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Nanodiamonds as an effective and novel matrix for immobilizing β galactosidase

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ABSTRACT

The present study demonstrates the biospecific adsorption of *Aspergillus oryzae* β galactosidase on glutaraldehyde functionalized nanodiamonds (NDs). Transmission electron microscopy showed that the synthesized NDs were of 20 nm size. Enzyme activity retained as a result of immobilization was 7420 U/gm of modified NDs. The optimal pH and temperature for soluble and immobilized β galactosidase was observed at pH 4.5 and at 50 °C, respectively. However, significant stability was observed at both higher and lower limits of pH and temperature for the enzyme immobilized on glutaraldehyde modified NDs. Moreover, our findings demonstrated that β galactosidase immobilized on surface functionalized NDs retained greater biocatalytic activity even after 2 months of storage and at higher galactose concentration, and upon repeated uses as compared to enzyme in solution. Modified NDs bound β galactosidase showed improved hydrolysis of lactose from solution in batch processes at various temperatures even after 10 h, thereby suggesting its use for hydrolyzing lactose in dairy products.

Keywords: β Galactosidase; Nanodiamonds; Glutaraldehyde; Lactose hydrolysis; Stability; Dairy industries

1. Introduction

Nanodiamonds (NDs) are a new member of the carbon nanoparticles family with a truncated octahedral architecture that exhibited superior characteristics of diamond such as chemical stability and hardness, apart from holding the advantages of nanoparticles like small size, large surface area and high adsorption capacity (Baidakova and Vul, 2007; Chao et al., 2007). Therefore, NDs have superior physical and chemical properties over conventional materials which brought their applications in electrochemical coatings, enzyme immobilization, polymer compositions, antifriction coatings, polishing, lubricants, biosensors, imaging probes, implant coatings and drug carriers (Huang et al., 2007; Pramatarova et al., 2007). Non toxic nature, biocompatibility and sensing, imaging and drug delivery options of NDs have yielded exciting advancement in the recent past (Chang et al., 2008; Xing and Dai, 2009).

Surface functionalization of NDs gives the opportunity to graft different functional moieties like bioactive structures, polymerizable monomers and radical initiators. Their surfaces can be modified either by covalent or non-covalent attachment of organic structures (Krueger and Lang, 2012). The carbon surface of NDs is initially covered by a variety of oxygen-containing functional groups, which serves as the foundation for covalently or permanently attaching other functional groups or bio active molecules. After homogenization of surface functional groups, the surface of NDs can be altered using variety of different chemical reactions, giving the platform incredible versatility which can be used in a wide variety of fields including biology, medicine and electronics

Abbreviations: NDs, nanodiamonds; ONPG, *o*-nitrophenyl β-D-galctopyranoside; SβG, soluble β galactosidase; IβG, immobilized β galactosidase (enzyme immobilized on glutaraldehyde modified NDs).

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(Bumb et al., 2013). Numerous studies have revealed that there is an increasing interest in utilizing surface-modified NDs for biosensor applications and for optical labeling and drug/gene delivery (Kaur and Badea, 2013). The ongoing technological advancements and impending biomedical needs are further using fabricating technology for functionalizing NDs as the promising optical nanoprobes for in vivo imaging and diagnostics (Purtov et al., 2010; Kaur et al., 2012). NDs exhibited exceptional biocompatibility and unique surface properties apart from tunable surface modification characteristics which brought their utilization in drug delivery, cancer therapy and polymer hybrid microfilms (Lam and Ho, 2009; Zhu et al., 2012).

β Galactosidase catalyzes the hydrolysis of lactose present in milk/whey to produce glucose and galactose. Presently, lactose-free dairy products are obtained as result of hydrolysis of immobilized β galactosidase on suitable and highly efficient matrices (Grosova et al., 2008; Panesar et al., 2010). These matrices should impart the enzyme high reusability, improvement of thermal stability, continuous operation, controlled product formation, high reactor productivity, and no contamination of product by the enzyme (Carlsson et al., 2014). In this regard, NDs can be utilized for enzyme immobilization since they offered greater surface area for binding higher amount of enzyme to matrix and prevents unfolding of protein thereby permitting greater flexibility for conformational changes required for enzyme activity. Moreover, their use will allow continuous operations and catalyst recycling apart from providing enhanced stability to the enzyme against physical and chemical denaturants.

Excellent characteristics and ease of modification of NDs encouraged us to exploit it for immobilizing β galactosidase. Moreover, glutaraldehyde is a bi-functional agent that proved to be a highly efficient crosslinking agent for stabilizing β-galactosidase. It enhanced thermal stability of the enzyme due to the formation of Schiff base with amine groups of enzyme and form a pendant like highly stable structure (Ansari and Husain, 2010; Ansari et al., 2012). This study involves the surface modification of NDs by glutaraldehyde for immobilizing *Aspergillus oryzae* β galactosidase. The result demonstrated that the enzyme immobilized on surface functionalized NDs retained greater biocatalytic activity against various physical and chemical denaturants and is more stable than enzymes in solution. Improved hydrolysis of lactose in batch reactors by β galactosidase covalently immobilized on glutaraldehyde modified NDs further confirmed its importance in producing lactose-free dairy products.

2. Materials and methods

2.1. Materials

Buffers of different pH values and *A. oryzae* β galactosidase were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Glutaraldehyde and *o*-nitrophenyl β-D-galctopyranoside (ONPG) was obtained from SRL Chemicals (Mumbai, India). All reagents were prepared in double distilled water with chemicals of analytical grade.

2.2. Synthesis and surface functionalization of NDs

Nanodiamonds were synthesized by decomposing graphitic C_3N_4 according to the procedure described by Fang et al. (2013) with slight modification. The obtained powder was compressed at room temperature followed by heating at 1800 °C for 15 min and left at room temperature for few hours. The morphological characterization of the synthesized NDs was carried out by transmission electron microscope. In order to functionalize the obtained NDs, they were washed with deionized water and recovered by centrifugation at 2000 rpm for 10 min. The washed NDs were then suspended in 100 mM glutaraldehyde in a shaker at 200 rpm for 4 h. The activated support was removed by centrifugation, washed twice with deionized water to remove traces of glutaraldehyde and subsequently washed with assay buffer (100 mM sodium acetate buffer, pH 4.5) and used for further studies.

2.3. Adsorption of β galactosidase on modified NDs

β Galactosidase (8500 U) was mixed with modified NDs (1.0 g) and this mixture was stirred overnight in sodium acetate buffer, pH 4.5 at 4 °C. Immobilized β galactosidase was collected by centrifugation at 250 rpm for 20 min. Matrix bound β galactosidase was washed thrice with 100 mM sodium acetate buffer, pH 4.5 and finally suspended in the same buffer and stored at 4 °C for further use.

2.4. Assay of β galactosidase

Hydrolysis of β galactosidase was analyzed by continuous shaking of an assay volume of 2.0 mL containing 1.79 mL of 100 mM sodium acetate buffer (pH 4.5), 100 μL suitably diluted β galactosidase (2.0 U) and 0.2 mL of 2.0 mM ONPG for 15 min at 40 °C. However, in case of immobilized enzyme, 0.02 mL of enzyme preparation was taken with 1.78 mL assay buffer and 0.2 mL ONPG was added, and incubated under identical conditions. The reaction was stopped by adding 2.0 mL of 2.0 M sodium carbonate solution and product formed was measured spectrophotometrically at 405 nm.

2.5. Desorption profile of β galactosidase covalently attached to the modified NDs

β Galactosidase immobilized on glutaraldehyde modified NDs was suspended in 100 mM NaCl at room temperature in a shaker at 200 rpm for 4 h. Immobilized β galactosidase was collected by centrifugation at 2000 rpm for 20 min after every 1.5 h, and activity of enzyme present in the supernatant was checked according to the procedure discussed above.

2.6. Determination of kinetic parameters

Line–Weaver Burk plot was used to determine the kinetic parameters of soluble and immobilized β galactosidase by measuring the initial rates of soluble and enzyme adsorbed on glutaraldehyde modified NDs at varying concentrations of ONPG in sodium acetate buffer (100 mM, pH 4.5).

2.7. Effect of physical and chemical denaturants

Enzyme activity of soluble and immobilized β galactosidase (20 μL) was assayed in 100 mM buffers of different pH (5.0–9.0). The buffers were sodium acetate (pH 4, 4.5, 5.0), potassium phosphate (6.0, 7.0) and Tris–HCl (pH 8.0, 9.0). Activity measured at pH 4.5 was taken as control (100%) for calculation of remaining percent activity.

Temperature-activity profile was determined by measuring the activity of soluble and immobilized β galactosidase (20 μL) at various temperatures (20–70 °C) while keeping the

other conditions same as discussed above. Enzyme activity at 50 °C was taken as control (100%) for calculating the activity of soluble and immobilized enzyme at other temperatures.

In another experiment, activity of free and immobilized 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 40 °C for 1.5 h. Activity of enzyme without added galactose was considered as control (100%) for calculation of remaining percent activity.

2.8. *Reusability and storage stability studies*

Immobilized β galactosidase (100 μL) was taken in triplicates for assaying its activity upon repeated uses. After each assay, immobilized enzyme was washed with 100 mM sodium acetate buffer, pH 4.5 by centrifugation at 2500 × *g* for 10 min. The obtained pellet was stored in assay buffer at 4 °C and this process was repeated for 7 successive days. Activity determined on first day was considered as control (100%) to calculate remaining percent activity after repeated uses. In another experiment, soluble and immobilized β galactosidase was stored at 4 °C in 100 mM sodium acetate buffer, pH 4.5 for 2 months. The aliquots from each preparation (20 μL) were taken in triplicates at the gap of 10 days and were then analyzed for the remaining activity. The activity determined on the first day was taken as control (100%) for the calculation of remaining percent activity.

2.9. *Lactose hydrolysis in batch process*

Lactose solution (200 mL, 100 mM) was independently incubated with soluble and immobilized β galactosidase (250 U) in water bath at 50 °C for various time intervals and stirred continuously. The aliquots were taken at different times and assayed for the formation of glucose by glucose oxidase-peroxidase assay kit.

2.10. *Estimation of protein*

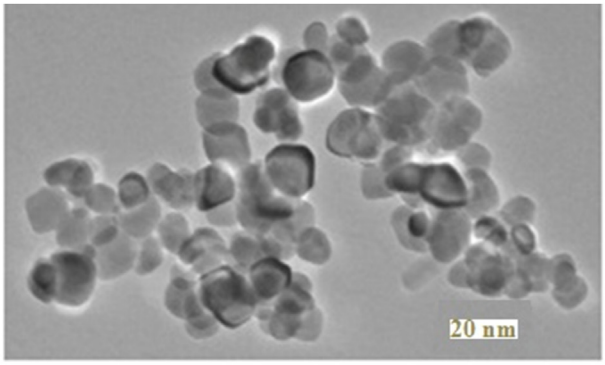
Protein concentration was determined according to the procedure described by Lowry et al. (1951) using bovine serum albumin as standard.

2.11. *Statistical analysis*

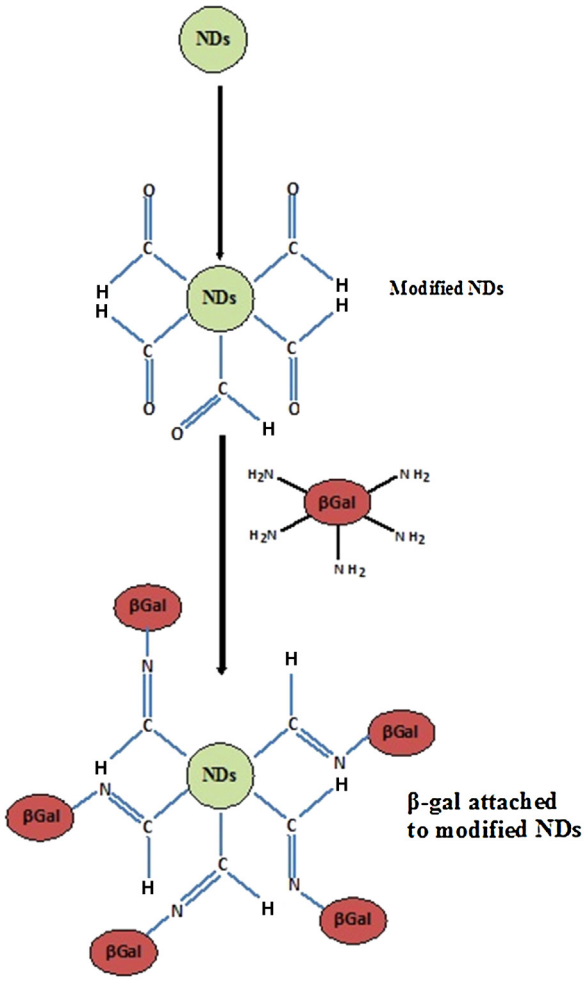
Each value represents the mean for three independent experiments performed in triplicates, with average standard deviations <5%. The data expressed in various studies was plotted using Sigma Plot-9.

3. Results and discussion

This article describes the synthesis and surface modification of NDs by glutaraldehyde as a crosslinking agent (Fig. 1), and basic experiments related to stability study of *A. oryzae* β galactosidase covalently attached to glutaraldehyde modified NDs against various physical and chemical denaturants which are pre-requisite for the construction of well defined and highly efficient biosensors. The modified nanodiamonds retained 95% enzyme upon immobilization (Table 1) and to the best of our knowledge, this is the highest immobilization yield for *A. oryzae* β-galactosidase ever reported. Efforts were raised to utilize this preparation for obtaining lactose-free dairy products in biotechnology industries.



(A) Transmission electron microscopy of NDs



(B) Surface modification of NDs by glutaraldehyde

Fig. 1 – Transmission electron microscopy of NDs and their surface modification by glutaraldehyde. (A) Transmission electron microscopy revealing the size of synthesized NDs as 20 nm. (B) Surface modification of NDs by glutaraldehyde for covalent attachment of β-galactosidase.

Kinetic studies showed that enzyme adsorbed on glutaraldehyde modified NDs exhibited increase in *Km* and reduction in V_{max} as compared to the soluble counterpart (Table 2). It is evident from the fact that as a result of immobilization, the affinity of the enzyme for its substrate and the velocity of enzymatic reaction decreased which can be explained due to the lower accessibility of the substrate to the active site of the immobilized β galactosidase and lower transporting of the substrate and products into and out the

Table 1 – Percentage yield of β galactosidase as a result of covalent attachment on glutaraldehyde modified NDs.

Enzyme activity loaded (X units)	Enzyme activity in washes (Y units)	Activity bound/g of glutaraldehyde modified NDs		Activity yield (%)
		Theoretical (X – Y) = A	Actual = B	B/A × 100
8500	689	7811	7420	95

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

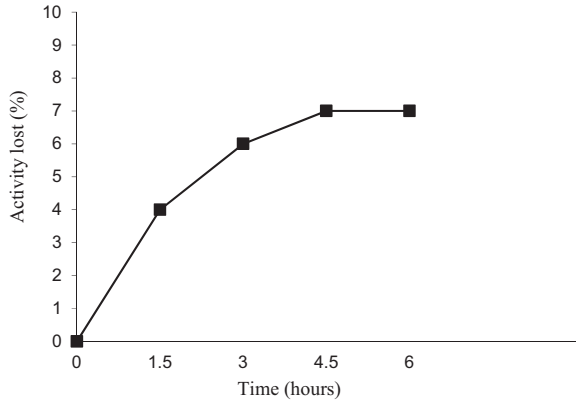


Fig. 2 – Desorption study of enzyme attached to modified NDs by 100 mM NaCl. Activity of enzyme immobilized on glutaraldehyde modified NDs was observed after an interval of 1.5 h for 6 h in presence of 100 mM NaCl at room temperature according to the procedure discussed in Section 2.5. Enzyme activity was determined as described in the text.

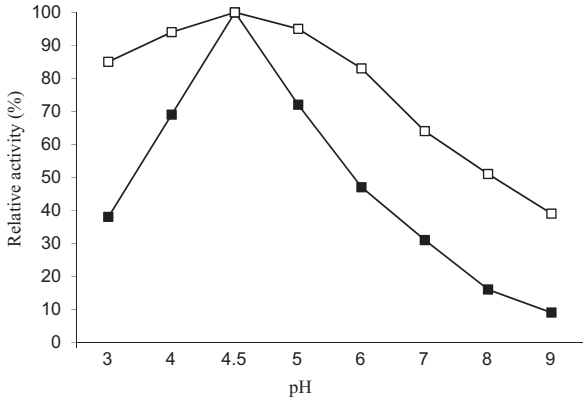


Fig. 3 – pH activity profiles for soluble and modified NDs bound β galactosidase. Activity of soluble and immobilized β galactosidase (20 μL) was measured at 40 °C in the buffers of various pH (3.0–9.0). The buffers used were glycine–HCl (pH 3.0), sodium acetate (pH 4.0, 4.5, 5.0), potassium phosphate (6.0, 7.0) and Tris–HCl (pH 8.0, 9.0). Molarity of each buffer was 100 mM. Activity at pH 4.5 was taken as control (100%) for calculation of remaining percent activity. Enzyme activity was determined as described in the text. Symbols show (■) soluble and (□) immobilized β galactosidase.

modified nanomatrix. These observations agree closely with the previous study in which *Kluyveromyces lactis* β galactosidase was immobilized on surface modified carbon nanotubes (Ansari et al., 2013).

It should be clearly noted that major disadvantages associated with immobilized β galactosidase include microbial contamination, protein adherence and channeling. The periodic washing and flow direction of substrate can solve this problem to great extent. The problem of microbial contamination can be solved by exploiting the antithermal property of immobilized β galactosidase (Cipolatti et al., 2014). Moreover, stability against various denaturing agents is an important factor when selecting an appropriate enzymatic system for any application. Hence, in the present study, effect of physical denaturants like pH (Fig. 3) and temperature (Fig. 4) was observed for soluble and glutaraldehyde modified NDs adsorbed enzyme. Enhanced pH stability and broad spectrum temperature stability of immobilized enzyme reflected its antithermal property which might be attributed to conformational stability attained by the enzyme as a result of bond formation between enzyme and matrix or lower restriction to substrate diffusion at higher temperatures (Kishore et al., 2012). Fig. 5 shows the inhibitory effect of galactose on the activity of soluble and immobilized β galactosidase. Soluble β galactosidase showed 40% activity in the presence of 3.0% galactose, while the immobilized enzyme exhibited

Table 2 – Determination of kinetic parameters.

Enzyme preparation	*Km* (mM)	V_{max} (mM/min)
Soluble β-gal	3.63 ± 0.37	2.51 ± 0.18
β-Gal covalently attached to glutaraldehyde modified NDs	3.71 ± 0.48	2.23 ± 0.52

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

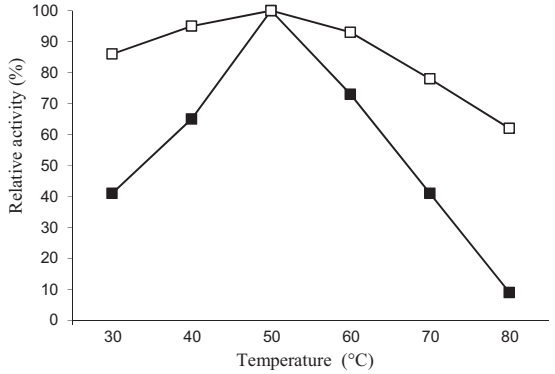


Fig. 4 – Temperature activity profiles for soluble and modified NDs bound β galactosidase. Activity of soluble and immobilized β galactosidase (20 μL) was assayed in 100 mM sodium acetate buffer, pH 4.5 at various temperatures (30–70 °C) for 15 min. Activity obtained at 50 °C was considered as control (100%) for calculation of remaining percent activity for soluble and immobilized enzyme. For symbols, refer to figure legends 3.

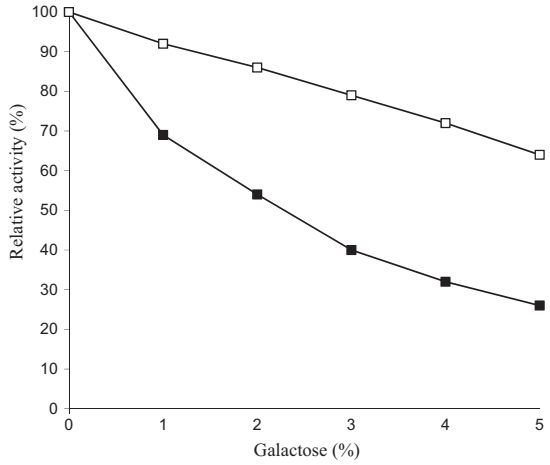


Fig. 5 – Effect of galactose on soluble and modified NDs bound β galactosidase. Effect of galactose on soluble and immobilized β galactosidase (20 μL) was measured in the presence of increasing concentrations of galactose (1.0–5.0%, w/v) in sodium acetate buffer, pH 4.5 for 1.5 h at 40 °C. Activity of enzyme without added galactose was considered as control (100%) for the calculation of remaining percent activity at other concentrations. For symbols, refer to figure legends 3.

much higher enzyme activity, 79% at the same concentration of galactose. Reusability of the immobilized β galactosidase has been shown in Fig. 6. Immobilized β galactosidase showed 87% and 82% of the initial activity, after its 5th and 6th repeated use, respectively.

Table 3 depicted the hydrolysis of lactose solution (200 mL, 0.1 M) in batch process for 10 h. The results showed that the rate of hydrolysis was more in case of free enzyme for first few hours as compared to β galactosidase adsorbed on glutaraldehyde modified NDs. Since the temperature-optima of *A. oryzae* β galactosidase was 50 °C, the greater percent of lactose hydrolysis was obtained by IβG at this temperature as compared to 40 °C. It has been observed that lactose hydrolysis obtained after 4 h was 69% by soluble β galactosidase at 50 °C while the maximum hydrolysis obtained by it was 77% after 8 h under identical conditions. Similarly, it was noticed that hydrolysis of lactose obtained by immobilized enzyme was 84% as compared to 65% by soluble enzyme after 8 h at 40 °C. It was due to the fact that soluble enzyme was more accessible

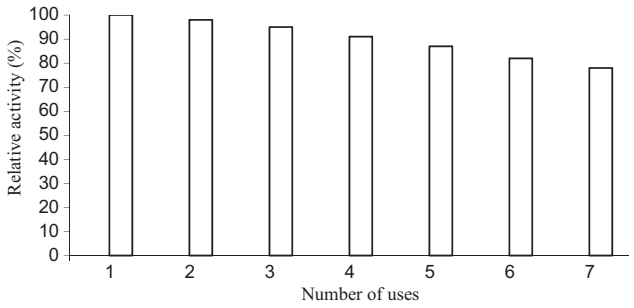


Fig. 6 – Reusability of modified NDs bound β galactosidase. Reusability of immobilized β galactosidase was monitored for six successive days. IβG (100 μL) was taken in triplicates and was assayed for remaining percent activity. Activity determined on the first day was taken as control (100%) for calculation of remaining activity after each use.

Table 3 – Hydrolysis of lactose in solution by soluble and immobilized β galactosidase in batch process.

Time (*h*)	Lactose hydrolysis			
	50 °C		40 °C	
	SβG	IβG	SβG	IβG
Control	0	0	0	0
1	54 ± 1.8	46 ± 2.1	47 ± 2.9	51 ± 1.8
2	59 ± 2.9	66 ± 2.4	53 ± 2.4	56 ± 2.2
3	65 ± 2.2	72 ± 3.2	55 ± 2.0	60 ± 1.5
4	69 ± 1.5	76 ± 2.4	60 ± 1.1	63 ± 1.2
5	72 ± 1.8	80 ± 1.4	62 ± 1.9	69 ± 2.3
6	74 ± 2.5	85 ± 3.4	64 ± 2.3	74 ± 2.4
7	77 ± 2.8	85 ± 1.9	65 ± 2.2	78 ± 2.6
8	77 ± 3.1	88 ± 2.6	65 ± 1.8	84 ± 1.9
9	77 ± 2.7	88 ± 1.1	67 ± 2.5	87 ± 1.8
10	77 ± 2.5	88 ± 0.8	67 ± 2.9	87 ± 2.7

Lactose hydrolysis was performed as described in text (Section 2.9). Each value represents the mean for three independent experiments performed in triplicates, with average standard deviations, <5%.

for hydrolysis of lactose for first few hours but after prolonged time intervals, the rate of lactolysis decreased much faster. This phenomenon of inhibition of β galactosidase by the product has been explained earlier by Mammarella and Rubiolo (2006). It was also observed that *K. lactis* β galactosidase adsorbed on glutaraldehyde modified graphite hydrolyzed 5% (w/v) lactose to 70% within 3 h at 37 °C but when temperature was increased to 50 °C, only 50% of lactose was hydrolyzed after similar time interval (Zhou et al., 2003).

Stability study (Fig. 7) and desorption profile (Fig. 2) further confirmed the excellent stability of enzyme adsorbed on glutaraldehyde modified NDs and supported that the enzyme did not leach out of the matrix appreciably, therefore such preparation could be exploited for the continuous conversion of lactose for longer durations in continuous stirred reactors in near future.

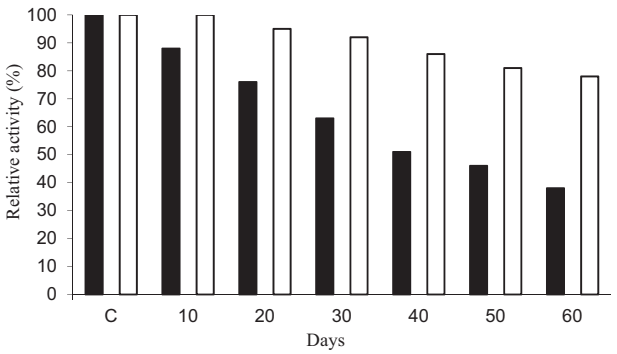


Fig. 7 – Storage stability of glutaraldehyde modified NDs bound β galactosidase. Soluble and immobilized β galactosidase was stored at 4 °C in 100 mM sodium acetate buffer, pH 4.5 for 2 months. The aliquots (20 μL) from each preparation were taken in triplicates at the gap of 10 days and were then analyzed for the remaining enzyme activity. The activity determined after first use was considered as control (100%) for the calculation of remaining percent activity at subsequent intervals. For symbols, refer to figure legends 3.

4. Conclusion

Nanodiamonds can be used as highly efficient and novel matrices for enzyme immobilization because they provide greater surface area for binding higher amount of enzyme to matrix and prevents unfolding of protein which permits greater flexibility for conformational changes required for enzyme activity against various physical and chemical denaturants. Other advantages involved with their use catalyst recycling (as observed by reusability experiment), enhanced stability against various physical (pH and temperature) and chemical denaturants (sodium chloride). Apart from offering greater activity to the enzyme against galactose mediated product inhibition, NDs provides easy separation of enzyme from reaction mixture, possible modulation of the catalytic properties (by surface modification) and much easier prevention of microbial growth.

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