Research article

Biotechnological application of *Aspergillus oryzae* β-galactosidase immobilized on glutaraldehyde modified zinc oxide nanoparticles

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A R T I C L E   I N F O

A B S T R A C T

The current research demonstrates the synthesis of zinc oxide nanoparticles (ZnO-NPs) via green nanotechnology approach (*Azatirachta indica* leaves). The size of the synthesized ZnO-NPs was confirmed as 27 nm by TEM. Glutaraldehyde was used to modify the surface of the developed ZnO-NPs in order to promote covalent binding of *Aspergillus oryzae* β-galactosidase. Enzyme activity was achieved as 93% on glutaraldehyde modified ZnO-NPs. The immobilized enzyme exhibited significant enhancement in activity under extreme temperature and pH variations, as compared to the soluble β-galactosidase (SβG). It was further observed that the immobilized enzyme retained 58% activity at 5% galactose concentration. However, under similar experimental conditions, SβG showed 27% activity. Reusability of immobilized enzyme revealed that it retained 89% activity even after fifth repeated use, and hence could be recovered easily by centrifugation for repeated use in biotechnological applications. Batch reactor experiment indicates that the immobilized enzyme displayed 81% and 70% lactose hydrolysis at 50 °C and 60 °C, respectively as compared to 70% and 58% lactose hydrolysis by soluble enzyme under identical conditions after 9 h.

1. Introduction

The commercial production of lactose-free commodities is of utmost importance due to the high prevalence of "lactose intolerant" population globally [1]. *β*-Galactosidase is specifically used for hydrolyzing the lactose into its basic components i.e. glucose and galactose. This property represents its promising application in food processing and dairy industries [2,3]. *β*-Galactosidase from *Aspergillus oryzae* is monomeric, having molecular weight of 105 kDa and a pI of 4.6. This enzyme showed pH optima of 4.5 with *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) and 4.8 with lactose [4]. Enzymatic hydrolysis of lactose is a popular technology used to produce lactose-free dairy products for consumption by lactose intolerant individuals whose metabolism exhibited a decline in the level of *β*-galactosidase activity [5].

The soluble form of enzyme is exploited for this purpose. However, the immobilized enzyme can be benefitted in continuous process and in batch operation. Moreover, the immobilization process makes the enzyme thermostable as well as reusable. Additionally, the enzymes become more resistant to harsh environmental variations as a result of immobilization [6–8]. Apart from these properties, immobilization imparts improved stability in biomedical and biotechnological applications. Highly efficient biocatalysts are therefore continuously developed to improve the enzyme stability protocols for producing lactose-free dairy products [9]. Due to

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limited scope of conventional biocatalysts, nanotechnology paves way by involving nanoparticles as carriers for enzyme immobilization. The drawbacks associated with conventional biocatalysts like long reaction time, mass transfer resistance, catalyst deactivation and poisoning (refers specifically to chemical deactivation rather than other mechanisms of catalyst degradation like thermal decomposition or physical damage) can be outstripped by the application of nanoparticles. Nanobiocatalysts improve the catalytic activity of enzyme by increasing surface to volume ratio for immobilization, thereby favoring enzyme loading in higher amount. It also imparts stability and reusability to the immobilized enzyme. The simplified downstream processing or continuous process operations and easy separation (soluble enzymes can contaminate the product and their removal may involve extra purification costs) from the reaction mixture are other cost-effective advantages offered by immobilization [10–14].

Recent years further witnessed the application of modern tools for obtaining the conditioned and high quality optimized nano-biocatalyst systems [15]. Green nanotechnology is one such promising technology that utilizes eco-friendly approach of using plant extracts to manufacture nanoparticles [16,17]. This procedure restricts the use of harmful and toxic chemicals which further extend their scope in diverse biological applications. Moreover, surface functionalization of nanoparticles favors covalent attachment of enzyme. It presents an advanced approach of retaining enzymes in excellent yield apart from avoiding the time consuming and expensive downstream processes required for enzyme immobilization [18–20].

Azadirachta indica (commonly known as neem) is a medicinal plant. It contains phytochemicals namely terpenoids and flavanones, which act as reducing as well as capping agent required for the stabilization of nanoparticles [21].

Hence, efforts were raised in the present study to synthesize ZnO-NPs by using neem leaves as a green nanotechnology based approach. The synthesized ZnO-NP was characterized by TEM. The surface of the ZnO-NPs was modified by glutaraldehyde to favor covalent mode of immobilization of *Aspergillus oryzae* β-galactosidase, for obtaining higher activity yield. Effect of various physical and chemical denaturants on the activity of soluble β-galactosidase (SβG) and glutaraldehyde modified ZnO-NPs (IβG) has been investigated. The effect of product inhibition by galactose on SβG and IβG was monitored while reusability of immobilized enzyme was also investigated. Moreover, lactose hydrolysis experiment was performed by the immobilized as well as soluble enzyme in batch process in stirred-batch reactors for 10 h at 50 °C and 60 °C.

2. Experimental

2.1. Materials

Aspergillus oryzae β-galactosidase, ONPG and zinc acetate dihydrate was obtained from Sigma Aldrich, USA. *Azadirachta indica* (neem) leaves were collected from nearby garden. All reagents were used without further purifications.

2.2. Synthesis of zinc nanoparticles (ZnO-NPs) by neem leaves and characterization by TEM

The leaves of neem *(Azadirachta indica)* were washed with distilled water three times. An aqueous extract was obtained by boiling fresh neem leaves (25 g) by boiling in glass beaker (250 ml). The process was extended by boiling the neem leaves at 60 °C in double distilled water (100 ml) for 20 min. The formation of aqueous solution was confirmed by the appearance of brown color. This extract was kept at room temperature for cooling and then filtered by Whatman filter paper 1. This extract was stored in a refrigerator to pursue the subsequent experiments [17].

The synthesis of ZnO-NP involves the mixing of neem leaf extract (25 ml) and 1 M $Zn(CH_3CO_2)_2 \cdot 2H_2O$ (25 ml) in 1:1 ratio. The solution was maintained at pH 7.0 by adding sodium hydroxide (0.5 M) dropwise at room temperature. This step leads to precipitate formation. The precipitate obtained was filtered followed by repeated washing with water, followed by ethanol for removing the remaining impurities. The resulting material was put in an oven (60 °C) overnight for drying, grounded to fine powder. Finally, calcination was performed (400 °C) for 1 h in Muffle furnace under standard conditions. JEOL JEM-2100F transmission electron microscope was used to evaluate the size of ZnO-NPs obtained by dropping the nanoparticle solution on carbon-coated copper grids at a 15 kV voltage under normal atmospheric conditions.

2.3. Surface modification of ZnO-NP by glutaraldehyde and immobilization of β-galactosidase on surface modified ZnO-NPs

ZnO-NPs obtained above were suspended in glutaraldehyde (0.5 M) for 4 h in a shaker at 200 rpm. Table top centrifuge was used to obtain the modified nanomatrix [21]. This step was followed by repeated washing with distilled water to remove traces of glutaraldehyde which were finally washed with assay buffer to remove any impurities (if present) to perform our experiments [22].

Glutaraldehyde modified ZnO-NPs (1 g) were mixed with β-galactosidase (2000 U) overnight (12 h) with slow stirring in 0.1 M sodium acetate buffer, pH 4.5 (assay buffer) at room temperature. Enzyme conjugated to the modified nanosupport was obtained by centrifugation (2000 rpm) for 20 min. Enzyme-nanoparticle conjugate obtained was centrifuged thrice with the assay buffer to remove even the minor impurities from the complex. Finally, the immobilized enzyme was stored at 4 °C for analyzing the enzyme stability.

2.4. β-Galactosidase assay

One unit of β-galactosidase activity is defined as the amount of enzyme that releases 1.0 μmole of *o*-nitrophenol/min under standard assay conditions [23]. Two milliliter reaction volume containing 1.79 ml of 0.1 M sodium acetate buffer (pH 4.5), 0.01 ml β-galactosidase (equivalent to 2.0 U) and 0.2 ml of ONPG (2.0 mM) was continuously stirred (200 rpm) at 50 °C for 15 min. The

reaction was terminated by 2 ml sodium carbonate solution (1 M) and product formed was measured at 405 nm (UV–Vis near-infrared spectrophotometers).

2.5. Enzyme activity at varying pH and temperature ranges

Enzyme activity of soluble and immobilized β-galactosidase preparations (20 μL) was assayed in the 0.1 M buffers of different pH (pH 3–7) at 50 °C. The used buffers were glycine-HCl (pH 3.0), sodium acetate (pH 4.0–6.0) and Tris-HCl (7.0). The activity expressed at pH 4.5 was considered as control (100%) for the calculation of remaining percent activity.

Effect of temperature on soluble and immobilized β-galactosidase preparations (20 μL) was studied by measuring their activity at various temperatures (30–70 °C) in 0.1 M sodium acetate buffer (pH 4.5). The activity expressed at 50 °C was considered as control (100%) for the calculation of remaining percent activity.

2.6. Galactose mediated product inhibition

The effect of various concentrations of galactose (1–5%, w/v) on the activity of soluble and immobilized β-galactosidase preparations (20 μL) was measured independently in 0.1 M sodium acetate buffer, pH 4.5 at 50 °C. The activity of enzyme without added galactose was considered as control (100%) for calculating the remaining percent activity.

2.7. Reusability study

Reusability of immobilized enzyme (100 μL) was performed for analyzing the stability of the obtained preparation. Assay buffer was used for washing the enzyme and the precipitate was obtained by centrifugation (2000 rpm for 10 min). The protocol was repeated for five successive days. The activity after every reuse was analyzed in proportion to the activity determined on first day (which was considered as 100%).

2.8. Batch conversion of lactose

Lactose solution (250 ml, 0.1 M) was stirred continuously with SβG and immobilized enzyme (250 U) at 50 °C and 60 °C in water bath for 10 h. The aliquots were drawn after every hour and assayed for the formation of glucose by glucose oxidase-peroxidase assay kit.

2.9. Statistical analysis

All the experiments were performed in triplicates. Sigma Plot-9 was used for data expression. P-values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Significance of green nanotechnology for synthesis of nanoparticles

Green nanotechnology is continuously inspiring the researchers to synthesize nanoscaled materials with improving chemical and physical properties by novel approaches. The goal of using this technique is to protect the environment from negative effects by using innocuous solvents and additives for synthesizing nanoparticles [24]. This is an eco-friendly approach to utilize plant extracts for
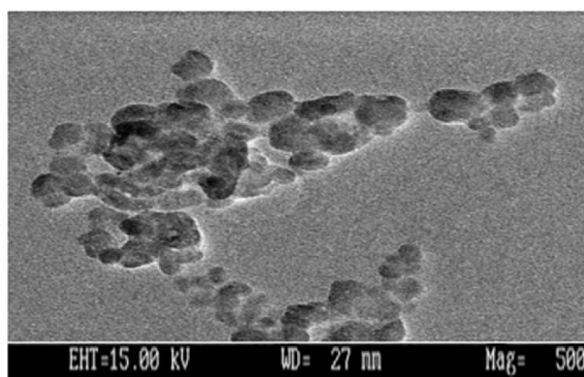

Fig. 1. TEM of ZnO nanoparticles. The size of synthesized ZnO-NPs was estimated by JEOL JEM-2100F transmission electron microscope by dropping the nanoparticle solution on carbon-coated copper grids at a 15 kV voltage under normal atmospheric conditions.

formulating nanoparticles which makes their application feasible in plethora of biological applications [25–28]. Hence, ZnO-NPs were prepared from neem leaves extract followed by their surface modification by glutaraldehyde. β-Galactosidase was immobilized on the developed nanosystem to suggest their possible exploitation in dairy industries for producing lactose-free products.

3.2. Characterization of ZnO-NPs

ZnO-NPs synthesized by above approach exhibited yellowish-brown color. Their size was confirmed as 27 nm by transmission electron micrographs [Fig. 1].

3.3. Surface modification of ZnO-NPs by glutaraldehyde and immobilization of β-galactosidase on the developed matrix

Importance of glutaraldehyde in obtaining highly stable biocatalysts has been excellently reviewed earlier in detail by Ansari and co-workers [29]. Moreover, it was recently observed by Ranjbari and coworkers that immobilization of *Penaeus vannamei* protease on glutaraldehyde activated graphene oxide nanosheets improved its characteristics and performance in biotechnological application [30]. In pursuit of this, β-galactosidase was immobilized on the surface of glutaraldehyde modified ZnO-NPs via covalent attachment [Fig. 2]. The modified nanomatrix exhibited 93% enzyme activity [Table 1] which makes its implementation cost-effective in the suggested biotechnological application. Moreover, it was observed by several investigators that immobilization of β-galactosidase on tannic acid stabilized AgNPs and chitosan-coated magnetic nanoparticles exhibited 83% and 98% enzyme activity yield, respectively [31,32].

3.4. Enzyme activity at varying pH and temperature ranges

Minor change in the enzyme tertiary structure is an indication of its diminished catalytic activity. When an enzyme loses its tertiary structure, it can no longer perform its function because of denaturation and loss in its biological activity [33]. Hence, enzyme immobilization is suggested to prevent its distortion and hence its catalytic activity. Fig. 3 showed pH profile of soluble and immobilized enzyme. The immobilized enzyme exhibited remarkable increase in its activity as compared to the native enzyme at varying pH ranges. It could be explained by the fact that covalent mode of enzyme immobilization prevented the deformation in the structure of β-galactosidase resulting due to basic and acidic environment. Similarly, temperature-activity profile exhibited 62% and 90%, enzyme activity for soluble and immobilized enzyme, respectively at 60 °C [Fig. 4]. It is noteworthy to mention that denaturation of β-galactosidase at greater temperatures results in significant downshift in enzyme activity which poses a major drawback and hence limits their application in biotechnological sectors.
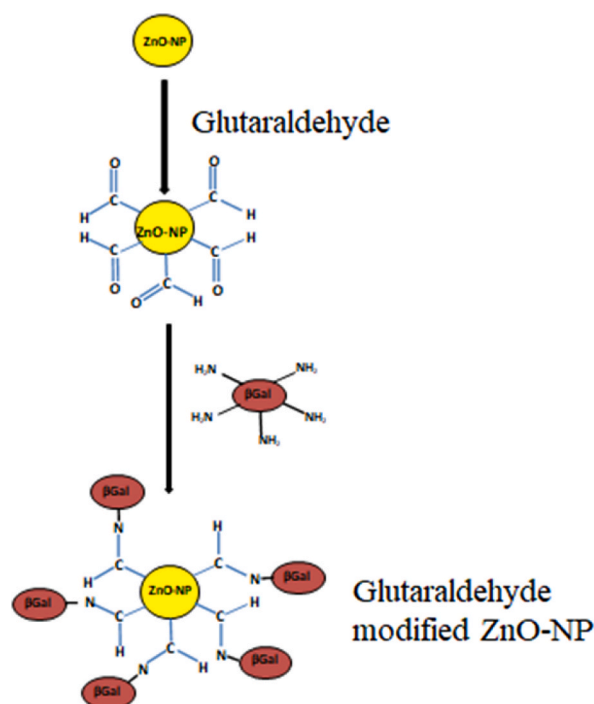

Fig. 2. Surface modification of ZnO-NPs by glutaraldehyde and immobilization of β-galactosidase on the developed nanomatrix. A step by step procedure demonstrating the surface functionalization of ZnO-NPs by glutaraldehyde (0.5 M) for 4 h followed by covalent attachment of β-galactosidase in 0.1 M sodium acetate buffer in slow stirring conditions at room temperature.

Table 1

Immobilization yield.

Enzyme activity loaded (X Units)	Enzyme activity in washes (Y Units)	Activity bound on glutaraldehyde modified ZnO-NPs		Activity yield (%)
		Theoretical (X–Y) = A	Actual = B	B/A × 100
2000	354	1646	1530	93 ± 2.3

Each value represents the mean for three independent experiments performed in triplicates, with average standard deviations, <5%.
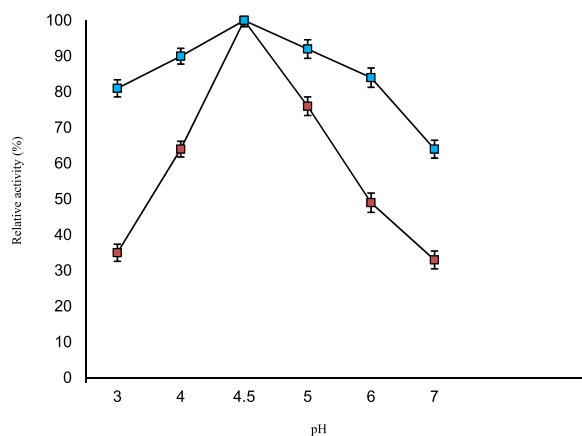
Fig. 3. pH-activity profiles. Activity of soluble and immobilized β-galactosidase (20 μL) was measured at 50 °C in different pH buffers (3.0–7.0) as mentioned in the text. Activity at pH 4.5 was taken as control (100%) for calculation of remaining percent activity. Enzyme activity was determined as described in the text. Symbols show (■) soluble and (■) immobilized β-galactosidase.
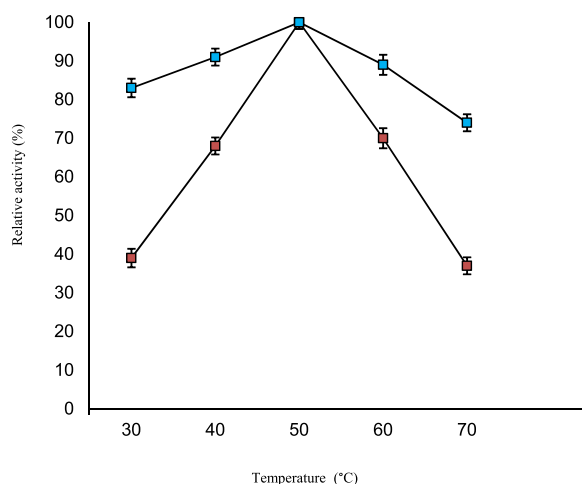
Fig. 4. Temperature-activity profiles. Activity of soluble and immobilized β-galactosidase (20 μL) was assayed in 0.1 M sodium acetate buffer, pH 4.5 at various temperatures (30–70 °C) for 15 min. Activity obtained at 50 °C was considered as control (100%) for calculation of remaining percent activity for soluble and immobilized enzyme. For symbols, please refer to figure legends 3.

3.5. Galactose mediated product inhibition

Lactose hydrolysis is affected significantly as a result of decrease in β-galactosidase activity offered by galactose as a product inhibitor [5] and this challenge was encountered positively by covalently attaching the enzyme on glutaraldehyde modified ZnO-NPs. This method of enzyme immobilization might have protected the active sites of β-galactosidase against the action of galactose. Fig. 5 exhibited 60% activity of immobilized enzyme upon 1 h incubation even at higher concentration of galactose (5%) in contrast to the soluble enzyme. These results predicted that the enzyme conjugated to the glutaraldehyde modified ZnO-NPs were resistant to galactose as compared to the soluble enzyme. Higher activity was retained for immobilized enzyme as compared to its soluble form at higher galactose concentration by earlier investigators, which explains the substrate accessibility and hence the efficiency of the
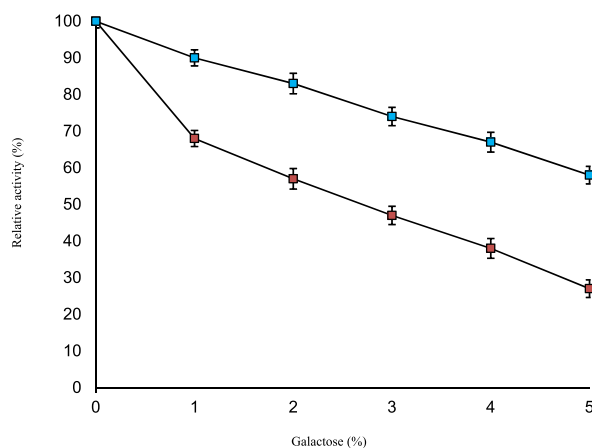
Fig. 5. Galactose mediated product inhibition. Effect of galactose on soluble and immobilized β-galactosidase (20 μL) was measured in the presence of increasing concentrations of galactose (1.0–5.0%, w/v) in 0.1 M sodium acetate buffer, pH 4.5 for 1 h at 50 °C. Activity of enzyme without added galactose was considered as control (100%) for the calculation of remaining percent activity at other concentrations. For symbols, please refer to figure legends 3.

immobilized enzyme in converting lactose in batch reactors [5].

3.6. Reusability studies

Enzyme attached to glutaraldehyde modified ZnO-NPs reflected 97% and 93% of the initial activity after its 3rd and 4th repeated use, respectively [Fig. 6]. Thus, the developed nanomatrix could serve as promising candidates in other biotechnological and biomedical relevance. Kessi and Arias had earlier concluded that β-galactosidase crosslinked on chicken eggshell by glutaraldehyde can be reused several times, thereby making it a good catalyst for dairy industries [34].

3.7. Batch conversion of lactose

Lactose solution was hydrolyzed at two different temperatures in batch process [Table 2]. Greater percent of lactose conversion was obtained by SβG initially in contrast to the immobilized enzyme. Enzyme mechanics explained this fact soluble enzyme was accessible to the substrate in greater amount and hence lead to higher percentage of lactose hydrolysis [27,28]. Moreover, there was a marked decrease in the hydrolysis of lactose for SβG after 5 h as compared to the immobilized enzyme. It is because of the galactose mediated product inhibition. Ansari and Husain [35] have earlier observed that β-galactosidase conjugated to concanavalin A-cellulose promotes 55% lactose conversion in 1 h. The study further revealed that in 5 h, 74% of lactose hydrolysis was achieved by SβG. After 6 h, lactose hydrolysis by SβG show insignificant increase while the lactose conversion by Con A-cellulose enzyme converts 90% lactose in 6 h. Kuribayashi and co-workers also obtained improved results for lactose hydrolysis by *Bacillus licheniformis* β-galactosidase [36]. Lactose hydrolysis results depend on β-galactosidase activity which in turn depends on optimum pH and temperature conditions along with enzyme concentration and processing time [37]. Our study indicated improved conversion of lactose by β-galactosidase conjugated to glutaraldehyde modified ZnO-NPs at 50 °C, owing to its temperature optima and hence its stability was more at 50 °C as compared to 60 °C. It was recently reported that *Kluyveromyces lactis* β-galactosidase immobilized with sodium alginate was able to hydrolyze 46% of the initial lactose content in 6 h [38]. Additionally, Hoppe and coworkers have obtained significant enhancement in the lactose hydrolysis when deep eutectic solvents were used in combination with β-galactosidase [39].

4. Conclusion

Green technology based approach was developed herein to synthesize ZnO-NPs by neem leaves. This step prevents the usage of expensive and toxic reagents. Covalent attachment of β-galactosidase on glutaraldehyde modified ZnO-NPs retained 93% enzyme activity. Moreover, the activity of enzyme immobilized on glutaraldehyde modified ZnO-NPs was significantly enhanced under the influence of harsh physical (higher and lower ranges of pH and temperature) and chemical (galactose mediated product inhibition) conditions. 93% of enzyme activity was retained on the prepared glutaraldehyde modified ZnO-NPs even after 4th repeated use. The batch conversion revealed that 81% of lactose was hydrolyzed even after 9 h by β-galactosidase conjugated to glutaraldehyde modified ZnO-NPs at 50 °C as compared to its 70% at 60 °C. It should be noted that the lactose conversion was more at 50 °C as it represents the temperature optima of this enzyme. It favors its application in biotechnology industries with cost effective advantages and at higher temperature ranges. However, research of the developed nanobiocatalyst is required for analyzing its stability in producing lactose-free dairy products in continuous reactors.
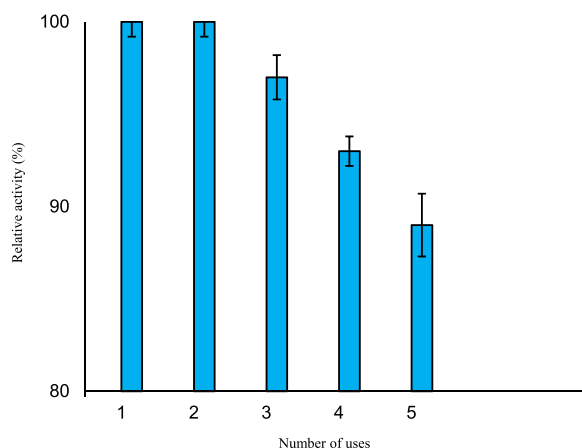
Fig. 6. Reusability studies. IβG (100 μL) was taken in triplicates for six successive days and the activity determined on the first day was taken as control (100%) for calculation of remaining activity after each use.

Table 2
Lactose hydrolysis in batch reactors.

Time (h)	Lactose hydrolysis (%)				
	60 °C			50 °C	
	SβG	IβG		SβG	IβG
Control	0	0		0	0
1	11 ± 2.3	8 ± 2.6		16 ± 4.3	12 ± 2.6
2	18 ± 3.1	17 ± 2.4		25 ± 2.4	32 ± 4.6
3	23 ± 4.6	33 ± 3.1		33 ± 3.4	44 ± 2.8
4	39 ± 1.9	48 ± 4.1		42 ± 3.7	57 ± 2.6
5	44 ± 2.1	51 ± 3.3		49 ± 2.9	65 ± 1.6
6	51 ± 2.3	62 ± 1.2		57 ± 1.3	70 ± 2.6
7	54 ± 3.2	66 ± 2.8		63 ± 1.5	74 ± 4.4
8	58 ± 1.7	70 ± 1.4		66 ± 1.9	74 ± 2.8
9	58 ± 3.5	70 ± 3.7		70 ± 4.3	81 ± 1.6
10	58 ± 2.8	70 ± 2.8		70 ± 3.0	81 ± 3.5

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

Author contribution statement

Shakeel Ahmed Ansari: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ahmed Abdelghany Damanhory: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

SβG	soluble β-galactosidase
IβG	β-galactosidase covalently bound to glutaraldehyde modified zinc oxide nanoparticles
ONPG	ortho-nitrophenyl-β-D-galactopyranoside
ZnO-NPs	zinc oxide nanoparticles

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