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Research Article

Effect of Different Additives on the Structure and Activity of β-Galactosidase Immobilized on a Concanavalin A–Modified Silica-Coated Titanium Dioxide Nanocomposite

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Academic Editor: Jose M. Palomo

Special Issue: Recent Trends in Biocatalysis

Catalysis Research
2022, volume 2, issue 4
doi:10.21926/cr.2204040

Received: August 06, 2022
Accepted: October 30, 2022
Published: November 17, 2022

Abstract

Interpreting the relationship between the activity and structure of β-galactosidase is necessary to perceive the impact of the enzyme's conformation on its catalysis. The current study thoroughly explains the effects of additives such as ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), and urea on β-galactosidase activity and structure. β-Galactosidase activity was determined at various ionic strengths and temperatures as a function of time. Structural studies evaluating changes in the secondary and tertiary structures of the enzyme in the presence of the additives were conducted using ultraviolet (UV)-visible and intrinsic fluorescence spectroscopy. The immobilized enzyme showed enhanced stability under different environmental conditions. Activity assays demonstrated concentration-dependent inactivation of β-galactosidase in the presence of SDS and urea, which suggests that hydrophobic and charged residues are present near the active site. In the presence of EDTA, loss in activity was noted, which confirms that β-galactosidase is a metalloenzyme. Enhancement in enzyme activity in the presence of DTT suggests the presence of a cysteine residue near the catalytic center. In UV-visible and intrinsic fluorescence spectroscopy studies, the native enzyme showed significant conformational

transitions in the presence of DTT, SDS, and urea and very few changes in the presence of EDTA. However, the immobilized enzyme could resist significant structural changes. In conclusion, this study provides a detailed description of the association between the activity and conformational stability of β-galactosidase.

Keywords
β-Galactosidase; structural stability; functional stability; additives; sodium dodecyl sulfate; ethylenediaminetetraacetic acid; dithiothreitol; urea

1. Introduction

Soluble enzymes are fragile and susceptible to denaturation under harsh environmental conditions associated with pH, temperature, and chemicals, among others. For the fabrication of an effective device for industrial-level application, it is crucial to improve its stability and handling. Hence, enzyme immobilization on a solid support is preferred. The immobilization process minimizes the drawbacks occurring with the use of soluble enzymes, especially in terms of enzyme instability under different reaction conditions and challenging recovery and reuse [1, 2].

Nanomaterials are mostly preferred as a support material because they possess a vast interfacial area per unit volume, which makes them an efficient enzyme carrier. Surface functionalization of these nanometric supports with different chemical groups may aid in efficient and high-yield enzyme binding [3]. The immobilization process confers protection to the enzymes against denaturing reaction conditions [4]. Hence, this process helps build a new system that provides scope for various applications in biotechnology, medical technology, and food processing [5]. Immobilization mainly aims to conserve the catalytic activity of an enzyme by preventing the development of structural changes [6].

For industrial-level application of enzymes, additional information on enzyme activity needs to be accumulated. The reaction conditions for the enzymes at the industrial level are quite different from the conditions in their natural sources. Additives have been generally used for enzyme stabilization [7]. However, the influence of additives on the properties of the enzyme is poorly understood because only very few systematic studies are available on this topic [8, 9]. Hence, it is of pivotal importance to analyze the effect of various solvents, non-optimal pH, high ionic strength, inhibitors, and elevated temperature on enzyme structure and activity, all of which lead to enzyme denaturation and loss of activity [10, 11]. Additives can either remarkably enhance or lower enzyme activity without causing any genetic or chemical modification [8, 12]. Therefore, it is crucial to accumulate comprehensive knowledge about the long-term stability of the enzyme in its free and bound forms in the presence of different chemicals and under varying environmental conditions [7, 13]. The substrate-hydrolyzing property of enzymes and their stability under different physiochemical conditions for an extended period are characteristic features essential for their commercial use [14]. A research group has explored the effect of different additives on the stability of native and immobilized α-chymotrypsin in different aqueous–organic solvent systems [15]. α-Chymotrypsin was immobilized on a silica-based support and demonstrated a twofold increase in its catalytic activity with sorbitol or glucose.

It is essential to treat milk with β-galactosidase before marketing the milk because it eliminates lactose crystallization and lowers the chances of lactose intolerance [16-18]. Milk is often adulterated with various compounds to increase its stability. β-Galactosidase has various applications in the commercial sector, such as for lactose elimination from milk for people with lactose intolerance, galactooligosaccharides (GOS) synthesis from lactose for preparing probiotic foods, bioremediation of dairy waste, and conversion of lactose into more fermentable sugars [19, 20]. β-Galactosidase interacts with various chemicals used in industrial processes, which may affect its catalytic activity. The stability of this enzyme in the reaction environment continues to remain concerning and requires improvement. Several schemes, including enzyme immobilization and the addition of some chemicals to the reaction media have been followed to overcome this drawback [21-23]. These chemicals not only interact with the proteins but also shield them against harsh microenvironments and sometimes modify the enzyme's catalytic activity [24-26].

This study aimed to determine the stability of soluble/native and immobilized β-galactosidase at various temperatures and pH with varying time intervals. Second, the effect of different additives (ethylenediaminetetraacetic acid [EDTA], sodium dodecyl sulfate [SDS], dithiothreitol [DTT], and urea) at varying concentrations on the activity of native and nanocomposite (NC)–bound β-galactosidase was evaluated. Finally, the influence of these additives on the secondary and tertiary conformation of free and bound β-galactosidase was assessed through ultraviolet (UV)-vis and intrinsic fluorescence spectroscopy studies.

2. Materials

Aspergillus oryzae β-galactosidase was purchased from Sigma-Aldrich Company (USA). The substrate *o*-nitrophenyl β-D-galactopyranoside (ONPG), sodium carbonate, SiO_2-TiO_2 nanoparticles, concanavalin A (Con A), magnesium chloride, calcium chloride, EDTA, DTT, SDS, and urea were purchased from SRL Chemicals (Mumbai, India). The other chemicals used were of analytical grade.

3. Methods

3.1 Enzyme Activity Assay

β-Galactosidase activity assay was performed with ONPG as the substrate, according to the procedure described previously [27]. The assay was performed in 0.1 M sodium acetate buffer at pH 4.5 and 37°C, and the amount of *o*-nitrophenol released was recorded by measuring the absorbance at 405 nm. Thus, one unit of β-galactosidase activity was defined as the amount of enzyme that released 1.0 µmol of *o*-nitrophenol (ε_m = 4500 L mol^{-1} cm^{-1}) min^{-1} under standardized conditions (37°C for 15 min).

3.2 Synthesis of Con A–Layered NC

Silica-coated TiO_2 nanoparticles (125 mg) were incubated with an appropriate amount of Con A in 0.1 M phosphate buffer (pH 7). The solution mixture was then stirred for 24 h at 4°C. Next, the NC-bound protein was separated by centrifugation at 8000 *x g* for 10 min, and the protein in the supernatant and washes was quantitated according to Lowry's method to determine the amount of bound protein [28]. Finally, the NC-bound protein was suspended in phosphate buffer (pH 7) and stored at 4°C.

3.3 Bioaffinity Layering of β-Galactosidase on Con A–Coated SiO_2-TiO_2 NC

The NC solution was sonicated for the dispersion of the particles in the medium. β-Galactosidase (0.4 U) was loaded onto a 125-mg Con A@SiO_2-TiO_2 NC suspended in 0.1 M phosphate buffer (pH 7). The binding process was initiated by keeping this mixture on the disc rotator at a constant speed for 6 h at room temperature. The solution was then centrifuged at 5000 *x g* for 10 min to collect the supernatant. The pellet was collected and washed thrice with phosphate buffer to remove any unbound enzyme molecules and the washes were collected. The supernatant, washes, and pellet were stored at 4°C until further use.

The procedure was repeated by varying all parameters (one at a time), such as enzyme concentration, NC concentration, and immobilization time, until maximum immobilization yield was obtained [29].

3.4 Functional Studies

3.4.1 Effect of Temperature as a Function of Time

The soluble/native and immobilized enzyme preparations were evaluated for thermal stability by incubating them at temperatures ranging from 30°C to 70°C for varying time intervals (0–180 min). Aliquots of equal amounts were withdrawn from the solutions every 30 min for the evaluation of the residual activity.

3.4.2 Effect of pH as a Function of Time

The soluble/native and immobilized enzyme preparations were incubated in buffers of varying pH (ranging from 4.0 to 7.0) for varying time intervals (0–180 min). The buffers used were sodium acetate (pH 4.0, 4.5, and 5.0) and sodium phosphate (pH 6.0 and 7.0). The molarity of both buffers was 0.1 M. Aliquots of an equal amount of both β-galactosidase preparations were taken after equal time intervals to calculate percent residual activity.

3.4.3 Effect of Additives

To understand the impact of SDS, DTT, EDTA, and urea on the activity of β-galactosidase, the soluble/native and immobilized enzyme preparations were pre-incubated with each additive individually for 60 min at 37°C. The results were recorded as the percentage of remaining activity and measured by using control preparations (without additives) running in parallel. Routine enzyme assay was conducted in 0.1 M sodium acetate buffer (pH 4.5) to determine enzyme activity. Additionally, to investigate the effect of EDTA, the enzyme preparations were pre-incubated with Mg^{2+} ions for 10 min. Enhancement in enzyme activity in the presence of Mg^{2+} ions has already been reported [27].

3.5 Structural Studies

3.5.1 UV-Visible Spectroscopy

UV-vis spectroscopy was reported using UV-vis spectrophotometer 1800 (Shimadzu, Japan). The influence of the aforementioned additives on the secondary structure of β-galactosidase was

assessed using the UV-vis spectra recorded with different concentrations of these additives. The soluble/native and immobilized enzyme preparations were incubated with three different concentrations (0.1 mM, 1 mM, and 10 mM) of SDS, EDTA, DTT, and urea at 37°C in 0.1 M sodium acetate buffer (pH 4.5) for 1 h. UV-vis absorption spectra were recorded in the absorbance range of 200–500 nm. The spectrum of the enzyme preparation without any additive was considered as control (100%).

3.5.2 Fluorescence Emission Spectroscopy

Intrinsic fluorescence spectra were recorded using RF-6000 Spectrofluorophotometer (Shimadzu, Japan). Changes in the enzyme's tertiary structure in the presence of different additives were determined by evaluating the intrinsic fluorescence spectra. This technique is widely used to analyze protein conformational changes because the intrinsic fluorescence is produced by the tryptophan (Trp) and tyrosine residues in the active site. An excitation wavelength of 380 nm was used to measure intrinsic fluorescence, and emission spectra were recorded from 350 to 500 nm using a quartz cuvette with a 1-cm path length. Subsequently, the native and immobilized enzyme preparations were incubated with additives at 37°C for 1 h before performing the tests.

3.6 Results and Discussion

3.6.1 Immobilization Yield and Stability Studies

After optimization of all reaction conditions (enzyme concentration, NC concentration, and immobilization time), an immobilization yield of 221.7% was achieved, as reported in our previous research [27]. The immobilized enzyme had better stability than the soluble enzyme. The immobilized enzyme had a change in its pH optima (from pH 4.5 to 5.0), whereas the temperature optima remained unchanged (50°C). When the kinetic parameters were evaluated, the increase in V_{max} values from 0.03 to 0.04 suggests efficient hydrolysis of the substrate. The immobilized enzyme demonstrated a decrease in K_m values from 0.5 to 0.46, which indicates a higher affinity of the immobilized enzyme toward its substrate. These studies along with the effect of different metal ions on the free and immobilized enzyme have been discussed in detail in our previous research [27].

3.6.2 Functional Studies

Effect of temperature as a function of time. The thermal stability of both β-galactosidase preparations was analyzed as a function of time. At 40°C, the native enzyme had 100% and 98% activity preserved °after 1 h and 3 h of incubation, respectively (Figure 1). At 50°C, the native enzyme had 90% activity preserved after 1 h of incubation, °but with prolonged incubation, a gradual loss of activity was noted, i.e., 63% activity was preserved after 3 h of incubation. The stability of the soluble/native enzyme decreased when incubated at higher temperatures, i.e., 60°C and 70°C, because only 40% and 24% activity were preserved after 3 h of incubation, respectively (Figure 1a).

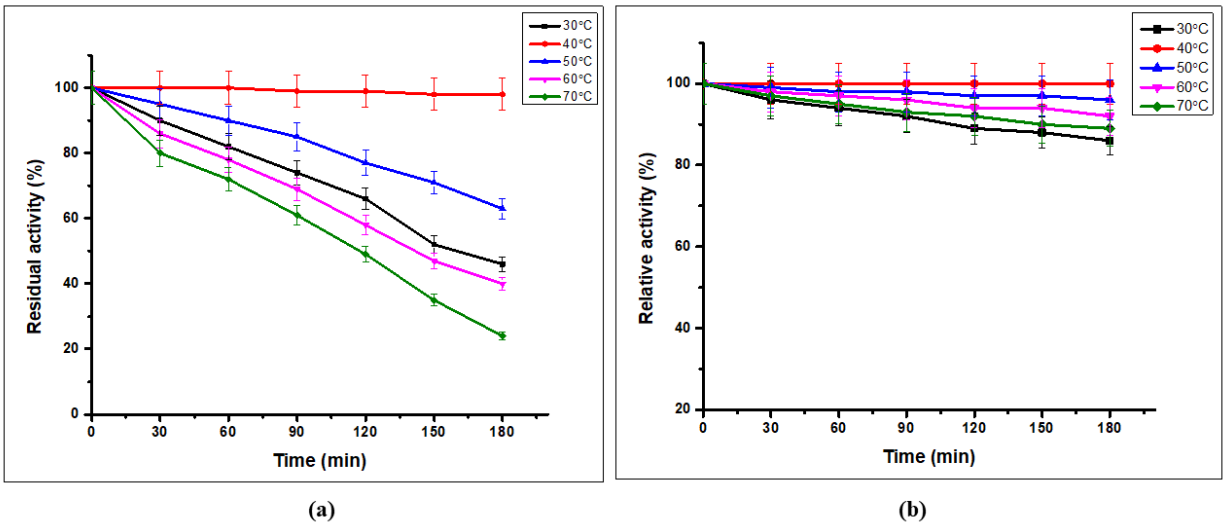

Figure 1 Effect of different temperatures as a function of time on the activity of (a) soluble β-galactosidase and (b) immobilized β-galactosidase.

The decrease in the catalytic activity of the native enzyme at higher temperatures can be imputed as enzyme deactivation at these temperatures. Initially, when the enzyme was incubated at higher temperatures, not much loss in activity was noted because the interactions had weakened during the incubation time. However, the kinetic energy of the molecules increased with time, which raises the rotation about the bonds resulting in the shifting of the catalysis-associated amino groups from their location. Consequently, the enzyme is inactivated at higher temperatures [30].

Similarly, at lower temperatures, the kinetic energy is significantly reduced, which decreases the number of effective collisions between the enzyme and the substrate, resulting in less enzyme activity. Thermal inactivation profiles of β-galactosidase derived from different microorganisms have been extensively studied previously. *Aspergillus nidulans*–derived β-galactosidase maintained stability at 40°C, and 100% activity was preserved even after 2 h of incubation. However, the activity was reduced by 40% and 95% at 50°C and 60°C after 2 h of incubation, respectively [31]. Recombinant β-galactosidase from *Bacillus licheniformis* KG9 displayed high stability at 60°C after 120 min of incubation [32]. Immobilized β-galactosidase showed enhanced thermal stability, with almost 100% activity being preserved at 40°C even after 3 h of incubation (Figure 1b). At 50°C and 60°C, the immobilized enzyme had 96% and 92% activity preserved °°when incubated for 3 h, respectively. Similarly, the immobilized β-galactosidase had approximately 86% and 89% activity preserved when incubated at 30°C and 70°C, respectively. The reason for the stability of the bound enzyme is that immobilization limits the thermal movement of the enzyme even at high temperatures [33]. The firm binding of the enzyme provides rigidity to the enzyme structure, which prevents structural changes in the active site of the enzyme. Lipase immobilized on multi walled carbon nanotube (MWCNT) showed improvement in thermostability when incubated at 60°C, 70°C, and 80°C compared with that of the native enzyme subjected to similar experimental conditions [34].

Effect of pH as a function of time. The soluble/native enzyme demonstrated high stability at pH 4.5, wherein almost 98% activity was preserved after 3 h of incubation, whereas at pH 4.0 and pH

5.0, 70% and 74% activity was preserved, respectively. However, enzyme activity dropped rapidly in buffers of pH 6.0 and pH 7.0, wherein 65% and 62% activity was preserved, respectively (Figure 2a).

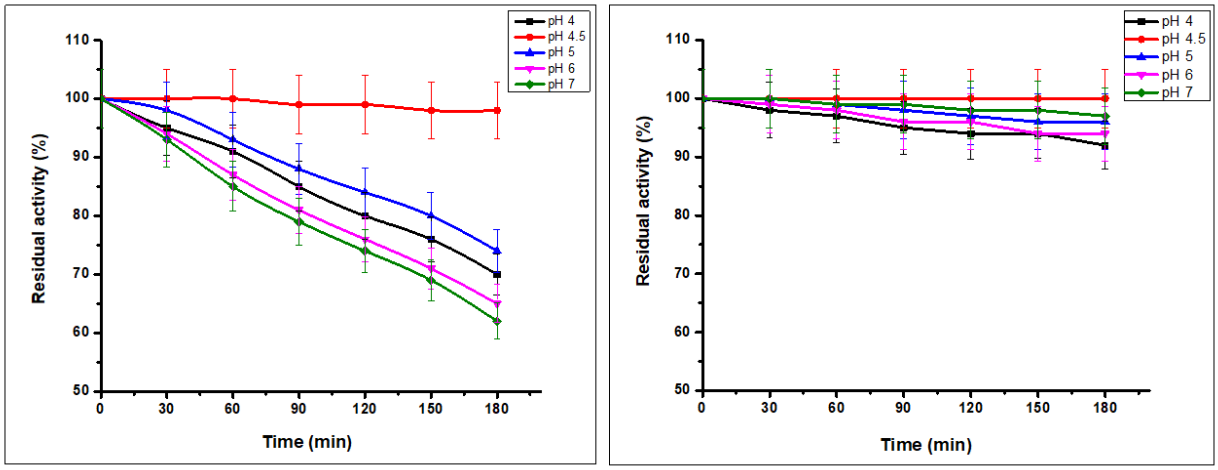

Figure 2 Effect of different ionic strengths (pH) as a function of time on the activity of (a) soluble β-galactosidase and (b) immobilized β-galactosidase.

Partially purified *Teratosphaeria acidotherma* AIU-BGA-1 showed stability in a pH range of 1.5–7.0, with 60% activity being preserved [35]. Similarly, *Aspergillus niger* β-galactosidase was stable at pH 5.0 when incubated at 50°C for 15 h [36]. However, the immobilized β-galactosidase displayed 100%, 95%, 92%, 94%, and 97% activity at pH 4.5, 5.0, 4.0, 6.0, and 7.0, respectively, and 95% activity after 3 h of incubation (Figure 2b).

Thus, the immobilized enzyme exhibited enhanced stability when incubated in buffers of different pH values. The possible explanation for the improved stability of the bound enzyme is the strong binding between the enzyme and the support, which enables the enzyme to resist any conformational changes in different ionic concentrations. Negligible changes in the activity of the bound β-galactosidase, when compared with those in the soluble enzyme, were observed when the enzyme was incubated in buffers of pH 2, 4.5, and 7 for 3 h [37]. Past research has shown the high stability of the immobilized enzymes in buffers of different pH values [23, 27, 38].

Effect of additives. Figure 3 shows the influence of various additives on the activity of soluble/native and bound β-galactosidase. EDTA is a common metal ion chelator. Therefore, in this research, the effect of EDTA on the activity of free and bound β-galactosidase was studied. A slight change in enzyme activity was noted in the presence of 0.1 mM and 1 mM EDTA. However, the activity of the free enzyme dropped drastically in the presence of 10 mM EDTA. The decrease in enzyme activity to 60.7% was because EDTA destabilizes β-galactosidase by forming complexes with Mg^{2+} ions. The enhancement in β-galactosidase activity in the presence of Mg^{2+} ions has already been reported earlier [27]. Therefore, reduced enzyme activity in the presence of EDTA suggests that β-galactosidase is a metalloenzyme. The sharp drop in the activity of β-galactosidase derived from *Lactobacillus reuteri* was observed in the presence of 10 mM EDTA [39].

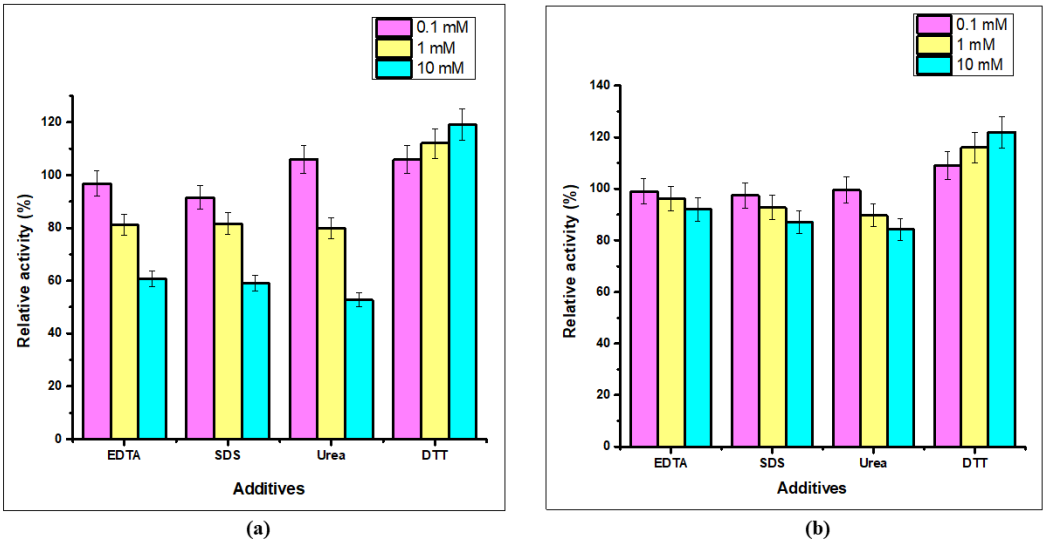

Figure 3 Effect of different concentrations of additives on the activity of (a) soluble and (b) immobilized β-galactosidase.

The negative effect of EDTA was seen in purified alkaline phosphatase from *Thermus caldophilus*, with its activity having reduced to 41% in the presence of 2 mM EDTA [40]. Past studies have shown the inhibitory effect of EDTA on the activity of xylanases [41, 42]. The activity of cutinase derived from *Fusarium verticillioides* was reduced by 31% in the presence of 5 mM EDTA [43]. The immobilized enzyme was significantly more stable in the presence of 10 mM EDTA and had 92% activity preserved. This is due to the restricted accessibility of EDTA to the Mg^{2+} ions bound by the immobilized enzyme.

SDS is an anionic detergent that forms complexes with proteins. The relationship between the relative activity of β-galactosidase and SDS concentration is shown in Figure 3. A decline in the residual enzyme activity was noted with a gradual increase in SDS concentration. For the free enzyme, 91.5% and 81.6% activity was preserved in the presence of 0.1 mM and 1 mM SDS, respectively. With further increase in SDS concentration to 10 mM, the residual activity reduced to 59.1%. This indicates a significant change in the active site conformation with increasing concentration, resulting in the inactivation of β-galactosidase. A remarkable decline was noted in xylanase activity in the presence of 5 mM SDS [14]. Furthermore, 2 mM SDS had a negative impact on the catalytic activity of alkaline phosphatase, with its activity dropping to 11% [40]. However, the immobilized enzyme had 87.1% activity preserved in the presence of 10 mM SDS.

Urea plays a vital role in protein folding or unfolding. Milk is often adulterated with urea and other compounds. Therefore, the influence of urea on the catalytic activity of soluble and bound β-galactosidase was estimated at varying concentrations of urea. Although urea is known to cause enzyme denaturation, it mediated the activation of purified β-galactosidase at a concentration of 0.1 mM, and 106% activity was preserved. However, a further increase in the urea concentration from 1 mM to 10 mM led to a decrease in enzyme activity from 79.8% to 52.7%. The most probable reason for this increase in β-galactosidase activity is the unfolding of the tertiary structure at a lower concentration of urea, and some of the buried active sites become exposed to the substrate, which leads to higher activity. Nevertheless, at higher urea concentrations, the enzyme structure further becomes unfolded, thereby undergoing denaturation. Nguyen et al. [39] reported a decline in the

β-galactosidase activity when the urea concentration was increased from 1 mM to 10 mM. The immobilized enzyme had 84.2% activity preserved at 10 mM, suggesting that immobilization promotes strong binding of the enzyme to the support, which prevents urea-induced unfolding of its structure.

DTT is a sulfhydryl group–containing reagent widely used in molecular biology assays for enzyme stabilization. DTT is used for maintaining the cysteine-containing protein in its active state. At 0.1 mM concentration, the enzyme activity did not show much change. However, at a high DTT concentration (1 mM and 10 mM), the enzyme had immense catalytic activity, i.e., 112% and 119% activity, respectively. This indicates that DTT confers protection to the enzyme from loss of activity due to oxidation of the sulfhydryl groups. Furthermore, enhancement in the activity of β-galactosidase derived from other sources in the presence of DTT has already been reported previously. Yalaz et al. [44] also reported the concentration-dependent stimulatory effect of DTT on β-galactosidase activity. An enhancement in catalytic activity to up to 172% was observed for alkaline phosphatase in the presence of 2 mM DTT [40]. Moreover, 1 mM DTT induced a threefold increase in the activity of alkaline phosphatase derived from *Thermus caldophilus* [45]. The immobilized β-galactosidase had 122% activity preserved in the presence of 10 mM DTT.

3.6.3 Structural Studies

UV-visible spectroscopy. To determine the influence of these additives on the secondary structure of the enzyme, UV-vis spectroscopy was performed.

In the presence of 0.1 mM EDTA, the spectra of the native enzyme did not show much change when compared with those of the control. However, with increasing EDTA concentration, a slight increase in A_{max} (absorption intensity) was seen (Figure 4a). This indicates that the aromatic amino acid residues are exposed to the solvent, which results in increased absorbance. On the contrary, the immobilized enzyme had significantly fewer changes in the absorption spectra, indicating the inaccessibility of EDTA to chelate metal ions, thereby preventing any conformational changes in the enzyme (Figure 4b).

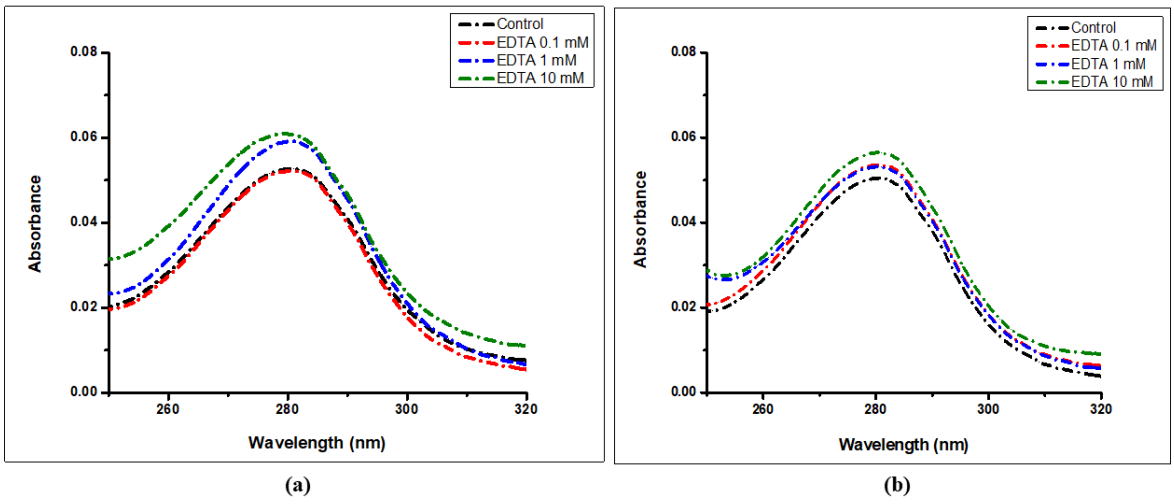

Figure 4 UV-visible spectroscopy of (a) soluble/native enzyme and (b) immobilized enzyme in the presence of different concentrations of ethylenediaminetetraacetic acid (EDTA; 0.1 mM, 1 mM, and 10 mM).

Figure 5 shows the changes in the UV-vis spectra of free and NC-bound enzymes in the presence of SDS. At a lower concentration (0.1 mM) of SDS, a minor change in the spectra was observed. However, with increasing SDS concentration from 1 mM to 10 mM, the A_{max} value increased. This is due to the enzyme denaturation at high SDS concentrations, which unwinds the enzyme structure and exposes the aromatic residues, leading to higher absorbance.

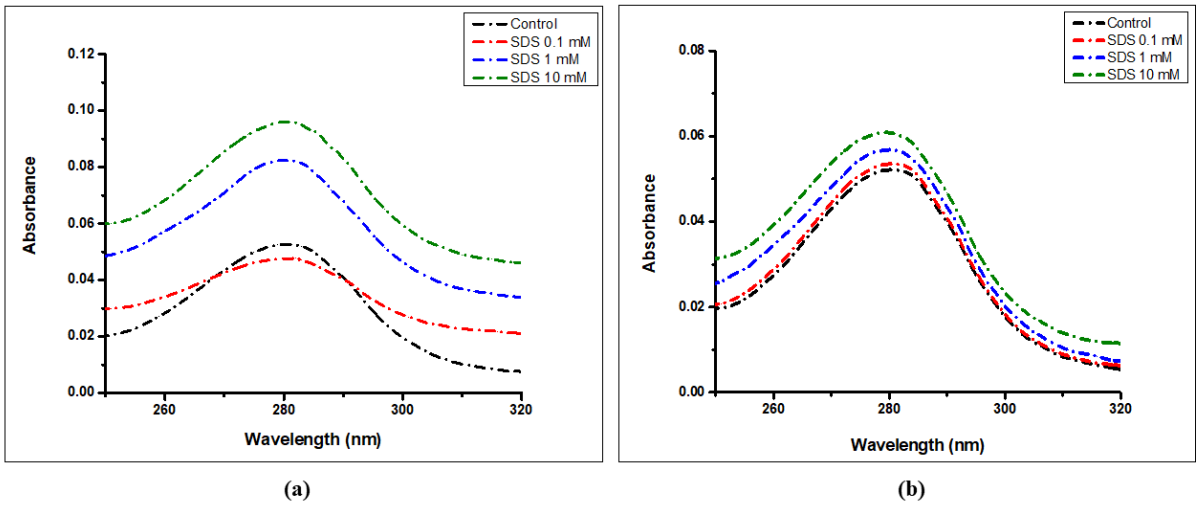

Figure 5 Ultraviolet-visible spectroscopy of (a) soluble/native enzyme and (b) immobilized enzyme in the presence of different concentrations of sodium dodecyl sulfate (SDS; 0.1 mM, 1 mM, and 10 mM).

The effect of urea on the structure of the soluble/native and NC-bound forms of β-galactosidase was evaluated at different urea concentrations (Figure 6). At 1 mM concentration, a slight increase in A_{max} was noted when compared with that of the control, which suggests restricted unfolding of the enzyme structure at lower concentrations and hence less exposure of the aromatic amino acid residues to the solvent. However, at a higher concentration (10 mM), a significant increase in A_{max} was noted, which confirms the unfolding/denaturation of the enzyme and interaction of the solvent with the aromatic amino acid residues, showing higher absorbance.

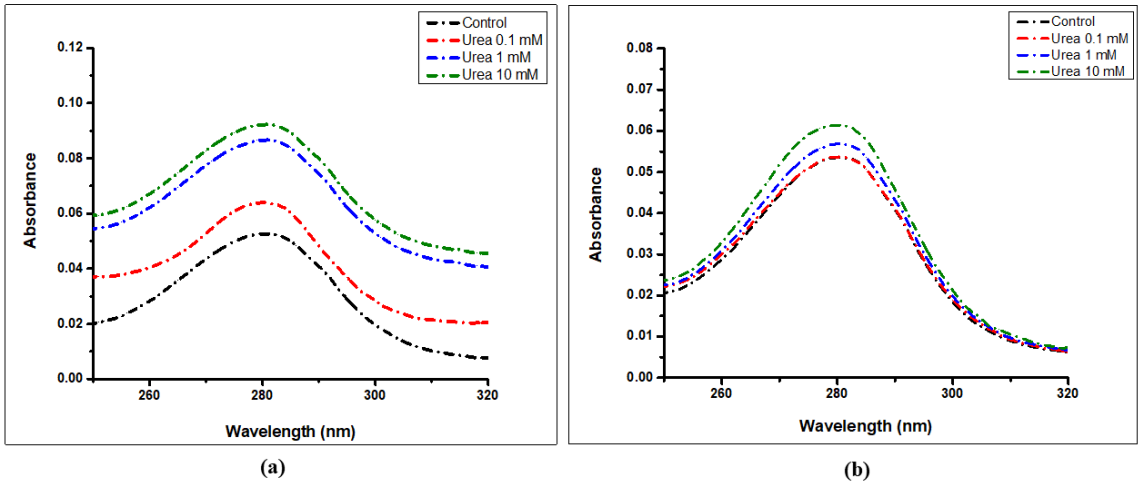

Figure 6 Ultraviolet-visible spectra of (a) soluble enzyme and (b) immobilized enzyme in the presence of different concentrations of urea (0.1 mM, 1 mM, and 10 mM).

Figure 7 shows DTT-dependent conformational changes in soluble and bound β-galactosidase. The A_{max} value decreased with increasing DTT concentrations. The enzyme forms a more compact structure with increasing DTT concentrations; hence, the solvent does not come in contact with the aromatic amino acid residues. This suggests that DTT acts as a stabilizing agent for the β-galactosidase structure.

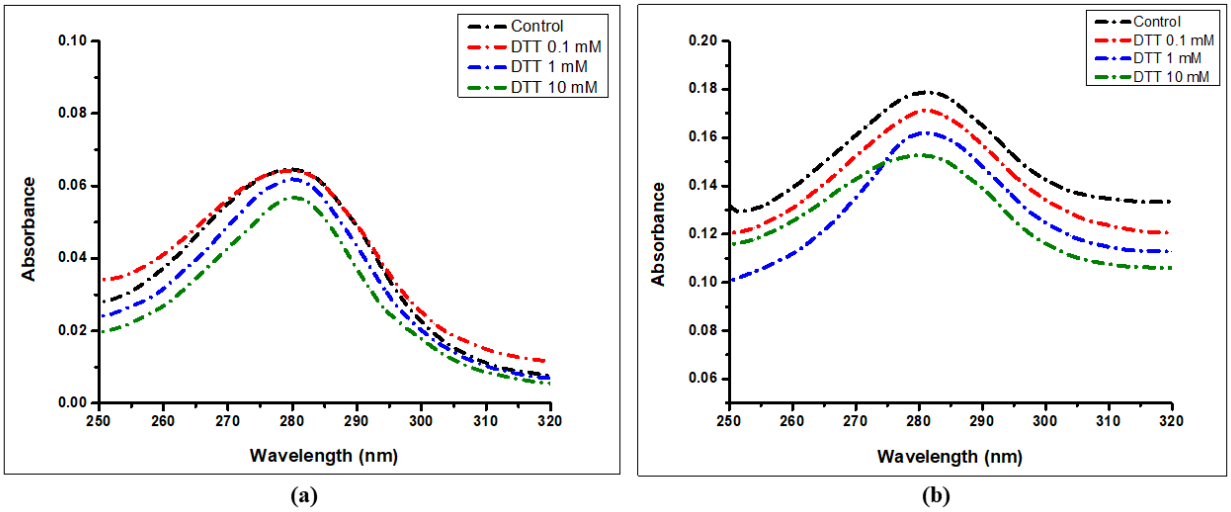

Figure 7 Ultraviolet-visible spectroscopy of (a) soluble enzyme and (b) immobilized enzyme in the presence of different concentrations of dithiothreitol (DTT; 0.1 mM, 1 mM, and 10 mM).

Intrinsic fluorescence spectroscopy. To further support our data and to determine the changes in the tertiary structure of β-galactosidase, intrinsic fluorescence spectra were recorded for the enzyme preparations (soluble/native and immobilized forms) in the presence of the additives.

No peak shift was observed at 0.1 mM and 1 mM EDTA concentrations, and a slight change was seen in the fluorescence intensity (I_{max}). However, at 10 mM concentration, EDTA chelates the metal ions, which causes a marginal alteration in the enzyme's conformation, as evident by the decrease in I_{max} (Figure 8). A slight change in the enzyme's conformation occurred in regions where the Trp residues might have come in contact with the solvent, and quenching in fluorescence was noted. Because immobilization limits, the accessibility of EDTA, no change in I_{max} was recorded in the immobilized enzyme when compared with that of the control. Slight changes in the conformation of *Aspergillus aculeatus*–derived endoglucanase were observed by far UV–circular dichroism and intrinsic fluorescence spectroscopy in the presence of increasing concentrations of EDTA [46].

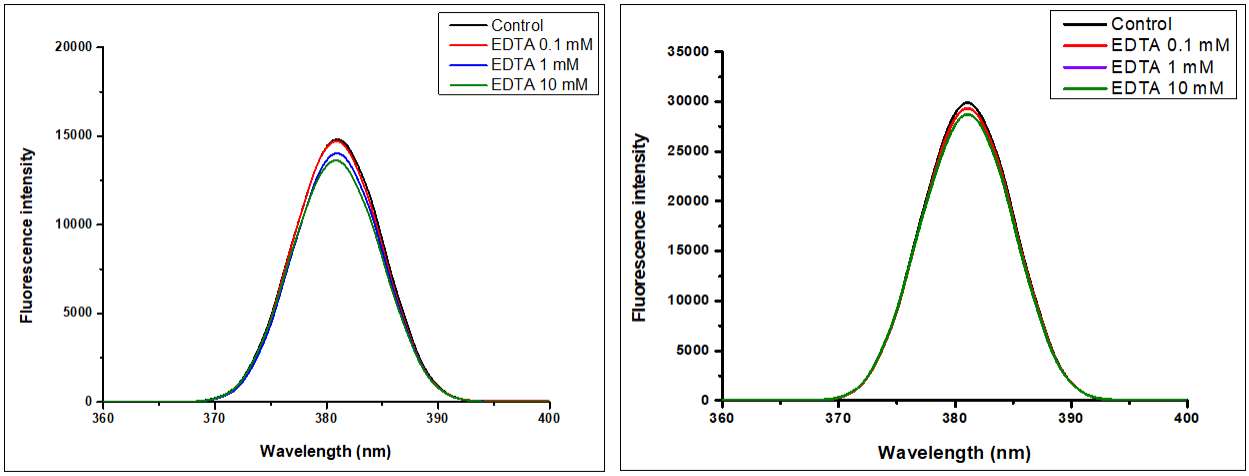

Figure 8 Emission spectra of (a) soluble enzyme and (b) immobilized enzyme in the presence of different concentrations of ethylenediaminetetraacetic acid (EDTA; 0.1 mM, 1 mM, and 10 mM).

In the presence of 0.1 mM and 1 mM SDS, a slight alteration in the fluorescence spectra (I_{max}) was noted; increasing the SDS concentration to 10 mM drastically influenced the I_{max} value when compared with that of the control (Figure 9). Thus, the decrease in I_{max} is attributed to the change in the enzyme's tertiary structure, wherein the Trp residues are exposed to the solvent, resulting in quenching of the fluorescence. This result suggests that the hydrophobic and charged residues present around the active site are essential for maintaining the enzyme's conformation. Denaturation and inactivation of xylanase have been reported to occur because of SDS-induced conformational changes, eventually leading to a decrease in enzyme activity [47].

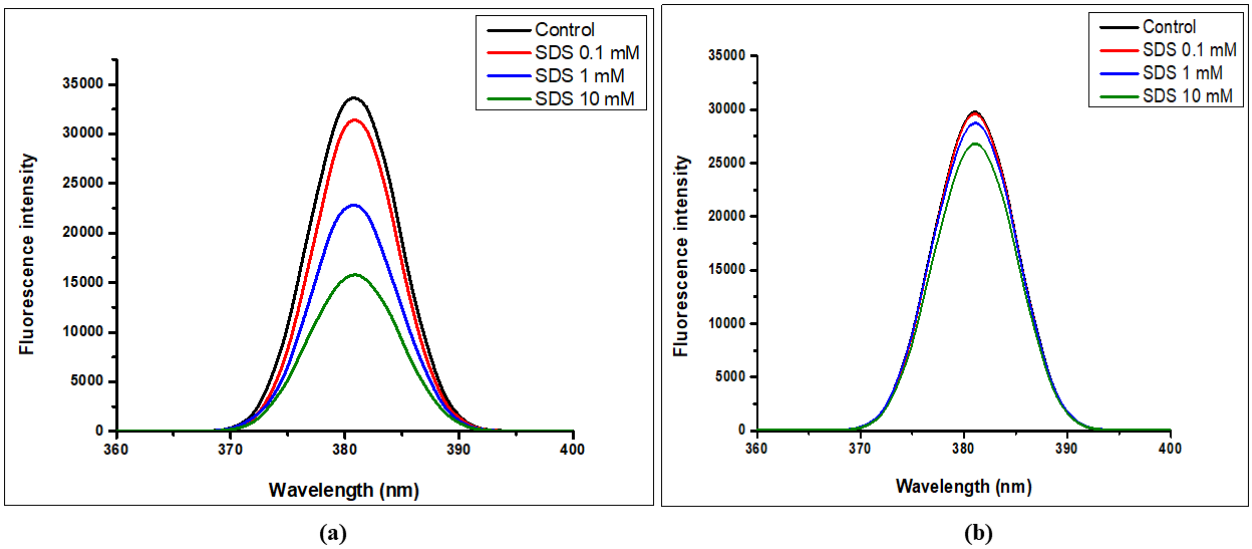

Figure 9 Emission spectra of (a) soluble enzyme and (b) immobilized enzyme in the presence of different concentrations of sodium dodecyl sulfate (SDS; 0.1 mM, 1 mM, and 10 mM).

Figure 10 shows the conformational changes occurring near the Trp residues during urea-induced unfolding of the β-galactosidase structure. Although no changes were recorded in the peak intensity ([I_{max}]) at 0.1 mM urea, the maximum fluorescence intensity decreased at 1 mM urea when compared with that of the control. With a further increase in the urea concentration to 10 mM, a greater decline in the I_{max} value was seen. This is due to the urea-induced unwinding of the enzyme's structure, which allows the solvent to gain access to the enzyme's active site, leading to fluorescence quenching. The degree of unwinding was much lower in the immobilized enzyme than in the soluble enzyme. Fluorescence quenching was seen for lysozyme, which is attributed to the unfolding of its native structure in different concentrations of urea [48].

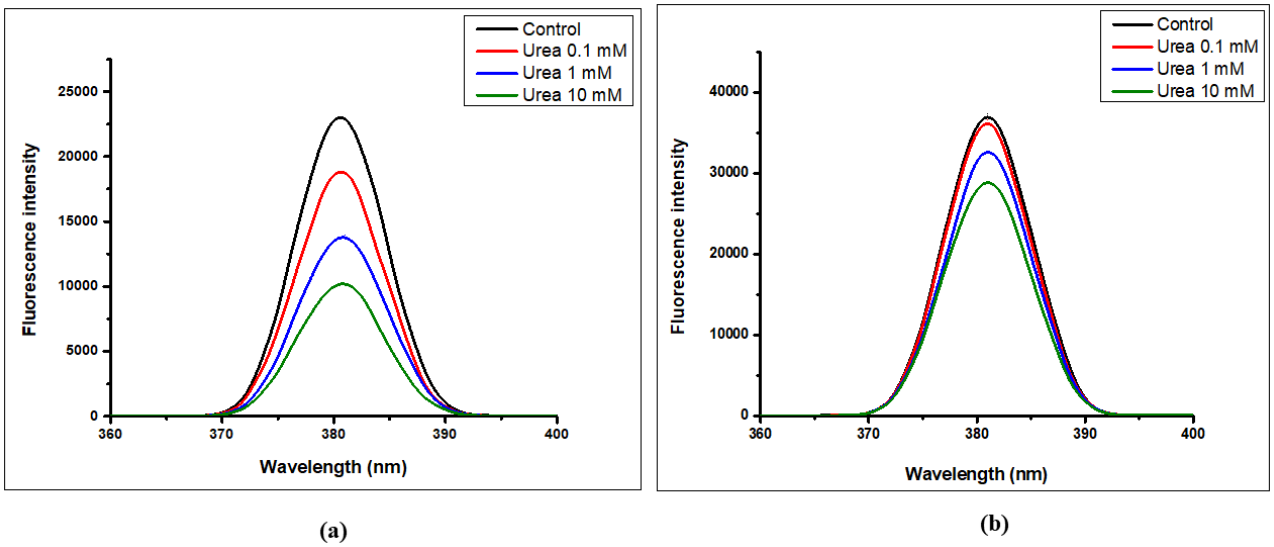

Figure 10 Emission spectra of (a) soluble enzyme and (b) immobilized enzyme in the presence of different concentrations of urea (0.1 mM, 1 mM, and 10 mM).

DTT protects the thiol groups, which stabilize the β-galactosidase structure. In the presence of 1 mM and 10 mM DTT, a slight increase in I_{max} was observed for the native enzyme, while the fluorescence intensity of the intrinsic fluorophores remained almost unchanged for the immobilized enzyme (Figure 11). The increase in I_{max} indicates confirmational changes in the enzyme, which causes internalization of the intrinsic fluorophores to the core of the enzyme.

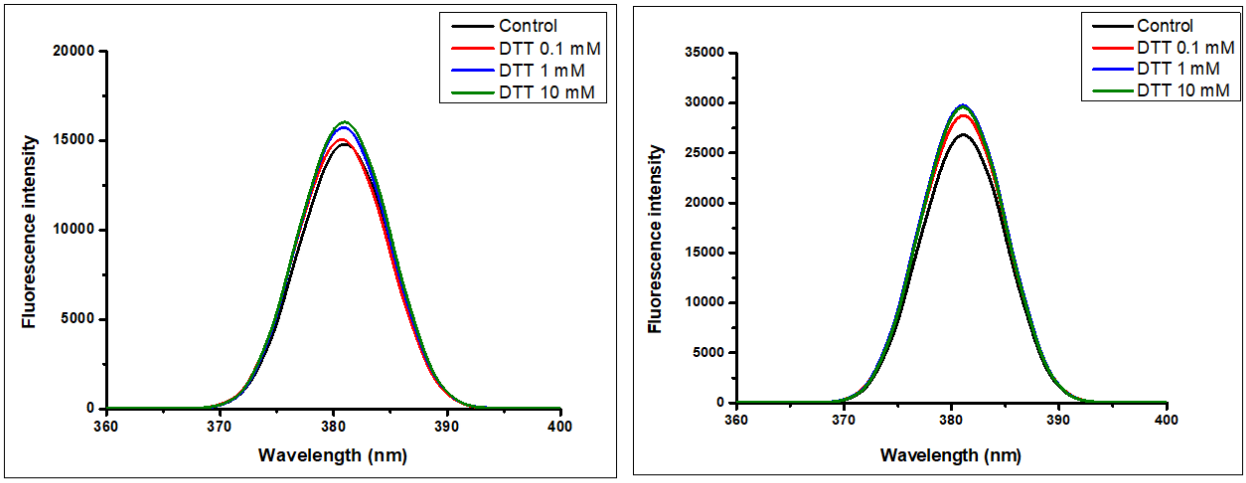

Figure 11 Emission spectra of (a) soluble enzyme and (b) immobilized enzyme in the presence of different concentrations of dithiothreitol (DTT; 0.1 mM, 1 mM, and 10 mM).

3.7 Conclusion

This study demonstrates the structure–activity relationship of β-galactosidase in its soluble and immobilized forms in the presence of different additives. Milk is often adulterated with urea, detergents, and other compounds to increase its shelf life. Hence, the effect of the additives EDTA, SDS, DTT, and urea on the catalytic activity of free and NC-immobilized β-galactosidase was measured. A significant drop in the activity of native β-galactosidase was observed in the presence of EDTA, SDS, and urea. This indicates that the soluble enzyme undergoes unfavorable changes in its secondary and tertiary structures, leading to a loss in its catalytic activity. However, β-galactosidase activity increased in the presence of DTT. Moreover, the immobilized β-galactosidase preparations demonstrated an insignificant loss in catalytic activity and active site conformation in the presence of these additives. The findings of this study provide new insight into the structure-activity relationship of β-galactosidase in the presence of different additives. This NBC can be used in the manufacture of nanobiosensors to detect lactose in dairy products. This study also helps to improve the stability of enzyme in the presence of various reagents which are used at industrial level which will eventually enhance the efficiency of the nanobiocatalyst. However, more techniques and chemicals are required for better analysis of the nano-immobilized enzyme.

Acknowledgments

AS is grateful to the Department of Biochemistry, AMU, Aligarh for conducting spectroscopic studies.

Author Contributions

AS made a substantial contribution to the concept or design of the article along with analysis and interpretation of the data for the article. QH revised the manuscript critically for important intellectual content and approved the version to be published.

Funding

AS is thankful to University Grant Commission (UGC), New Delhi, India, for providing financial assistance through non-net fellowship.

Competing Interests

The authors declare that they have no competing interests.

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