

**CARBOXYLATION OF SILVER NANOPARTICLES FOR THE IMMOBILIZATION OF  $\beta$ -GALACTOSIDASE AND ITS EFFICACY IN GALACTO-OLIGOSACCHARIDES PRODUCTION**Shakeel Ahmed Ansari<sup>a,\*</sup>, Rukhsana Satar<sup>b</sup> and Syed Kashif Zaidi<sup>a</sup><sup>a</sup>Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah-21589, Kingdom of Saudi Arabia<sup>b</sup>Department of Biochemistry, Ibn Sina National College for Medical Studies, Jeddah-21418, Kingdom of Saudi Arabia

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The present study investigated the carboxylation of silver nanoparticles (AgNPs) by 1:3 nitric acid-sulfuric acid mixtures for immobilizing *Aspergillus oryzae*  $\beta$ -galactosidase. Carboxylated AgNPs retained 93% enzyme upon immobilization and the enzyme did not leach out appreciably from the modified nanosupport in the presence of 100 mmol L<sup>-1</sup> NaCl. Atomic force micrograph revealed the binding of  $\beta$ -galactosidase on the modified AgNPs. The optimal pH for soluble and carboxylated AgNPs adsorbed  $\beta$ -galactosidase (I $\beta$ G) was observed at pH 4.5 while the optimal operating temperature was broadened from 50 °C to 60 °C for I $\beta$ G. Michaelis constant, Km was increased two and a half fold for I $\beta$ G while Vmax decreases slightly as compared to soluble enzyme.  $\beta$ -galactosidase immobilized on surface functionalized AgNPs retained 70% biocatalytic activity even at 4% galactose concentration as compared to enzyme in solution. Our study showed that I $\beta$ G produces greater amount of galacto-oligosaccharides at higher temperatures (50 °C and 60 °C) from 0.1 mol L<sup>-1</sup> lactose solution at pH 4.5 as compared to previous reports.

Keywords:  $\beta$ -galactosidase; food industry; functionalization; galacto-oligosaccharides; silver nanoparticles.

**INTRODUCTION**

$\beta$ -galactosidase (E.C. 3.2.1.23) is an important enzyme that has attracted the attention of the enzymologists due to its dual nature of producing lactose-free dairy products by catalyzing the hydrolysis of lactose into glucose and galactose, and in producing galacto-oligosaccharides by favoring transgalactosylation reaction when lactose acts as an acceptor.<sup>1,2</sup> An excellent review has appeared lately in which  $\beta$ -galactosidase from various sources including psychrophilic, mesophilic and thermophilic organisms were utilized by previous researchers for obtaining galacto-oligosaccharides and lactose-free dairy products.<sup>3</sup>

Galacto-oligosaccharides (GOS) are non-digestible food ingredients that are obtained from lactose as a result of transgalactosylation reaction catalyzed by  $\beta$ -galactosidase. They benefit the host by selectively stimulating the proliferation of bifidobacteria and lactobacilli in the intestine which are considered to be beneficial to human health.<sup>4-6</sup> Moreover, with the emergence and increase of microbial contamination in the immobilized system and the continuous emphasis on health care costs, many researchers have tried to develop new and effective nanoparticle based  $\beta$ -galactosidase immobilized system that are free of microbial resistance and reduced product inhibition which could facilitate the continuous and long-term processing of the biocatalyst, and ultimately reduce their cost in biotechnology industries.<sup>7,8</sup>

Numerous carriers and technologies have been implemented by researchers for improving the immobilization of enzyme in order to enhance their activity and stability to decrease the enzyme biocatalyst cost in industrial biotechnology.<sup>9,10</sup> These include cross-linked enzyme aggregates, microwave-assisted immobilization, click chemistry technology, recombinant enzymes and nanoparticle-based immobilization of enzymes.<sup>11,12</sup>

Last decade witnessed the importance of AgNPs in the field of chemistry, physics and biology due to their unique optical, electrical and photothermal properties.<sup>13</sup> They are used widely for imparting stability to various bioactive substances including peptides, enzymes, antibodies and DNA due to their greater porosity and interconnectivity

for enzyme immobilization.<sup>14</sup> The fabrication technology has further solved the problems related to the toxicity of nanoparticles, and provides them a shield against harsh environmental conditions like pH variation, temperature alteration and shaking condition.<sup>15,16</sup> Previously, AgNPs have been modified by formamide,<sup>17</sup> phosphoryl disulfides,<sup>18</sup> titanium implant surface,<sup>19</sup> glutaraldehyde,<sup>20</sup> carbon nanotube/polyaniline film,<sup>21</sup> carbonate,<sup>22</sup> cysteamine,<sup>23</sup> polyaniline,<sup>24</sup> to name a few, to increase the catalytic efficiency of enzymes for various biomedical and biotechnological applications.

GOS production has been obtained in the recent past by immobilizing  $\beta$ -galactosidase from various sources including *Bacillus circulans*,<sup>2,25</sup> *Bifidobacterium longum*<sup>26</sup> and *Kluyveromyces lactis*.<sup>27</sup> However, these systems suffered from drawbacks either in terms of enzyme stability/reusability or exhibiting relatively lesser sensitivity toward glucose and galactose. In some cases, enzymes were unable to convert free galactose for GOS formation. Furthermore, the rate of the reaction was reduced when galactose was added in the lactose solution and hence maximum GOS concentration obtained as a result of partial hydrolysis of newly formed oligosaccharides was not observed with immobilized enzyme.

These studies encouraged us to exploit modified AgNPs as an immobilization matrix which can allow multiple and continuous use of enzyme along with minimum reaction time, high stability, improved process control, easy product separation apart from being less labor intensive and more cost effective to the biotechnology industries. Therefore, *Aspergillus oryzae*  $\beta$ -galactosidase was immobilized on the modified AgNPs. Effect of various physical and chemical denaturants, product inhibition by galactose, kinetic parameters and reusability study was investigated for carboxylated AgNPs adsorbed  $\beta$ -galactosidase (I $\beta$ G). The potential biotechnological application of I $\beta$ G has been shown by galacto-oligosaccharides production at 50 °C and 60 °C.

**EXPERIMENTAL****Materials**

Coomassie Blue Brilliant G-250, buffers of different pH

\*e-mail: shakeel.cegmr@gmail.com

values and commercial grade *Aspergillus oryzae*  $\beta$ -galactosidase (Activity: 1200 U/gm) were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Nitric acid, sulfuric acid and *o*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) was obtained from Merck. All reagents were prepared in double distilled water with chemicals of analytical grade.

### Carboxylation of silver nanoparticles

Silver nanoparticles (AgNPs) were prepared as described in our previous study.<sup>20</sup> Briefly, 1.0 mmol L<sup>-1</sup> silver nitrate solution was magnetically stirred in ice bath for 15 min before adding sodium borohydride (2.0 mmol L<sup>-1</sup>) to it. The transformation of color from transparent to golden yellow indicates the formation of AgNPs. The obtained powder was analyzed by XRD and TEM as discussed in our studies before and was observed to be of 26 nm. The washed AgNPs (1.0 gm) were carboxylated by incubating them in 10 mL of 1:3 HNO<sub>3</sub>/H<sub>2</sub>SO<sub>4</sub> (v/v) mixture in a shaker at 30 °C at 150 rpm for 8 h. The resulting carboxylated AgNPs (cAgNPs) obtained were continuously washed by distilled water and then dried overnight in an oven at 100 °C.

### $\beta$ -galactosidase immobilization and leaching of enzyme

$\beta$ -galactosidase (2400 U, equivalent to 2 mg) prepared in assay buffer (0.1 mol L<sup>-1</sup> sodium acetate buffer, pH 4.5) was suspended with cAgNPs (1 g) overnight at 32 °C with slow stirring. The unbound enzyme was removed by washing it thrice with assay buffer. In another experiment, immobilized enzyme preparation was suspended in 100 mmol L<sup>-1</sup> NaCl in a shaker at 50 °C and collected by centrifugation at 2000 rpm after a gap of 30 minutes. Activity of enzyme and the supernatant was checked according to the procedure discussed below.

### Enzyme assay

Hydrolysis of  $\beta$ -galactosidase was calculated by continuously shaking an assay volume of 2.0 mL containing 1.79 mL of 100 mmol L<sup>-1</sup> sodium acetate buffer (pH 4.5), 100  $\mu$ L suitably diluted  $\beta$ -galactosidase and 0.2 mL of 2.0 mmol L<sup>-1</sup> ONPG for 15 min at 40 °C. The reaction was stopped by adding 2.0 mL of 1.0 mol L<sup>-1</sup> sodium carbonate solution and product formed was measured spectrophotometrically at 405 nm.<sup>20</sup>

### Atomic force microscopy and determination of kinetic parameters

Tapping mode AFM experiments of cAgNPs adsorbed  $\beta$ -galactosidase was performed using commercial etched silicon tips as AFM probes by exposing the nanomatrix with the same protein-free buffer as the enzyme-contacted surfaces with typical resonance frequency of ca. 300 Hz (RTESP, Veeco, Japan). In another experiment, kinetic parameters of soluble and immobilized enzyme was investigated from Line-Weaver Burk plot by measuring their initial rates at varying concentrations of ONPG in 100 mmol L<sup>-1</sup> sodium acetate buffer at pH 4.5, 40 °C.

### Physico-chemical characterization and reusability study of cAgNPs attached $\beta$ -galactosidase

Enzyme activity of soluble and immobilized  $\beta$ -galactosidase (20  $\mu$ L) was assayed in buffers of different pH (pH 3.0-8.0). The buffers used were glycine-HCl (3.0), sodium acetate (pH 4.0-6.0)

and Tris-HCl (7.0, 8.0). Molarity of the buffer was 0.1 mol L<sup>-1</sup>. The activity expressed at pH 4.5 was considered as control (100%) for the calculation of remaining percent activity. In another experiment, effect of temperature on soluble and immobilized  $\beta$ -galactosidase (20  $\mu$ L) was studied by measuring their activity at various temperatures (30-70 °C). The enzyme was incubated at various temperatures in 0.1 mol L<sup>-1</sup> sodium acetate buffer, pH 4.5 for 15 min and the reaction was stopped by adding 2.0 ml of 2.0 mol L<sup>-1</sup> sodium carbonate solution. The activity obtained at 50 °C was considered as control (100%) for the calculation of remaining percent activity.

IBG (20  $\mu$ L) was taken in triplicates for assaying the activity of enzyme. After each assay, immobilized enzyme was taken out from assay tubes and was washed and stored in 0.1 mol L<sup>-1</sup> sodium acetate buffer, pH 4.5 overnight at 4 °C for 6 successive days. The activity determined on the first day was considered as control (100%) for the calculation of remaining percent activity.

### Effect of product inhibition and production of galacto-oligosaccharides

The activity of free and immobilized  $\beta$ -galactosidase (20  $\mu$ L) was determined in the presence of increasing concentrations of galactose (1.0-5.0%, w/v) in 0.1 mol L<sup>-1</sup> sodium acetate buffer, pH 4.5 at 40 °C for 1 h. The activity of enzyme without added galactose was considered as control (100%) for the calculation of remaining percent activity.

The formation of oligosaccharides was analyzed by high performance liquid chromatography (Shimadzu, Japan) which consists of a LC-10AT pump, a SPD-10AVP, PDA detector, phenomenex C18 (250 mm  $\times$  4.6 mm, 5  $\mu$ m) column, a Phenomenex, HPLC grade cartridge system and a class Nuchrom software. The pH of the mobile phase was checked on microprocessor based water proof pH tester while the overall illumination at the point of sample placement was tested by a calibrated lux meter. EDTA calcium disodium (60 mg/L) was dissolved in Milli-Q water and used as mobile phase at a flow rate of 0.6 mL min<sup>-1</sup>. The temperatures of the column oven and the detector were maintained at 75 °C and 35 °C, respectively.

### Estimation of protein

Protein concentration was determined by using bovine serum albumin as a standard.<sup>28</sup>

### 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. Data was analyzed by one-way ANOVA. P-values <0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

The present study demonstrates the successful immobilization of *Aspergillus oryzae*  $\beta$ -galactosidase on a highly efficient and selectively modified nanosupport, carboxylated AgNPs. The resulting carboxylated AgNPs (cAgNPs) exhibited 93% of the enzyme attached to the modified nanosupport (Table 1). Excellent yield may be attributed to the presence of large number of functional groups attained on the support surface after carboxylation step which ultimately provides large surface for enzyme immobilization.

Figure 1 showed the schematic representation of the step by step functionalization of AgNPs by the acid mixture and enzyme

**Table 1.**  $\beta$ -galactosidase retainment on modified AgNPs

Enzyme activity yield loaded (X Units)	Enzyme activity in washes (Y Units)	Activity bound/g of modified AgNPs		Activity (%)
		Theoretical (X-Y) = A	Actual=B	B/A x 100
2400	307	2093	1946	93 $\pm$ 3.7

immobilization. Moreover, integrity of the enzyme on the modified AgNPs has been illustrated by atomic force microscopy (Figure 2). AFM image exhibited covalent attachment of  $\beta$ -galactosidase on the carboxylated AgNPs and showed its efficiency as an immobilization matrix. The modified AgNPs generated excellent supports for enzyme immobilization due to their small size and large surface area. These molecules influence mechanical properties like stiffness and elasticity and reduce diffusion limitations to maximize the functional surface area needed for enzyme immobilization in a uniform manner. Finally, the enzymatic activity measurements for the immobilized enzyme confirmed the suitability of the optimized protocol which was demonstrated by the retainment of greater amount of enzyme on the designed nanomatrix (Table 1).

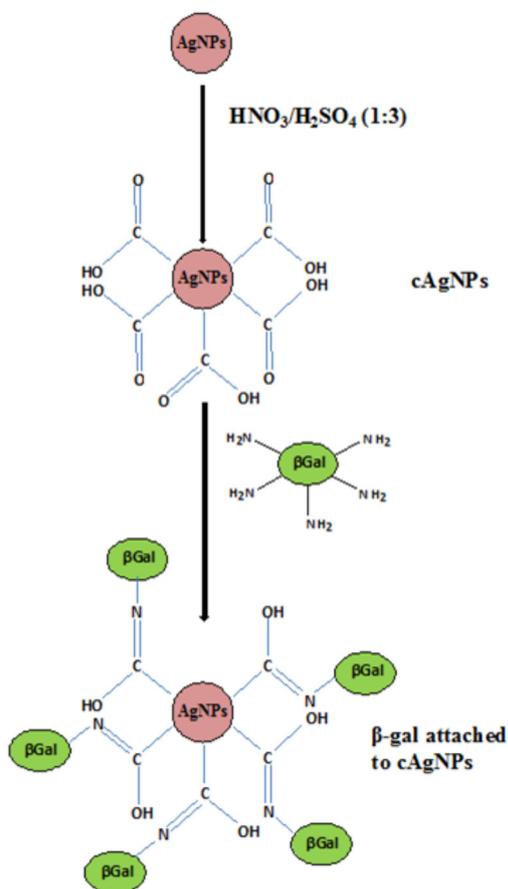
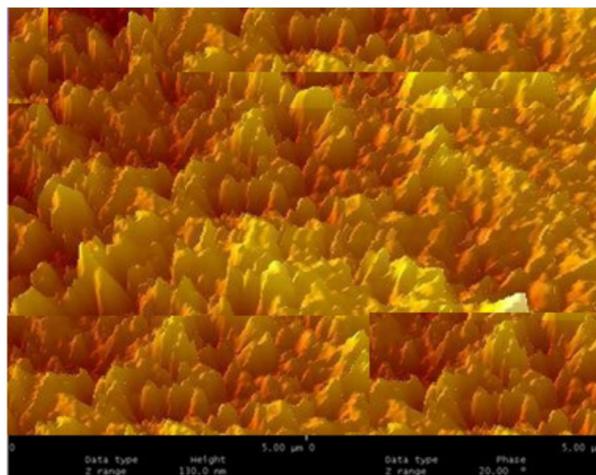
**Figure 1.** Step by step functionalization of AgNPs and attachment of  $\beta$ -galactosidase on carboxylated AgNPs

Table 2 suggested that as a result of immobilization,  $K_m$  was increased to 6.24 mmol L<sup>-1</sup> as compared to 2.46 mmol L<sup>-1</sup> of soluble  $\beta$ -galactosidase. However,  $V_{max}$  does not change significantly. It means that the affinity of the immobilized enzyme for its substrate and the velocity of enzymatic reaction decreased which occurred due to the lower accessibility of the substrate to the active site and lower transporting of the substrate and products into and out the modified nanomatrix (Figure 3). These observations are in agreement with *Aspergillus oryzae*  $\beta$ -galactosidase immobilized on

**Figure 2.** AFM image of  $\beta$ -galactosidase covalently attached to cAgNPs**Table 2.** Kinetic parameters for soluble  $\beta$ -galactosidase (S $\beta$ G) and enzyme immobilized on surface functionalized AgNPs (I $\beta$ G)

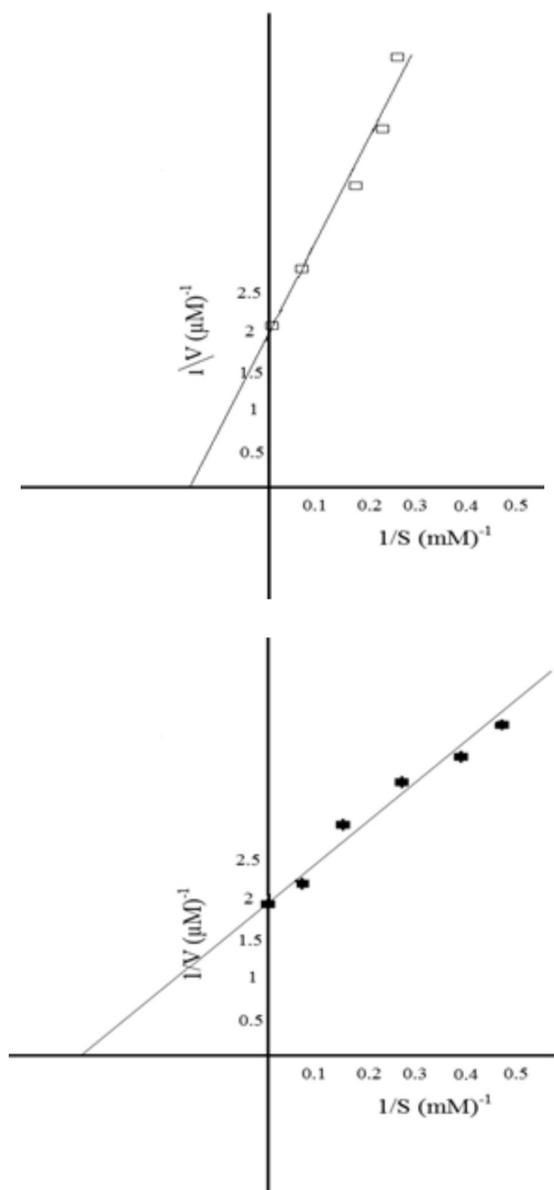
Enzyme preparation	$K_m$ (mmol L <sup>-1</sup> )	$V_{max}$ (mmol L <sup>-1</sup> min <sup>-1</sup> )
S $\beta$ G	2.46 $\pm$ 0.24	509 $\pm$ 3.2
I $\beta$ G	6.24 $\pm$ 0.27	487 $\pm$ 3.8

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

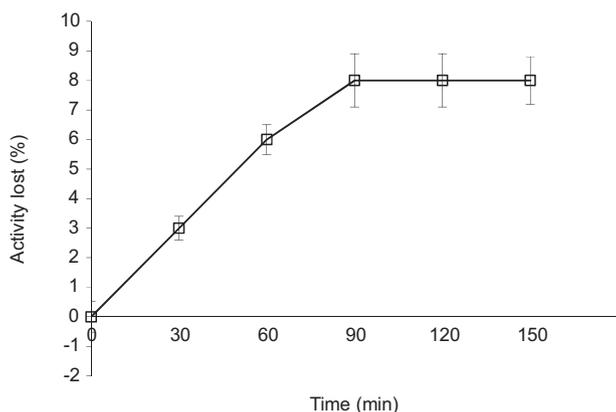
magnetic polysiloxane-polyvinyl alcohol<sup>7</sup> and *Kluyveromyces lactis*  $\beta$ -galactosidase attached to glutaraldehyde modified multiwalled carbon nanotubes.<sup>29</sup>

In order to investigate the possibility of leaching of enzyme from the developed nanosystem, we evaluated the activity of I $\beta$ G in the presence of 100 mmol L<sup>-1</sup> NaCl at 50 °C (Figure 4). It showed that only 8% of the enzyme leached out even after 2.5 hours of incubation with the leachant. This might be due to the presence of small quantities of physically adsorbed enzyme on the developed nanosystem. Needless to mention, covalent attachment resulted in strong bond formation between  $\beta$ -galactosidase and cAgNPs, and hence leaching occurs to a negligible extent even at high temperature.

The stability of enzyme might increase or decrease as a result of immobilization depending upon the properties of matrix used. Moreover, the catalytic activity of enzyme depends on conformational structure of the protein, even minor alterations in the tertiary structure of the protein resulted in loss of its catalytic activity.<sup>9</sup> Hence, we studied the effect of various denaturants on the activity of immobilized enzyme. Figure 5 depicts the pH-activity profiles for soluble and cAgNPs bound  $\beta$ -galactosidase. The optimal pH for soluble and immobilized  $\beta$ -galactosidase was observed at pH 4.5. However, greater fractions of catalytic activity were observed for I $\beta$ G at both lower and higher pH ranges. It should be noted that the optimal operating temperature was broadened from 50 °C to 60 °C for the immobilized enzyme (Figure 6). The probable reason for this may be that covalent binding and crosslinking provided more rigid external backbone for  $\beta$ -galactosidase. Similar results have been achieved earlier for *Aspergillus oryzae*  $\beta$ -galactosidase immobilized on concanavalin A-cellulose.<sup>30</sup>

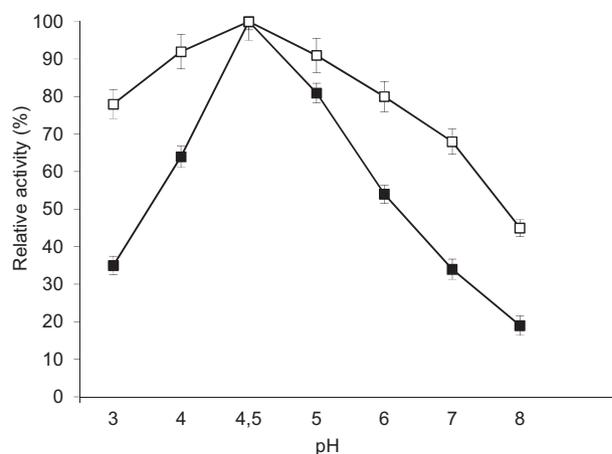


**Figure 3.** Double reciprocal plots to determine constants for ONPG hydrolysis by free ( $\square$ ) and immobilized  $\beta$ -galactosidase ( $\blacksquare$ )

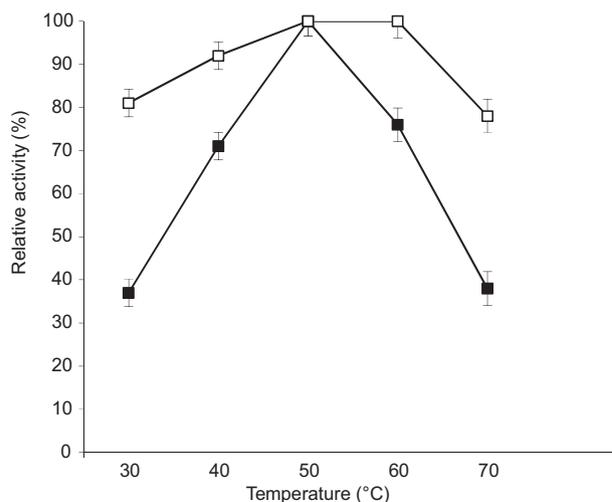


**Figure 4.** Leaching profile of cAgNPs adsorbed  $\beta$ -galactosidase

Figure 7 demonstrated the production of GOS at 50 °C and 60 °C from 0.1 mol L<sup>-1</sup> lactose solution at pH 4.5. Maximum GOS production achieved by cAgNPs adsorbed enzyme was 34% at 60 °C after 7 hours. The probable reason for obtaining greater percent of GOS



**Figure 5.** pH- activity profiles for soluble and immobilized  $\beta$ -galactosidase



**Figure 6.** Temperature-activity profiles for soluble and immobilized  $\beta$ -galactosidase

by  $\beta$ g at higher temperature might be due to its increased stability obtained as a result of its broadening in temperature-optima. In previous studies, maximum GOS production achieved was 30% by using 241 U of immobilized  $\beta$ -galactosidase at pH 6, 40 °C.<sup>2</sup> In another study, maximum formation of GOS achieved by *Aspergillus oryzae*  $\beta$ -galactosidase immobilized on magnetic polysiloxane-polyvinyl alcohol was 26% w/v of total sugars at 55% lactose conversion at pH 4.5 and 40 °C.<sup>7</sup> However, a decrease in GOS production was achieved after certain maximum value as a result of galactose mediated product inhibition. Immobilized enzyme was affected less by this inhibition as compared to soluble  $\beta$ -galactosidase because immobilization prevented the dissociation of enzymes from cAgNPs in the presence of various physical and chemical denaturants like galactose and high temperature.

The feasibility of regeneration of cAgNPs attached  $\beta$ -galactosidase and consequent reuse of the support has been shown in Figure 8.  $\beta$ g retained 85% activity even after its sixth repeated use, hence it can provide economic benefits for its industrial application. Galactose acts as a strong competitive inhibitor for *Aspergillus oryzae*  $\beta$ -galactosidase catalyzed reactions and thus can bring down the process in terms of function and quality of the products obtained.<sup>7</sup> Hence, it is difficult to achieve complete reaction due to these product inhibitors which decreased or even stops the reaction completely. Our results suggested that cAgNPs bound  $\beta$ -galactosidase showed promising resistance to inhibition mediated by galactose as compared

to its soluble counterpart even at higher concentration. The result indicated that soluble enzyme exhibited 34% activity as compared to 70% activity retained by I $\beta$ G at 4% galactose concentration (Figure 9). Moreover,  $K_{iapp}$  value of immobilized  $\beta$ -galactosidase was  $326 \times 10^{-6} \text{ mol L}^{-1}$  while the soluble enzyme exhibited lower  $K_{iapp}$  value,  $163 \times 10^{-6} \text{ mol L}^{-1}$  at 3% galactose concentration (Table 3). Thus, it can be concluded that immobilization of  $\beta$ -galactosidase on cAgNPs proved as a versatile approach and exhibited improvement in catalytic property and stability of enzyme for the production of GOS.

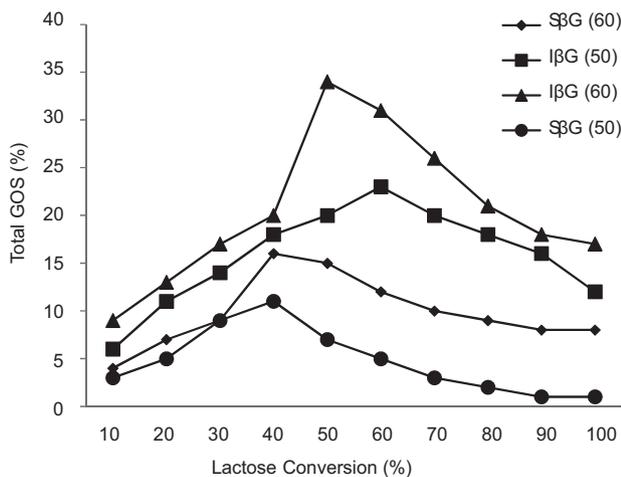


Figure 7. GOS production by soluble and cAgNPs bound  $\beta$ -galactosidase

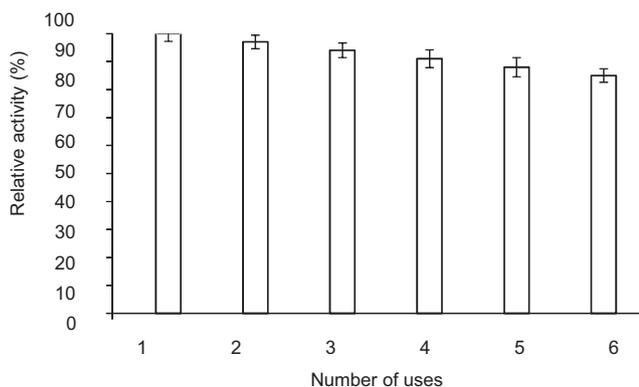


Figure 8. Reusability of cAgNPs bound  $\beta$ -galactosidase

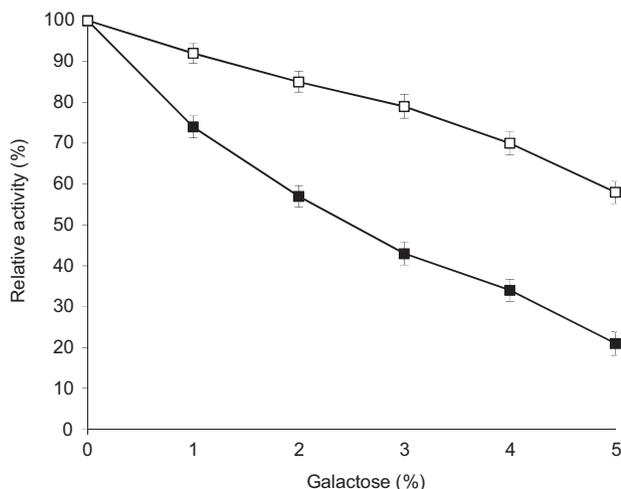


Figure 9. Galactose mediated inhibition on soluble and cAgNPs bound  $\beta$ -galactosidase

Table 3.  $K_{iapp}$  values for soluble  $\beta$ -galactosidase (S $\beta$ G) and enzyme immobilized on surface functionalized AgNPs (I $\beta$ G) in the presence of galactose

Concentration of galactose (%)	S $\beta$ G ( $\times 10^{-6} \text{ mol L}^{-1}$ )	I $\beta$ G ( $\times 10^{-6} \text{ mol L}^{-1}$ )
1	236 $\pm$ 0.146	546 $\pm$ 0.159
2	189 $\pm$ 0.188	379 $\pm$ 0.122
3	163 $\pm$ 0.128	326 $\pm$ 0.310
4	151 $\pm$ 0.139	215 $\pm$ 0.198
5	143 $\pm$ 0.167	170 $\pm$ 0.265

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

## CONCLUSION

The present study describes a simple, inexpensive and novel procedure of modifying silver nanoparticles from  $\text{HNO}_3/\text{H}_2\text{SO}_4$  mixture and exploiting it as a nanomatrix for immobilizing *Aspergillus oryzae*  $\beta$ -galactosidase. Covalently linked enzyme exhibited great immobilization efficiency and markedly improved stabilization against various physical and chemical denaturants. Moreover, immobilized enzyme system was not restricted by diffusional limitations and hence can be exploited in biotechnological process for producing galactosachharides from transgalactosylation of lactose in a convenient and cheaper way.

## SUPPLEMENTARY MATERIAL

Experiment related to immobilization of soluble  $\beta$ -galactosidase on unmodified AgNPs has been mentioned in our previous manuscript<sup>20</sup>. Since the immobilization yield of enzyme was less on unmodified AgNPs (80%) as compared to the functionalized AgNPs (93%), we continued our further studies with the carboxylated AgNPs.

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