 5 min. The enzyme was brought to room temperature and the above described enzyme assay was performed. The activity of enzyme without incubation at 60°C was defined as control and attributed 100% relative activity for each of their respective reactions [22].

2.4.4. Effect of galactose

The activity of free and immobilized β-galactosidase (0.04 U) was independently measured with increasing concentrations of galactose (1.0–5.0%, w/v) in 0.1 M sodium acetate buffer, pH 4.5 at 37°C. The activity of enzyme without galactose addition was considered as control (100%) for the calculation of remaining percent activity [22].

2.4.5. Reusability of immobilized enzyme

Both types of the immobilized enzyme preparations (0.04 U) were taken in triplicates for assaying the activity of the enzyme after repeated uses. The reaction was performed in 1.5 mL eppendorf tubes for 10 min at 37°C. Immediately, after the incubation, the mixture was centrifuged at 3000×g for another 10 min at 4°C. Supernatant was decanted and 0.1 M sodium carbonate was added into it, to completely terminate the reaction. After each assay, immobilized enzyme was washed with 0.1 M sodium acetate buffer, pH 4.5 by centrifugation at 3000×g for 10 min. The obtained pellet was stored in assay buffer at 4°C and this process was repeated for 10 successive days. The activity determined after the first cycle was

considered as control (100%) for the calculation of the relative activity after repeated uses [21].

2.4.6. Determination of kinetic parameters

Kinetic parameters K_m and V_{max} of free and immobilized *β*-galactosidase were evaluated from Michaelis-Menten plot by measuring their initial rates by varying the concentrations of the substrate, ONPG (0.1 mM -1.0 mM) in 0.1 M sodium acetate buffer, pH 4.5 [23].

2.5. Genotoxicity assessment of PANI/Co/MWCNTNC

Plasmid nicking assay was performed according to the procedure described by Khan et al [24]. Reaction mixture (30 µL) containing 10 mM Tris–HCl buffer (pH 7.5), pBR^{322} DNA plasmid (0.5 µg) with PAN/Co/CNTNC (50 µg), and immobilized enzyme (10 µL) were incubated for 2 h at 37°C. Ten micro litre of the solution containing 40 mM EDTA, 0.05% (w/v) bromophenol blue (tracking dye) and 50% (v/v) glycerol was added after incubation and the solution was then subjected to electrophoresis in submarine 1.0% (w/v) agarose gel. Ethidium bromide stained gel was then viewed and photographed on a UV-transilluminator

2.6. Assay of β-galactosidase

The ONPG hydrolyzing activity of free and bound *β*-galactosidase was assayed by measuring the release of *o*-nitrophenol from ONPG at 405 nm [25]. The reaction was performed by continuous shaking in a total volume of 2.0 mL containing 1.60 mL, 1.65 mL and 1.68 mL of 0.1 M sodium acetate buffer for free, simply adsorbed and covalently immobilized enzymes, respectively, pH 4.5, 0.04 U *β*-galactosidase (Free- 100 µL, adsorbed - 50 µL, cross-linked – 20 µL) and 0.3 mL of 20 mM ONPG at 37°C for 15 min. The reaction was stopped by the addition of 2.0 mL of 1.0 M sodium carbonate solution and product formation was spectrophotometrically read at 405 nm.

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

2.7. Estimation of protein

Protein concentration was determined according as described by Lowry et al [26]. Bovine serum albumin was used as a standard.

2.8. Statistical analysis

Each value represents the mean for three independent experiments and statistical analysis for comparison between free and both types of immobilized *β*-galactosidase were performed by one way ANOVA test. The data expressed in various studies were plotted using origin 6.1 and expressed with standard deviation of error (±). *p*-values ≤0.05 were considered statistically significant.

3. Results and discussion

3.1. Characterization

Native and enzyme bound PANI/Co/MWCNTNC was characterized by TEM, SEM and FT-IR. The TEM images of the NC alone and the enzyme bound NC are illustrated in **Fig. 2(a-c)**. The CNT can be seen clearly in the form of network. The coating of PANI on the CNT can be confirmed by the presence of thin border all along the tubular structures [27]. The particle size of the NC was found in the range of 18-23 nm. The incorporation of Co can be visualized in TEM images by the presence of small black spots between the PANI coated nanotubes. The binding of the enzyme to the NC by adsorption and covalent binding methods has been compared in **Fig. 2 (b & c)**. The covalently linked enzyme was found to bind more efficiently as compared to the adsorbed enzyme. The selection area diffraction pattern

indicates that the NC is crystalline in nature. However, the immobilization process is also responsible for alteration in the SAED pattern [24]. After immobilization, the adsorption procedure made the NC highly crystalline in nature with concentric rings visible in the SAED pattern as seen in Fig. 2 (b), whereas in the case of glutaraldehyde treated NC, its crystallinity decreases. Adsorption method is simple and does not involve any use of chemicals, while the covalent binding necessitates the use of glutaraldehyde and covalent bonds are formed in between the enzyme and the matrix. The surface morphology of the bound and free NCs was studied by SEM. According to the SEM images, the surface characteristic of the NC was significantly changed on immobilization of the enzyme by both the methods **(Fig. 3)**. Rigid binding was observed in case of cross linked enzyme.

The FT-IR spectra were recorded for free PANI/Co/MWCNTNC, adsorbed and covalently attached enzymes to the NC in the range of 500 cm^{-1} to 4000 cm-^{1} **(Fig. 4).** The characteristic peaks obtained at 1493 cm^{-1} and 1570 cm^{-1} were specific for benzenoic and quinonic type rings, respectively and signifies the presence of PANI in the NC. Giri et al [18] also recognised the peaks at 1560 cm^{-1} and 1490 cm^{-1} as characteristic peak of the stretching vibration of the N=Q=N ring and N-B-N respectively (where B refers to the benzenoic-type rings and Q refers to the quinonic-type rings. Similar results have been obtained by Zhao et al [27]. Peak at 1300 cm^{-1} corresponds to C-N stretching bond. The band which appeared at 1238 cm^{-1} in the spectrum obtained for NC, indicate that Co interacts with the nitrogen atoms in the PANI chain [20]. The characteristic peak for MWCNT was observed at 1148 cm^{-1} which corresponds to C=C. The binding of β-galactosidase on PANI/Co/MWCNTNC was confirmed by the absorption bands obtained for adsorbed and covalently attached enzyme. The stretching of carbonyl group of β-galactosidase was observed in both types of immobilized enzyme by broadening of peaks from 3131 cm^{-1} to 3653 cm^{-1}. Such broadening of peaks was also reported by Ansari and Husain [28]. The peak at 1647 cm^{-1} further signifies

the presence of amide linkage. However, the peak intensity decreased in case of covalently immobilized enzyme ensuring a strong interaction between the protein and the support. The decreased intensity has been earlier discussed by Bachhav *et al.* [29].

3.2. Binding studies of adsorbed and covalently attached β-galactosidase

The adsorption of protein onto the CNT involves both hydrophobic and ion exchange interactions. Hydrophobic interactions take place through interaction of the side chains of hydrophobic amino acids of the proteins with the sidewall of CNT. ~~As for the electrostatic interaction, the π electrons on the surface of the CNT will interact with the π electrons of the aromatic ring of amino acids (e.g., phenylalanine and tryptophan).~~ ~~Furthermore,~~ Whereas for ion exchange, the negatively charged groups on the support may interact with the positively charged groups on the enzyme. This type of immobilization is also referred to as immobilization by ionic exchange. Apart from electrostatic and ion exchange interactions, hydrogen bonds, and nonspecific adsorption can also play a role in enzyme adsorption onto CNT [30].

Optimization of the immobilization conditions is necessary in order to achieve maximum yield. During adsorption, two parameters were examined, the enzyme concentration and the NC concentration. The NC concentration was increased from 2 - 12 mg of dry weight, maintaining the enzyme concentration at 1 mg/mL. The results showed an increase in the immobilization yield with increase in NC concentration upto 10 mg of dry weight. On further increasing, no significant change was observed in the yield indicating that maximum binding of the enzyme to the support has taken place [31]. The enzyme concentration was also varied from 1 - 5 mg/mL keeping the NC concentration fixed at 10 mg. In this case, 93% yield was obtained at 2 mg/mL which gradually decreased to 84% at 5 mg/mL probably due to aggregation of enzyme molecules at high enzyme concentrations during which the accessibility of substrates to the active site is reduced [3,32].

Glutaraldehyde is one of the most widely used chemical for surface modification of enzyme as well the support. It interacts with the primary amino groups of protein and intermolecularly crosslink the enzyme molecules immobilized in any type of support [33]. There are two methods of using glutaraldehyde as cross-linking agents. First, is to adsorb the enzyme by ionic exchange on the aminated support and then treating this composite with glutaraldehyde. By this method, all the primary amino groups of the support and the enzyme will be activated and cross linking between the enzyme and support will be exhibited. The advantage of this method is to provide rigidity and multipoint covalent attachment while the drawback lies in the loss of the activity and stability of the enzyme, as all the surface amino groups on the enzyme will be modified. The second method involves the use of pre-activated support during which first the aminated support is treated with glutaraldehyde and this composite is then reacted with the enzyme [6].

In this manuscript, we have employed the second method in which we have used glutaraldehyde first to create reactive aldehyde groups on the support i.e. the NC and then free aldehyde groups present on the activated support subsequently reacts with free amino groups of the enzyme. The glutaraldehyde reacts with the amino group of proteins giving monomers and dimers and that depends on the concentration of glutaraldehyde [34]. In the covalent method, two parameters were analysed. Firstly, the NC concentration was increased from 2 - 10 mg, keeping the concentration of glutaraldehyde fixed (2%, v/v) and then the glutaraldehyde concentration was varied from 0.5% to 3.0% (v/v) maintaining the NC concentration at 6 mg. The maximum immobilization yield, 97% was achieved at 6 mg of dry weight of support and 2% (v/v) glutaraldehyde solution. On increase of glutaraldehyde concentration above 2%, there was a slight decrease in the yield, probably due to greater number of aldehyde groups which tend to cross link together or with the protein. The resulted conformational changes in the protein molecules might decrease its activity [35].

Table 1 demonstrates the calculation of the immobilization yield in both immobilized enzyme preparations. Several earlier workers have shown the use of glutaraldehyde as cross-linking agent in the process of immobilization [35,36]. In this study, we have compared the immobilization yield as well as all the stability parameters by both the methods of binding. The use of glutaraldehyde as cross linker has given an advantage of preventing leaching of enzyme from the support. There was a marginal increase in the immobilization yield in case of covalently bound enzyme. Ahmad and Sardar [37] have immobilized cellulose from *Aspergillus niger* on titanium dioxide nanoparticles using adsorption and covalent methods. The immobilization yields obtained were 76 and 93 % respectively.

3.3. Stability and kinetic studies of free and immobilized enzymes

3.3.1. Effect of pH and temperature

Fig.5 (a) illustrates the pH-activity profiles of soluble and immobilized *β*-galactosidase. The soluble enzyme exhibited sharp pH-optima at 4.5 while immobilized enzyme showed broad pH- optima. The covalently attached enzyme demonstrated much broader pH-optima as compared to adsorbed enzyme. At pH 2, the free enzyme retained only 19% of the original activity while the adsorbed enzyme lost 51% activity and the covalently bound has retained 68% activity under similar experimental conditions. Shift in pH-optima after immobilization of enzymes has been previously reported by other co-workers [38,39]. Depending on the nature and surface charges on the support as well as the enzyme, the pH in the vicinity of enzyme molecule may differ and hence alter the pH-optima of the immobilized enzyme.

Effect of temperature on the activity of free, adsorbed and covalently attached *β*-galactosidase was investigated in the range of 20- 80^{o}C **(Fig. 5 b)**. Temperature-optima for soluble and adsorbed enzymes was found to be same at 50^{o}C. However, the covalently bound *β*-galactosidase exhibited maximum activity at 60^{o}C. The soluble enzyme lost 90% activity

when incubated at 80ºC for 15 min while the adsorbed enzyme retained 50% activity and the covalently immobilized enzyme retained about 80% of the maximum activity under identical incubation conditions. Ansari and Husain [22] have earlier shown that there was a shift in temperature optima from 50ºC to 60ºC in case of cross-linked Con-A-cellulose adsorbed β-galactosidase. The stability and activity retention exhibited by the immobilized enzyme in a broad range of temperature can be explained by the phenomenon of multipoint attachment which provides rigidity to the enzyme, thereby preventing enzyme conformational changes. The increased enzymatic activity under those conditions, however, is not caused by the generation of a more active enzyme form, but by avoiding distortion of the enzyme structure [5]. Guidini and co-authors [40] have reported that the covalently bound enzyme exhibited greater stability against the extreme conditions of pH and temperature as compared to simply adsorbed enzyme.

Fig. 6 (a) demonstrates the thermal denaturation plots for soluble and immobilized enzymes. The soluble enzyme lost almost 95% of the initial activity on incubation at 60ºC for 2 h. The adsorbed enzyme retained 46% activity while the covalently immobilized *β*-galactosidase exhibited 78% activity at the end of 2 h under similar experimental conditions. The immobilization matrix again imparts rigidity to the enzyme, thus it makes the enzyme more resistant against thermal denaturation.

3.3.2. Effect of galactose

For the construction of biosensors, greater emphasis is given on the activity control of immobilized enzyme in the presence of inhibitors. Galactose has proved to be a competitive inhibitor for *β*-galactosidase catalyzed reaction by various workers [41,42]. The stability of the soluble, adsorbed and covalently attached enzymes in the presence of increasing concentrations of galactose has been evaluated and compared **(Fig.6 b)**. The covalently

bound β-galactosidase was found to be most resistant against galactose inhibition and thus it retained 70% activity in the presence of 5% galactose. Adsorbed enzyme, however, lost 66% enzymatic activity and lastly, the soluble counterpart showed only 19% activity under similar exposure. The interaction between the matrix and the enzyme plays a vital role in enzyme inhibition. The possible reason for decreased inhibition in case of immobilized enzyme can be rigidification of the enzyme due to multipoint covalent attachment which makes the immobilized protein more resistant against galactose inhibition due to less conformational changes [5].

3.3.3. Kinetic parameters

The K_i, K_m and V_{max} values obtained for soluble and immobilized enzyme preparations are listed in Table 2. The K_{iapp} values were found to significantly increase in the covalently bound β-galactosidase. These values infer that the covalently immobilized enzyme was comparatively more stable as compared to the adsorbed or the soluble enzyme and less affected by galactose inhibition. The K_m values were found to decrease from 0.5 mM to 0.4 mM for adsorbed enzyme and 0.3 mM for covalently attached enzyme, respectively, indicating higher substrate-enzyme affinity [41]. A slight decrease in V_{max} values was also observed in case of immobilized enzymes suggesting a decrease in activity retention by the enzyme during binding. K_m values were unaffected while V_{max} was declined when lipase was immobilized on chitosan-tethered poly(acrylonitrile-co-maleic acid) hollow fiber membrane [43].

3.3.4. Reusability assay

Reusability of enzyme which provides cost effectiveness for its use in an economically viable enzyme catalyzed process [1]. The PANI/Co/MWCNTNC adsorbed enzyme retained about 74% activity while the cross-linked β-galactosidase exhibited 92% of its initial activity after

the 10th successive reuse **(Fig. 7 a)**. The covalently immobilized enzyme was found to be more rigid as compared to the adsorbed enzyme because of the covalent linkages formed between the matrix and the enzyme which subsequently reduces the chances of leaching of the enzyme from the support or stabilizing native conformation. The activity of the glutaraldehyde treated enzyme has been shown to withstand after several cycles of reuse by various investigators [4,44].

3.4. Genotoxicity assessment

To assess the genotoxicity of the support, plasmid nicking assay was performed. It is one of the sensitive assays to detect the effect of various toxicants on the integrity of the DNA. The pBR^{322} plasmid was incubated in the presence of the NC alone as well as both types of immobilized enzyme. As shown in **Fig. 7 (b)** the bands observed in all the cases were found to be insignificantly different from that of the control. The NC did not induce any kind of breakage in the pBR^{322} DNA indicating that the matrix we have used for the immobilization of β-galactosidase is non-toxic and biocompatible in nature and can be safely employed in the biotechnology as well as the food industry. Khan *et al*. [24] have also noticed identical findings.

4. Conclusion

This study deals with the successful preparation of PANI/Co/MWCNTNC and subsequent immobilization of β-galactosidase onto this support by means of physical adsorption and covalent binding methods. The immobilization yield obtained for adsorbed and covalently bound enzyme was found to be 93% and 97%, respectively under optimized conditions. The stability against pH, temperature and galactose as an inhibitor was also assessed. The cross-linked enzyme markedly showed greater stability against all the parameters. In addition, this support also revealed a non-toxic nature when subjected to pBR^{322} plasmid. Furthermore, the

covalently immobilized β-galactosidase showed 92% activity, while the simply adsorbed enzyme retained 74% activity after successive utilization of 10 cycles. In view of the increased activity retention, higher stability and excellent reuse characteristics offered by covalently immobilized β-galactosidase as compared to the native and adsorbed enzyme, this matrix can be efficiently used as a promising nanoscaffold for the construction of nanobiosensor for β-galactosidase and subsequently, lactose in milk and its products can be easily detected. Utility of such biosensor also shows great promise in the outcome of therapeutic agents as novel targeted drug delivery for lactose intolerant patients worldwide.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

MK gratefully acknowledges the financial assistance in terms of 'SRF' by 'UGC-MANF, New Delhi'. USIF and the chemistry instrumentation, AMU, are duly acknowledged for providing the facilities of TEM, SEM and FT-IR respectively.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Method of immobilization	Enzyme activity loaded, X, (U)	Enzyme activity in washes, Y, (U)	Enzyme activity bound on PANI/Co/MWCNTNC		Immobilization yield (%), B/A×100
			Theoretical (X-Y) = A	Actual/Immobilized =B	
Adsorption	24.67	0.087	24.58	22.84	93±0.73
Covalent binding	24.67	0.002	24.66	24.00	97±0.54

Table 1

	K_i (mM)	K_m (mM)	V_{max}(µmoles/min/ml)
Free *β*-galactosidase	0.5±0.14	0.5±0.12	0.04±0.005
Adsorbed *β*-galactosidase	1.1±0.32	0.4±0.08	0.04±0.004
Cross linked *β*-galactosidase	11.1±1.3	0.3±0.05	0.03±0.002

Table 2

Legends to tables:

Table 1: Immobilization of *β*-galactosidase on PANI/Co/MWCNTNC. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation of ≤5%.

Table 2: Kinetic parameters of free and immobilized enzymes. Each value represents the mean of three independent experiments performed in duplicates, with average standard deviation of ≤5%.

Legends for figures:

Graphical abstract/ Fig.1. Over all reaction steps involved in the functionalization of PANI/Co/MWCNT and subsequent immobilization of *β*-galactosidase by physical adsorption and covalent attachment.

Fig.2. TEM images and SAED patterns observed of **(a)** PANI/Co/MWCNTNC, **(b)** NC adsorbed *β*-galactosidase, **(c)** covalently immobilized *β*-galactosidase on the NC.

Fig.3. **SEM images** of **(a)** PANI/Co/MWCNTNC, **(b)** NC adsorbed enzyme and **(c)** covalently bound enzyme on the NC.

Fig.4. **FT-IR spectra** of PANI/Co/MWCNTNC, adsorbed and covalently attached *β*-galactosidase on the PANI/Co/MWCNTNC.

Fig.5. (a) pH–activity profiles for free and immobilized *β*-galactosidase. The activity of free, NC adsorbed and covalently immobilized *β*-galactosidase (0.04 U) was measured in the buffers of varying pH 2.0–9.0. The buffers used were glycine–HCl (pH 2.0 & 3.0), sodium acetate (pH 4.0 & 5.0), sodium phosphate (6.0 & 7.0) and Tris–HCl (pH 8.0 & 9.0). Molarity for each buffer used was 0.1 M. The activity at pH 4.5 was considered as control (100%) for the calculation of residual percent activity. Data represents mean ±S.D of three individual experiments performed in duplicates, *p*-value ≤0.05. **(b) Temperature-activity profiles** for free and immobilized *β*-galactosidase. The activity of free, NC adsorbed and covalently attached *β*-galactosidase (0.04 U) was measured in 0.1 M sodium acetate buffer, pH 4.5 at different temperatures (20–80^{o}C) for 15 min. The activity obtained at 50^{o}C was considered as control (100%) for the calculation of residual percent activity. Data represents mean ±S.D of three individual experiments performed in duplicates, *p*-value ≤0.05. The symbols show free (■), NC adsorbed enzyme (●) and covalently bound enzyme to the NC (▲).

Fig.6. (a) Thermal denaturation of free and immobilized *β*-galactosidase. Free, NC adsorbed and covalently bound *β*-galactosidase (0.04 U) was incubated at 60ºC in 0.1 M sodium acetate buffer, pH 4.5 for 2 h. Aliquots of each preparation (20 µL) were taken out at an interval of every 15 min and chilled quickly in crushed ice for 5 min. The activity of the enzyme obtained without incubation at 60°C was taken as control (100%) for the calculation of residual percent activity. Data represents mean ±S.D of three individual experiments performed in duplicates, *p*-value ≤0.05. **(b) Effect of galactose on the activity of free and NC immobilized *β*-galactosidase.** The activity of free, NC adsorbed and covalently immobilized *β*-galactosidase (0.04 U) preparations was measured 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 37ºC. The activity of the enzyme in the absence of galactose was considered as control (100%) for the calculation of residual percent activity at different concentrations of galactose. Data represents mean ±S.D of three individual experiments performed in duplicates, *p*-value ≤ 0.05. For symbols, refer to legend of Fig. 4.

Fig.7. (a) Reusability assay: reusability of NC adsorbed and covalently attached *β*-galactosidase was assessed for 10 successive days. The aliquots from each preparation were taken in triplicates and the activity determined on the first day was taken as control (100%) for the calculation of residual activity after each use. *p*-value ≤0.05 was considered statistically significant. **(b) Plasmid nicking toxicity assay**: Lane A denotes pBR^{322} DNA alone, Lane B & C signify pBR^{322} DNA with PANI/Co/MWCNTNC (50 µg), Lane D & E denotes pBR^{322} DNA incubated with adsorbed enzyme, while Lane F & G contain pBR^{322} DNA with covalently bound *β*-galactosidase.