BIOTECHNOLOGICAL APPLICATION OF SURFACE MODIFIED CERIUM OXIDE NANOPARTICLES

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Abstract - Re-engineering of chemical materials at the nanoscale level that employ modification and improvement in their physical and chemical properties has been constantly pursued for application in biomedical and biotechnology industries. Moreover, immobilization of catalysts on these bio/chemically modified nanomaterials improved the performance of enzymes in a plethora of industrial uses. Hence, in this study, cerium oxide nanoparticles (CNPs) were synthesized and their morphology was investigated by TEM and UV-spectra. They were modified by carboxylation and glutaraldehyde to achieve highly efficient surface functionalized nanomatrices for immobilizing *Aspergillus oryzae* β-galactosidase for producing lactose-free products in dairy industries. Enzyme activity for soluble and immobilized enzyme was observed in different pH and temperature ranges, and on galactose mediated competitive inhibition offered by the substrate. It was observed that all the enzyme preparations exhibited temperature-optima at 50 °C and pH-optima at pH 4.5, respectively. Michaelis-Menten K_m (mmole/L) values were 2.40, 5.88, 6.02 and 6.11 for soluble β-galactosidase, and enzyme immobilized on CNPs, carboxylated CNPs and glutaraldehyde modified CNPs, respectively. However, V_{max} (mmole/L/min) was found to be 518, 507, 495 and 480 for these enzyme preparations under identical conditions. Immobilized enzyme demonstrated excellent reusability even after seven repeat uses. The bioconversion rates of lactose from solution in continuous batch reactors revealed the remarkable catalytic efficiency of β-galactosidase immobilized on glutaraldehyde modified CNPs in comparison to other enzyme preparations.
Keywords: Cerium nanoparticles; Dairy industries; Enzyme stability; Lactose hydrolysis; Surface modification.

INTRODUCTION

The term "industrial vitamin" has been given to rare earth elements owing to their rich source of *de novo* materials which can be employed in biomedical and biotechnology industries. These elements differ from other elements mainly due to the nature of their 4f orbitals, which lie deep inside the atom and are protected by 4d and 5p electrons (Daane, 1966; Xu and Qu, 2014; Charbgoo et al., 2017). These characteristics impart to them unique electrical, magnetic and catalytic properties. If exploited, an exciting range of applications are expected that are not observed easily with other metals. Cerium represents the first element of the lanthanide group possessing 4f electrons, and has attracted attention from physicists, chemists, biologists and materials science researchers due to its wider application ranges (Kumaravel et al., 2015; Liying et al., 2015).

Nanoparticles serves as excellent immobilization matrices by reducing product inhibition and providing large surface area to volume ratio for retaining a greater amount of enzyme for improving traditional enzyme immobilization. More significantly, they enhance the activity and loading of enzyme, thereby augmenting their utility in biotechnological applications by decreasing the

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enzyme biocatalyst cost in industry (Zdarta et al., 2018). Hence, nanotechnology has shown great impact on the development of successful immobilized enzyme systems for biotechnological and biomedical applications during the last decade (Vaghari et al., 2016; Kim et al., 2018). Moreover, recent research on rare earth elements has gained the attention of nanotechnologists to utilize cerium oxide nanoparticles (CNPs) in industrial and commercial products because they possess excellent catalytic activity obtained as a result of quick and expedient cycling between Ce^{4+} and Ce^{3+} oxidation states (Zhang et al., 2010; Xu and Qu, 2014).

Recently, surface modification of nanoparticles for enzyme immobilization was suggested for eliminating/reducing agglomeration between the particles for providing considerable surface area and enhancing the binding of enzyme to the matrix (Mohamad et al., 2015; Sonawane and Nimse, 2016). The technology also helped to prevent the unfolding of proteins. Besides, it offered enough flexibility in conformational changes vital for any enzyme activity. Furthermore, it assists in balancing various factors that are important in determining the efficiency of biocatalysts, which include surface area, mass transfer resistance and effective enzyme loading (Ansari et al., 2014). Such an approach for enzyme immobilization speaks for high enzyme activity without modification and distortion of the active site of the enzyme. As the active site is hindered least by the nanomatrix, such steric accessibility ensures greater access of incoming substrate as well as outgoing products (Bernales et al., 2018). Other advantages involved with the use of enzymes immobilized on surface-modified nanoparticles include enhanced stability, continuous operations, catalyst recycling and improvement in their catalytic action (Samui et al., 2016; Zhang et al., 2017).

Keeping these applications and advantages in mind, an attempt was made to synthesize CNPs and modify their surface by glutaraldehyde and carboxyl groups, independently. This was envisaged to offer a controlled and greater amount of adsorption of β-galactosidase onto the obtained modified nanosupports. The comparative analysis of the stability of enzyme immobilized on these surface modified-nanomatrices was analyzed against denaturants of physical and chemical nature. Kinetic parameters, stability and reusability studies were also investigated for the immobilized enzyme. The potential biotechnological application of the immobilized β-galactosidase preparations was shown by hydrolyzing lactose in batch reactors at 50ºC with an aim of its possible exploitation in dairy industries for manufacturing lactose-free products.

MATERIALS AND METHODS

Materials

Aspergillus oryzae β-galactosidase and various pH buffers used in the present study were procured from Sigma Chem. Co. (St. Louis, MO, USA). Glutaraldehyde, nitric acid, sulfuric acid and o-nitrophenyl β-D-galctopyranoside (ONPG) were supplied by Merck. Cerium nitrate hexahydrate, sodium hydroxide and ethanol were obtained from SRL Chemicals (Mumbai, India). All reagents were prepared in double distilled water with chemicals of analytical grade.

Synthesis and characterization of CNPs

CNPs were prepared by the hydroxide method. This approach involves the drop by drop addition of sodium hydroxide (0.3 M) to cerium nitrate hexahydrate solution (0.1 M) under constant stirring and heating for 2-3 hours until a pinkish white precipitate is obtained. The resulting precipitate was centrifuged at 9000 rpm for 10 minutes. The pellet so obtained by discarding the supernatant was washed thrice with distilled water with final washing in ethanol and drying in an oven (80ºC for 1h) followed by annealing (270ºC). This allowed the formation of yellow-colored CNPs. The size and morphology of CNPs were probed by transmission electron microscope (JEOL JEM-2010 at 120 kV), while the UV-vis spectrum was obtained on a Shimadzu UV-3600 UV-Vis spectrophotometer.

Surface modification of CNPs

The surface of CNPs was modified independently by a carboxylation method and glutaraldehyde treatment for retaining greater load of enzyme. In the carboxylation approach, CNPs (0.5 gm) were incubated in 5 mL of a 1:3 HNO_3/H_2SO_4 (v/v) mixture with mild shaking at 30ºC for 8h. The carboxylated CNPs (CCNPs) thus obtained were thoroughly and continuously washed using distilled water and dried overnight in an oven (80ºC). In another experiment, glutaraldehyde modified CNPs (GCNPs) were obtained by suspending CNPs in 0.5 M glutaraldehyde with mild shaking for 8h. The modified nanosupport was segregated by centrifugation, and then washed twice with distilled water to remove traces of glutaraldehyde, followed by overnight drying in an oven (80ºC).

Immobilization of β-galactosidase on the synthesized and surface modified CNPs

β-galactosidase solution was prepared in sodium acetate buffer (0.1 M, pH 4.5) and then suspended independently overnight (30ºC) in CNPs, CCNPs and GCNPs. The resulting solutions were centrifuged at 1000 rpm for 5 min. The solutions were re-centrifuged (1000 rpm, 5 min) and the supernatant was discarded. The unbound enzyme in the pellet was removed by washing thrice with assay buffer. The pellets obtained for CNPs, CCNPs and GCNPs were stored independently in the assay buffer for carrying out all the experiments. The activity of the enzyme and the supernatant were checked as per the assay procedure.

Assay of β-galactosidase

The product formation obtained as a result of β-galactosidase hydrolysis was evaluated by continuously shaking an assay volume of 2.0 mL containing 1.79 mL of 0.1 M sodium acetate buffer (pH 4.5), 100 μL of suitably diluted enzyme and 0.2 mL of 2.0 M ONPG for 15 min at 40°C. The reaction was terminated by adding 2.0 mL of 1.0 M sodium carbonate solution, and the product formed was spectrophotometrically analyzed and measured at 405 nm (Ansari et al., 2018).

Enzyme yield and kinetic parameters

The yield of enzyme immobilization was calculated according to the following equations:

Enzyme activity loaded (X Units)	Enzyme activity lost in washes (Y Units)	Activity bound on nanosupports		Yield (%)
		Theoretical (X-Y) = A	Actual=B	B/A x 100

The kinetic parameters of soluble β-galactosidase and of the enzyme immobilized on CNPs, CCNPs and GCNPs was investigated using Lineweaver Burk plots by measuring the initial rates at varying concentrations of ONPG in the assay buffer at 40°C.

Effect of physical and chemical denaturants on soluble and immobilized β-galactosidase

Soluble β-galactosidase and the enzyme immobilized on CNPs, CCNPs and GCNPs (20 μL) were assayed in 0.1 M buffers at different pH (3.0-9.0). The buffers prepared were comprised of glycine-HCl (3.0), sodium acetate (4.0-6.0) and Tris-HCl (7.0-9.0). The activity expressed at pH 4.5 was treated as control (100%) for the calculation of the remaining percent activity. In a separate experiment, the effect of temperature on soluble and immobilized β-galactosidase preparations (20 μL) was estimated by measuring their activity at varying temperatures (30-80°C) in 0.1 M sodium acetate buffer (pH 4.5, 15 min).The reaction was terminated by adding 2.0 mL of 1.0 M sodium carbonate solution. The activity obtained at 50°C was considered as control (100%) for estimating the remaining percent activity.

Product inhibition

For evaluating the activity of various enzyme preparations against galactose-mediated product inhibition, their activity (20 μL) was determined in the presence of 1.0-5.0% w/v concentrations of galactose in 0.1 M sodium acetate buffer (pH4.5, 40°C) for 1 h. The activity of enzyme obtained without adding galactose was considered as the control (100%) for the calculation of remaining percent activity.

Reusability studies

Twenty microliters of immobilized enzyme (CNPs, CCNPs and GCNPs) were independently taken in triplicate for assaying the activity of the enzyme. After each assay, the immobilized enzyme preparations were removed from the assay tubes, washed and stored in 0.1 M sodium acetate buffer, pH 4.5 overnight at 4°C and re-assayed for seven successive days. The activity determined on the first day was taken as control (100%) and used for calculating the percent activity.

Lactose hydrolysis in batch reactors

Lactose solution (500 mL) was incubated with 250 U of soluble β-galactosidase and enzyme immobilized on the nanomatrices (CNPs, CCNPs and GCNPs) independently, and stirred continuously for 10 h at 50°C in a shaking water bath. The sample aliquots were taken at various time intervals and subjected to the assay for the formation of glucose with the glucose oxidase-peroxidase assay kit (Ansari et al., 2018).

Estimation of protein

Protein concentration was determined with the help of bovine serum albumin taken as a standard (Bradford et al., 1976).

Statistical analysis

Each value represented the mean for three independent experiments performed in triplicate, with average standard deviations <5%. The data from observations was plotted with the help of Sigma Plot-9. One-way ANOVA and Tukey test were carried out at the significance level of P-values <0.05.

RESULTS AND DISCUSSION

Synthesis and characterization of cerium oxide nanoparticles

Several methods that have been exploited in the past to synthesize CNPs, including sol-gel, hydrothermal, flame-spray pyrolysis and solvothermal approaches (Charbgoo et al., 2017). However, these methods suffered from major disadvantages as they depend on high pressure and temperature, and the use of capping agents for their synthesis. Hence, the present study involves their synthesis by an inexpensive, novel and simple hydroxide method. The size of CNPs as observed by TEM was 50 nm (Fig 1), while the adsorption spectra of the synthesized CNPs dispersed in water is shown in Fig 2. It exhibited a strong absorption band below 400 nm and a well-defined absorbance peak around 320 nm for the synthesized CNPs.

Matrix selectivity and surface modification ligands

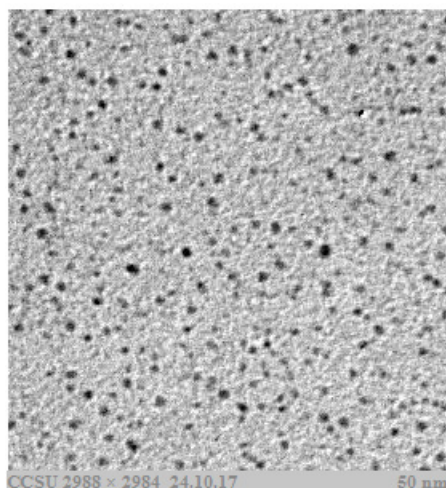CNPs, recognized as commercially important nanoparticles, have already shown a wider range of

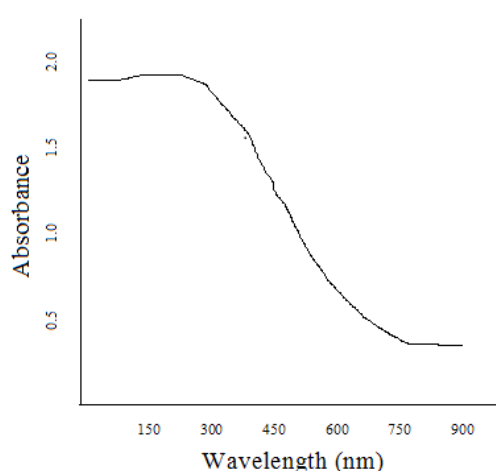**Figure 1.** TEM image of cerium oxide nanoparticles.

Figure 2. UV spectrum of cerium oxide nanoparticles.

biomedical and biotechnological applications and catalytic utility for sensors and permeation membranes. Besides, they have been used in solid oxide fuel cells, for glass-polishing and as an ultraviolet absorbent (Das et al., 2013; Ge et al., 2015).The biotechnological importance of glutaraldehyde as an activator/stabilizer/ surface modification ligand/crosslinking agent for the immobilization of β-galactosidase on a plethora of immobilization matrices has been excellently reviewed rather recently (Johnson et al., 2011; Satar et al., 2017). Moreover, the modification of matrices via carboxylation prior to enzyme immobilization ensures anchoring of the enzyme molecules to the matrices efficiently and in greater amount with improved stability either by physisorption or chemisorption (Ansari et al., 2015; Alexander et al., 2016).

Analysis of immobilization yield and kinetic parameters, and superior stability of GCNPs for β-galactosidase immobilization over other immobilized enzyme preparations

The role of nanoparticles as the supporting material for immobilizing enzymes is well established. This is due to their higher surface area, vital for greater enzyme loading. Also, their lower mass transfer resistance and selective non-chemical separation from the reaction mixture are other added advantages. In the present study, the yields of *Aspergillus oryzae* β-galactosidase immobilized on CNPs, CCNPs and GCNPs were 74%, 82% and 91%, respectively (Table 1). It should be noted that the surface modification of CNPs by the ligands used, i.e., carboxylation and glutaraldehyde, enhanced the binding of single or even multi-enzymes to the introduced functional groups. Moreover, crosslinking of β-galactosidase by glutaraldehyde further stabilizes the attached enzymes to a greater extent on the modified nanosupport. Enzyme stabilization by these ligands under different conditions has been reported by Vazquez-Ortega et al. (2018). The kinetic parameters of free and immobilized enzyme preparations are displayed in Table 1. The K_m and V_{max} value were 6.11 mmole/L and 480 mmole/L/min for the enzyme immobilized on glutaraldehyde-modified CNPs, respectively. However, a minor statistical difference between the kinetic parameters of the free and immobilized enzymes was observed. The slight increase in K_m after immobilization indicates that the immobilized enzyme has lower affinity for the substrate, which can be explained due to restricted access for the active site in the immobilized enzyme, as a consequence of immobilization.

Table 1. Enzyme immobilization yield and kinetic constants of soluble and immobilized β-galactosidase preparations.

Enzyme preparation	Enzyme yield (%)	K_m (mmole/L)	V_{max} (mmole/L/min)
SβG	-	2.40±2.8	518±3.9
CNPs-βG	74±2.30	5.88±0.80	507±2.75
CCNPs-βG	82±3.75	6.02±1.30	495±3.50
GCNPs-βG	91±1.80	6.11±4.50	480±3.90

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

Stability in various ranges of pH and temperature

The catalytic activity of enzyme is mainly due to the conformational structure of the protein. Therefore, a minor change in tertiary structure is manifested in loss of catalytic activity. Figure 3 shows pH-activity profiles for soluble and immobilized β-galactosidase. Immobilized enzyme registered a clear edge and exhibited remarkable broadening in the pH-activity profiles in comparison to the native enzyme. The free enzyme showed 56% activity at pH 6.0 while β-galactosidase immobilized on CNPs, carboxylated CNPs and glutaraldehyde-modified CNPs retained 69%, 75% and 84%, activity, respectively, under similar conditions. It could be due to greater alteration/ distortion produced in the tertiary structure of the free enzyme in highly acidic and basic solutions than that of
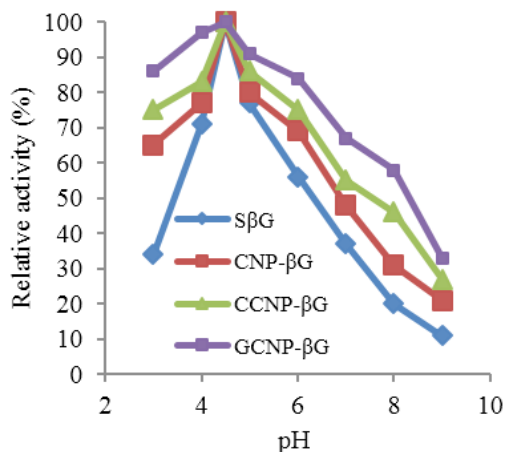
Figure 3. pH-activity profile of soluble and immobilized β-galactosidase preparations.

the immobilized enzyme (Ansari et al., 2018). Figure 4 suggests that the temperature-optima was 50°C for soluble and immobilized enzyme preparations. However, the activity retained by CNPs-βG, CCNPs-βG and GCNPs-βG was 56%, 69% and 77%, respectively, at 50°C whereas SβG exhibited only 32% activity under similar experimental conditions. The loss in enzyme activity at higher temperatures apparently can be attributed to denaturation of enzyme molecules (Migneault et al., 2004). Since glutaraldehyde reacts with enzyme by aldol condensation or Michael-type addition, GCNPs-βG showed greater resistance to high pH and temperature ranges as compared to other immobilized enzyme preparations.
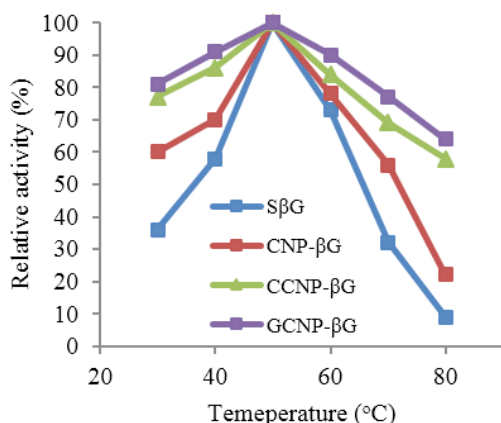

Figure 4. Temperature-activity profile of soluble and immobilized β-galactosidase preparations.

Product inhibition

It is a well known fact that complete hydrolysis of lactose is difficult to achieve as a result of inhibition of β-galactosidase by the products, i.e., galactose and glucose, and these products can reduce the hydrolysis rate or even completely halt the reaction (Park and Oh, 2010b). Hence, several strategies have been suggested to reduce the enzyme inhibition by the products. These include enzyme immobilization and mutation of the

inhibitor binding site in β-galactosidase that promotes the reduction in inhibition as a result of the support to the inhibitor's access to the binding site (Kim et al., 2011; Ansari et al., 2018). Our study suggested that GCNPs-βG showed 71% activity even after 1 hour at 5% galactose concentration. However, under similar incubation conditions, SβG, CNPs-βG and CCNPs-βG retained 28%, 49% and 66% activity, respectively (Fig 5). Hence, immobilized enzyme preparations showed promising resistance to inhibition mediated by galactose as compared to their soluble counterpart, suggesting the utilization of these immobilized enzyme preparations in biomedical/biotechnological applications.
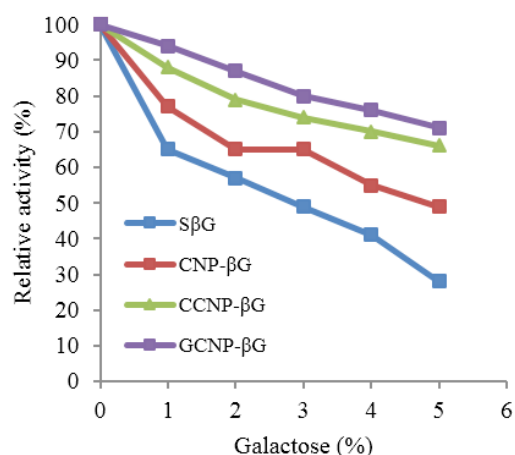

Figure 5. Effect of galactose on the activity of soluble and immobilized β-galactosidase preparations.

Reusability studies

An additional desirable feature of the process developed herein is the ability to reuse the immobilized enzymes under sufficiently mild conditions such that their activities would be maintained maximally. GCNPs-βG retained 85% of the initial enzyme activity consecutively after its 6th reuse as against 82% and 78% for CCNPs-βG and CNPs-βG, respectively, thereby emphasizing the importance of this study (Fig 6).
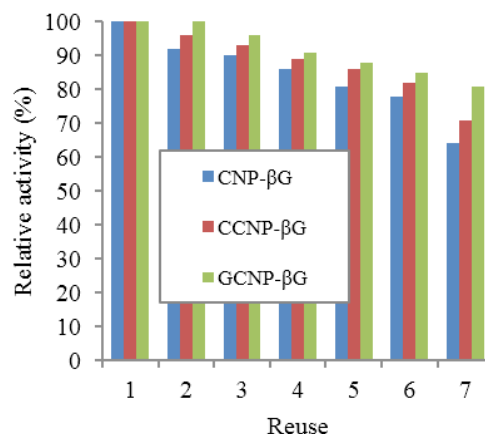

Figure 6. Reusability of immobilized β-galactosidase preparations.

Batch reactors

The batch reactor is a device that is designed for carrying out preliminary reactions on a lab scale under controlled conditions for obtaining specified products so that their utility can be exploited efficiently at the industrial level. These reactors may strongly differ in dimensions and structure depending on the experiment and product needed. However, major features that need to be considered for deriving a mathematical model for their quantitative description include their mode of operation, maintenance of reaction conditions (temperature, pH and humidity), and the quality of substrate mixing (Fang et al., 2012). Keeping in view the industrial application of the immobilized enzyme preparations developed in this study, batch reactor experiments were performed at 50°C, indicating their efficacy for hydrolyzing lactose hydrolysis industrially. It was observed that a greater percent of lactose hydrolysis was achieved for SβG initially against the other immobilized enzyme preparations (Table 2). This was due to the fact that the soluble enzyme was more accessible for the hydrolysis of lactose during the initial few hours. However, after prolong incubation, the rate of lactolysis by soluble enzyme decreased much faster as a result of the galactose-mediated competitive inhibition effect on the enzyme (Verma et al., 2012; Ansari and Husain, 2013). Table 2 shows that 64% lactose hydrolysis was achieved in 5h by SβG, while CCNPs-βG and GCNPs-βG showed 71% and 77% lactose hydrolysis after a similar time interval. Moreover, the maximum hydrolysis obtained with the GCNPs-βG was 95% after 9h, as compared to 86% hydrolysis by the enzyme immobilized on CNPs-βG.

Table 2. Hydrolysis of lactose by soluble and immobilized β-galactosidase preparations in batch reactors at 50 °C.

Time (h)	SβG	CNPs-βG	CCNPs-βG	GCNPs-βG
0	NA	NA	NA	NA
1	41±0.12	35±0.23	32±0.24	30±0.31
2	45±0.31	42±0.30	40±0.31	38±0.26
3	53±0.22	58±0.18	61±0.29	65±0.18
4	58±0.18	64±0.22	67±0.21	72±0.23
5	64±0.26	71±0.19	71±0.19	77±0.19
6	71±0.28	77±0.32	76±0.17	81±0.18
7	74±0.28	80±0.16	85±0.22	87±0.17
8	76±0.13	83±0.13	88±0.32	94±0.31
9	76±0.27	86±0.15	90±0.13	95±0.22
10	76±0.21	86±0.25	90±0.22	95±0.17

Each value represents the mean for three independent experiments performed in triplicate, with average standard deviations <5%.
*NA: Not Analyzed.

CONCLUSION

Modified CNPs may prove to be an important matrix for immobilizing other industrially important enzymes due to their low-cost, large surface area and less diffusion limitation provided in transporting substrate and product for enzymatic reactions. In view of their stability and utility against various physical and chemical denaturants, and lactose hydrolysis in batch reactors, such preparations could be exploited for the continuous conversion of lactose from milk and whey for longer durations in a reactor in a more convenient and cheaper way.

ABBREVIATIONS

CNPs	Cerium oxide nanoparticles
CCNPs	Carboxylated cerium oxide nanoparticles
GCNPs	Glutaraldehyde modified cerium oxide nanoparticles

REFERENCES

Alexander, S., Gomez, V., and Barron, A.R., Carboxylation and decarboxylation of aluminum oxide nanoparticles using bifunctional carboxylic acids and octylamine. J. Nanomat., 2016, 1-8 (2016).

Ansari, S.A., Ahmad, S.I., Jafri, M.A., Naseer, M.I., and Satar, R., Utility of functionalized agarose nanoparticles in hydrolyzing lactose in batch reactors for dairy industries. Quim. Nov., 41, 429-433 (2018).

Ansari, S.A., Satar, R., Chibber, S., and Khan, M.J., Enhanced stability of *Kluyveromyces lactis* β-galactosidase immobilized on glutaraldehyde modified multiwalled carbon nanotubes. J. Mol. Catal. B: Enzym., 97, 258-263 (2013). https://doi.org/10.1016/j.molcatb.2013.09.008

Ansari, S.A., Satar, R., Panda, D.S., Zaidi, S.K., Chibber, S., Khan, M.J., Khan, T.A., Jafri, M.A., and Alqahtani, M.H., Surface engineering of multifunctional nanocomposites for biomedical applications: a brief update. Iran. J. Biotechnol., 12, 1-7 (2014). https://doi.org/10.5812/ijb.11124

Ansari, S.A., Satar, R., and Zaidi, S.K., Carboxylation of silver nanoparticles for the immobilization of β-galactosidase and its efficacy in galacto-oligosaccharides production. Quim. Nov., 38, 387-392 (2015).

Bernales, V., Ortuno, M.A., Truhlar, D.G., Cramer, C.J., and Gagliardi, L., Computational design of functionalized metal-organic framework nodes for catalysis. ACS Cent. Sci., 4, 5-19 (2018). https://doi.org/10.1021/acscentsci.7b00500

Charbgoo, F., Ahmad, M.B., and Darroudi, M., Cerium oxide nanoparticles: green synthesis and biological applications. Int. J. Nanomed., 12, 1401-1413 (2017). https://doi.org/10.2147/IJN.S124855

Daane AH., Chemistry of the rare-earth elements. J. Am. Chem. Soc., 88, 5938-5938 (1966). https://doi.org/10.1021/ja00976a049

Das, S., Dowding, J.M., Klump, K.E., McGinnis, J.F., Self, W., and Deal, S., Cerium oxide nanoparticles: applications and prospects in nanomedicine. Nanomedicine, 8, 19-28 (2013). https://doi.org/10.2217/nnm.13.133

Fang, S.M., Wang, H.N., Zhao, Z.X., and Wang, W.H., Immobilized enzyme reactors in HPLC and its application in inhibitor screening: A review. J. Pharm. Anal., 2, 83-89 (2012). https://doi.org/10.1016/j.jpha.2011.12.002

Ge, X., Li, Z., and Yan, Q., 1D ceria nanomaterials: versatile synthesis and bio-application. J. Mater. Sc. Technol., 31, 645-654 (2015). https://doi.org/10.1016/j.jmst.2015.01.008

Johnson, P.A., Park, H.J., and Driscoll, A.J., Enzyme nanoparticle fabrication: magnetic nanoparticle synthesis and enzyme immobilization. Meth. Mol. Biol., 679, 183-191 (2011). https://doi.org/10.1007/978-1-60761-895-9_15

Kim, K.H., Lee, O.K., and Lee, E.Y., Nano-immobilized biocatalysts for biodiesel production from renewable and sustainable resources. Catalysts, 8, 68-76 (2018). https://doi.org/10.3390/catal8020068

Kim, Y.S., Yeom, S.J., and Oh, D.K.., Reduction of galactose inhibition via the mutation of β-galactosidase from *Caldicellulosiruptor saccharolyticus* for lactose hydrolysis. Biotechnol. Lett., 33, 353-358 (2011). https://doi.org/10.1007/s10529-010-0445-z

Kumaravel, M.S., Murali, A.P., Sekar, S., and Mathiyalagan, P., Cerium oxide nanoparticles in engineering, biology and medicine. Int. J. Appl. Eng. Res., 10, 34473-34475 (2015).

Liying, H.E., Yumin, S.U., Lanhong, J., and Shikao, S.H.I., Recent advances of cerium oxide nanoparticles in synthesis, luminescence and biomedical studies: a review. J. Rare Earths, 33, 791-799 (2015). https://doi.org/10.1016/S1002-0721(14)60486-5

Migneault, I., Dartiguenave, C., Bertrand, M.J., and Waldron, K.C., Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. Biotechniques, 37, 790-802 (2004). https://doi.org/10.2144/04375RV01

Mohammad, N.R., Marzuki, N.H.C., Buang, N.A., Huyop, F., and Wahab, R.A., An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnol. Biotechnol. Equip., 29, 205-220 (2015). https://doi.org/10.1080/13102818.2015.1008192

Samui, A., Chowdhuri, A.W., Mahto, T.K., and Sahu, S.K., Fabrication of a magnetic nanoparticle embedded NH2-MIL-88B MOF hybrid for highly efficient covalent immobilization of lipase. RSC Adv., 6, 71-77 (2016). https://doi.org/10.1039/C6RA10885G

Satar, R., Jafri, M.A., Rasool, M., and Ansari, S.A., Role of glutaraldehyde in imparting stability to immobilized β-galactosidase systems. Braz. Arch. Biol. Technol., 60, 1-12 (2017). http://dx.doi.org/10.1590/1678-4324-2017160311

Sonawane, M.G., and Nimse, S.B., Surface modification chemistries of materials used in diagnostic platforms with biomolecules. J. Chem. 2016, Article ID 9241378, 19 pages (2016).

Vaghari, H., Jafarizadeh-Malmiri, H., Mohammadlou, M., Berenjian, A., Anarjan, N., Jafari, N. and Nasiri, S., Application of magnetic nanoparticles in smart enzyme immobilization. Biotechnol. Lett. 38, 223-233 (2016). https://doi.org/10.1007/s10529-015-1977-z

Vazquez-Ortega, P.G., Alcaraz-Fructuoso, M.T., Rojas-Contreras, J.A., Lopez-Miranda, J., and Fernandez-Lafuente, R., Stabilization of dimeric β-glucosidase from *Aspergillus niger* via glutaraldehyde immobilization under different conditions. Enzym. Microb. Technol. 110, 38-45 (2018). https://doi.org/10.1016/j.enzmictec.2017.12.007

Verma, M.L., Barrow, C.J., Kennedy, J.F., and Puri, M., Immobilization of β-galactosidase from *Kluyveromyces lactis* on functionalized silicon dioxide nanoparticles: characterization and lactose hydrolysis. Int. J. Biol. Macromol. 50, 432-437 (2012). https://doi.org/10.1016/j.ijbiomac.2011.12.029

Xu, C., and Qu, X., Cerium oxide nanoparticle: a remarkably versatile rare earth nanomaterial for biological applications. NPG Asia Mater. 6, 1-16 (2014). https://doi.org/10.1038/am.2013.88

Zdarta, J., Meyer, A.S., Jesionowski, T., and Pinelo, M., A general overview of support materials for enzyme immobilization: characteristics, properties, practical utility. Catalysts 8, 92-100 (2018). https://doi.org/10.3390/catal8020092

Zhang, W., Yang, H., Liu, W., Wang, N., and Yu, X., Improved performance of magnetic cross-linked lipase aggregates by interfacial activation: a robust and magnetically recyclable biocatalyst for transesterification of jatropha oil. Molecules 22, 2157-2172 (2017). https://doi.org/10.3390/molecules22122157

Zhang, Z., Yu, L., Liu, W., and Song, Z., Surface modification of ceria nanoparticles and their chemical mechanical polishing behavior on glass substrate. Appl. Surf. Sc. 256, 3856-3861 (2010). https://doi.org/10.1016/j.apsusc.2010.01.040