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A novel polyurethane/nano ZnO matrix for immobilization of chitinolytic enzymes and optical sensing of chitin

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ABSTRACT

Purified chitinase from *Vigna mungo* and N-acetyl β glucosaminidase (NAGase) from *Canavalia ensiformis* were immobilized on to the novel polyurethane (PU)/zinc oxide nanoparticles (nano ZnO) composite matrix with a conjugation yield of 0.785 ± 0.01 mg/cm^2 and 96.19 ± 0.85% retention of specific activity. Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR) also confirmed the presence of nano ZnO and enzymes on the PU support. Thus synthesized PU/nano ZnO/chitinase/NAGase conjugates were optimized with respect to pH, temperature and substrate concentration and successfully employed for development of an absorbance based optical biosensor for chitin determination in stored wheat grains. The limit of detection was 0.01 mM with linearity from 0.1 to 10.0 mM. The% recoveries of added chitin (0.1 and 0.2 mM) were >95.0% and >96.5% respectively and within-day and between-day coefficients of variations were 1.03% and 1.78% respectively. The method showed good correlation ($R^2 = 0.996$) with the popular 3,5-dinitrosalicylic acid method. PU/nano ZnO bound chitinase/NAGase showed good thermal and storage stabilities and could be reused 10 times without any appreciable loss of activity.

1. Introduction

Among the different pathogens affecting crop plants, occurrence of fungal contamination during pre and post – harvest is the most threating one, that alone contributes to 25% loss of food crops and stored food grains throughout the world [1]. Fungi under dormant conditions are retained as spores in the food grains and with the advent of favorable conditions releases off odors, mycotoxins, halts the germination and causes discoloration and loss in weight of grains [2]. Consumption of these contaminated crops or food products adversely affects the human health causing growth disorders, liver and kidney damage, skin diseases, cancer and other mutations [3]. Hence, large-scale issues of health and safety warrant the analysis of a given sample for fungal contamination at short intervals during the storage and also before using it for consumption.

A growing approach for fungal invasion detection includes quantification of chitin contents as chitin (a biopolymer of β-1, 4-N-acetylglucosamine) is the main constituent of the cell walls of most fungi and fungal spores. Though, chemical methods for chitin quantification were successful in checking the quality of stored cereals but use acid and alkaline hydrolysis along with lengthy protocols of about 4–5 h and hence becomes impractical when rapid results are required [4,5]. Other techniques such as HPLC, GC–MS and IR spectroscopy have also been used but are interference prone, involve lengthy sample preparations and also use expensive instruments [6–8]. Chitin determination based on chitinolytic enzymes is not only environment-friendly, rapid and simple but also highly sensitive and specific. Two main chitinolytic enzymes, chitinase and N–acetyl β glucosaminidase (NAGase) together digest chitin, in a sequential manner by catalyzing the hydrolytic cleavage of the β-1, 4-glycosidic bond present in the chitin and release reducing sugar, N-acetyl glucosamine, that may be detected by a colorimetric reaction with 3, 5-dinitro salicylic acid [9]. Further, the use of chitinolytic enzymes in immobilized form is advantageous in terms of their reusability, stability and separation from the reaction mixture [10,11]. Consequently, over the years, a number of supports such as CM-Sephadex, AH-Sepharose, CNBr-Sepharose, AS-Alumina, tannin sepharose and tannin chitosan [12], chitosan beads [13] and cashew gum polysaccharide/poly vinyl alcohol [14] film have been used for immobilizing chitinase for developing fungal growth inhibition or detection assays.

Over the last one-decade, various metal oxide nanoparticles have also been extensively explored as supports for enzyme immobilization since they provide a conducive microenvironment to the

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bound enzyme [15]. In this regard, zinc oxide nanoparticles (nano ZnO) also create favorable conditions for immobilization as they have a tendency to aggregate, present high surface area to volume ratio and high electron mobility [16,17]. Additionally, nano ZnO has been classified as a multifunctional material owing to its high photostability, biocompatibility, biodegradability and chemical stability [18,19]. However, separation of nano ZnO/enzyme conjugates from the reaction mixture is difficult since they remain suspended in the solution and can be separated only by cold centrifugation, making centrifugation an integral component of each enzyme assay [20]. Hence, it is highly desirable to adhere the nanoparticles to a macro scale support, which is physically distinct, visible and easily separable from the reaction mixture. Polyurethanes (PU) with strong binding properties, biocompatible nature and good resistivity to environmental factors are suitable to be used for this purpose [21], which will not only affix the nano ZnO to their surface but will also immobilize the enzymes by multiple covalent interactions. Altogether, a composite support prepared by annexing nano ZnO to the PU surface is expected to yield an easy to handle immobilized enzyme preparation and improve immobilization due to its biocompatible nature, augmented surface area and binding properties.

Hence, the present study explores the development of a simple, sensitive and inexpensive colorimetric sensor for determination of total fungal load of a sample based on digestion of chitin by immobilized chitinolytic enzymes. To achieve this, chitinase and NAGase were co-immobilized onto PU/nano ZnO composite support, optimized with respect to working conditions of pH, temperature and substrate concentration and employed for quantification of chitin contents in the stored wheat grains. Few properties of the free chitinase, NAGase and chitinase/NAGase were also ascertained to understand the impact of immobilization on enzymes. Data on reusability, thermal and storage stabilities of the free and co-immobilized enzymes has also been included.

2. Experimental

2.1. Reagents

N–acetyl β glucosaminidase (NAGase, EC 3.2.1.14, ≥230 units/mg protein) from jack bean (*Canavalia ensiformis*), 4-nitrophenyl N-acetyl-β-D-glucosaminide (NP-GlcNAc), ammonium sulfate and Sephadex G-100 was purchased from Sigma-Aldrich Co. (St. Louis, USA). Chitinase (EC 3.2.1.14) was purified from the urd bean (*Vigna mungo*) sprouts [22]. Chitin from shrimp shell, Tris base, sodium hydroxide, sodium acetate, glacial acetic acid, sodium carbonate, sodium potassium tartrate, 3,5-dinitrosalicylic acid, zinc nitrate and sodium azide was procured from Hi-Media, Mumbai, India. Locally available polyurethane sold under the trade name "Vetaseal" was used as such. Rest all the chemicals were of analytical reagent grade.

2.2. Purification of chitinase

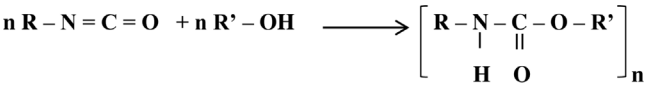
Chitinase was purified from urd bean seeds soaked previously in distilled water for 48 h following previously described protocols [22] with slight modifications. Briefly, the soaked seeds were homogenized in 0.01 M sodium acetate buffer (pH 5.4), centrifuged and the crude solution was stored under cold conditions. Further, the enzyme was purified to constant specific activity i.e. homogeneity, using ammonium sulfate precipitation (0–80%), dialysis and molecular exclusion chromatography with Sephadex G-100. The specific activity of purified chitinase was found to be ≥1305 units/mg protein.

2.3. Synthesis and characterization of nano ZnO

To prepare nano ZnO, a standardized protocol was followed with slight modifications [23]. In a typical reaction, aqueous solutions of zinc nitrate (0.45 M) and sodium hydroxide (0.9 M) were prepared separately in distilled water. Sodium hydroxide solution was heated up to a temperature of 55 °C and then zinc nitrate solution was added drop by drop to it, over a time span of 60 min, under continuous stirring. Afterward, the beaker was sealed and left undisturbed for nearly 2 h. The precipitated nano ZnO were cleaned with deionized water and ethanol and air dried at about 60 °C. The size of nano ZnO was determined using Transmission Electron Microscopy (FEI Tecnai S Twin) at Sophisticated Advanced Instrument Facility (SAIF), Department of Anatomy, AIIMS, New Delhi. Crystal structure and phase purity of the nanoparticles was confirmed by recording the X-ray diffraction pattern in the 2θ range of 10°–85° (XRD, Rigaku Ultima-IV, X-ray powder diffractometer with CuK α radiations) at Department of Chemistry, M.D.University, Rohtak.

2.4. Immobilization of chitinase/NAGase

PU having poly isocyanate and polyol groups was layered evenly over a polyethylene strip of size 4cm × 4 cm and allowed to polymerize at room temperature for 2 min. During polymerization isocyanate group reacted with the hydroxyl group, thereby yielding a urethane or the basic unit of PU (Eq. (1)).



Polyisocyanate Polyol Polyurethane (1)

1.0 ml of nano ZnO emulsion (10.0 mg/ml) was spread over the top of polymerized PU layer and air dried for 5 min. 1.0 ml of purified chitinase (98.50 units) and 1.0 ml of NAGase (83.33 units) solution in sodium acetate buffer (0.02 M, pH 5.4) was mixed together and poured over the PU/nano ZnO support. The preparation was kept at 20 °C for 48 h to allow the enzymes to develop maximum bonded or non-bonded interactions with the support. Thereafter, the enzymes bound PU/nano ZnO support was washed with sodium acetate buffer (0.02 M, pH 5.4) several times. The amount of enzyme immobilized on to the support was calculated by subtracting the total protein content of the washings from the protein content of the enzyme solution [24]. In order to know the influence of nanoparticles on immobilization efficiency, PU activated support without nano ZnO was also prepared and utilized for enzyme immobilization in the same manner, except for the addition of nanoparticles to the PU layer. The enzymes-bound PU and PU/nano ZnO chitin conjugates were stored in 0.02 M sodium acetate buffer pH 5.4 at 4 °C when not in use.

2.5. Characterization of support bound chitinase/NAGase

Bare as well as nano ZnO and enzymes bound PU support was characterized by Scanning Electron Microscopy (LEO 435 VP 501 B-SEM, Philips) at Sophisticated Advanced Instrument Facility (SAIF), Department of Anatomy, AIIMS, New Delhi, to study the changes in surface morphology of the support after modification with nanoparticles and enzymes. In order to know the chemistry and bond present in the bare PU, PU/nano ZnO and PU/nano ZnO/chitinase/NAGase, Fourier Transform Infrared Spectroscopy (FTIR Alpha, Bruker, Germany) was done at Department of Genetics, Maharishi Dayanand University, Rohtak. The surface scanning and profile imaging of the bare and modified PU supports were done in contact mode by atomic force microscopy (AFM, NT-MDT

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Solver P47-Pro instrument) at CSIR – National Physical Laboratory, New Delhi. Computation of surface roughness and visualization of three-dimensional images was done using the freeware Gwyddion 2.47.

2.6. Enzyme assays

Chitinase/NAGase activities were determined by measuring the reducing end group, N-acetyl glucosamine produced after enzymatic digestion of colloidal chitin according to the method of Boller and Mauch (1988) with slight modifications [25]. In order to perform the assay, 3.0 ml of reaction mixture consisting of 1.0 ml of 1.0% (w/v) colloidal chitin (100 mM, pH 5.4) prepared by the method of Sheng et al. (2002) [26], 0.2 ml of 3.3 mM sodium azide, 0.8 ml of sodium acetate buffer (0.02 M, pH 5.4) and 1.0 ml enzyme solution having 49.25 units of chitinase (98.50 units/ml) and 41.65 units of NAGase (83.33 units/ml) was incubated at 50 °C for 15 min. Chitinases catalyze the hydrolytic cleavage of chitin into N-acetyl glucosamine and N-acetyl-chito-oligosaccharides. The released oligosaccharides were further broken down into beta-linked N-acetyl glucosamine monomers by the action of NAGase. Further, 2.0 ml of 3,5-dinitrosalicylic acid reagent was added to the reaction mixture and it was heated in boiling water for 5 min to terminate the reaction. The yellow to orange colored solution thus obtained was subjected to spectrophotometric measurement at 540 nm. The activity of PU/nano ZnO bound chitinase/NAGase was determined in the same way with the modification that the PU/nano ZnO/chitinase/NAGase conjugates replaced the soluble enzymes and the amount of sodium acetate buffer (0.02 M, pH 5.4) was increased by 1.0 ml in the reaction mixture. Chitinase activity assay was also similar to the chitinase/NAGase assay except for replacing the free NAGase solution with the same volume of reaction buffer. One unit of chitinase or chitinase/NAGase activity is defined as the amount of enzyme that liberates 1.0 μg N-acetyl amino-glucose per minute at pH 5.4 and 50 °C.

NAGase assay was based on the protocol of Shibata and Yagi (1996) [27], which involves the hydrolysis of substrate, NP-GlcNAc to *p*-nitrophenol and N-acetyl-β-D-glucosamine (NAG). The released *p*-nitrophenol upon ionization in the basic pH formed the yellow p-nitrophenylate ion. The absorbance of the p-nitrophenylate ion was measured at 405 nm and used to calculate NAGase activity as given below:

$$\text{Units/ml} = \frac{A_{405}\ \text{sample} \times \text{final reaction volume (3.0ml)} \times \text{Enzyme dilusion factor}}{\text{Extinction coefficient for p} - \text{nitrophenolate (18.3 mM)} \times \text{Time (min)} \times \text{sample volume (ml)}}$$

The reaction mixture containing 0.50 ml of 10.0 mM NP-GlcNAc (prepared in 0.1 M citrate buffer), 1.0 ml of 50.0 mM sodium acetate buffer (pH 5.4), and 0.5 ml of enzyme solution in sodium acetate buffer (0.02 M, pH 5.4) (41.65 units) was incubated at 37 °C for 5 min. After incubation, the reaction was stopped by adding 5 ml of 1.0 M sodium carbonate solution. p-nitrophenol becomes intensely colored after addition of the stop reagent. The increase in absorbance at 405 nm after addition of the stop reagent is directly proportional to the enzyme activity. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per min at pH 5.4 at 37 °C.

2.7. Optimization of working conditions

All experiments related to maximization of immobilized PU/nano ZnO/chitinase/NAGase activity were repeated three times and mean was calculated as a measure of central tendency. Standard error was used to estimate the accuracy of mean in all graphs. To determine optimum pH, the co-immobilized enzymes were assayed at 50 °C using 0.02 M sodium acetate buffer in the pH range of 4.0–6.0, 0.01 M sodium phosphate buffer at pH of 6.5 and 7.5 and 0.01 M borate buffer at pH 8.0–9.0. Experiments related to optimization of working temperature were carried out at optimum pH and in the temperature range of 20–80 °C with a gap 5 °C. The energy of activation (E_a) was evaluated from Arrhenius plot by plotting inverse of temperature (in degree Kelvin) vs. log of enzyme activity. Under optimum conditions of pH and temperature, the concentration of chitin was varied from 1.0 mM to 15.0 mM. Kinetic parameters K_m and V_{max} were calculated by Lineweaver-Burk plot. k_{cat} values and k_{cat}/K_m ratios were also calculated to determine the turnover number and catalytic efficiency of immobilized chitinase/NAGase. For comparison purposes, parallel studies on optimization were also carried out for free chitinase, free NAGase and free chitinase/NAGase.

2.8. Stability studies

Thermal stabilities of chitinase/NAGase and PU/nano ZnO/chitinase/NAGase were measured by keeping the preparations for 60 min at various temperatures ranging from 20 to 80 °C and then measuring the residual activity under optimal assay conditions. A peltier system attached with the UV–vis spectrophotometer was used for maintaining nearly constant temperatures with negligible fluctuations of 0.05 °C. To work out the storage stabilities, activities after storage in 0.02 M sodium acetate buffer (pH 5.4) at 5 °C were measured on alternate days for four months. For operational stability, the co-immobilized enzyme preparation was repeatedly assayed at optimum conditions till the point its activity was significantly lost. After each reaction run, washing of PU/nano ZnO/chitinase/NAG conjugates was done with 0.02 M sodium acetate buffer (pH 5.4) to remove any residual activity. The concentration of chitin was kept as 100 mM for all the assays.

2.9. Colorimetric sensing of chitin

Polyurethane/nano ZnO/chitinase/NAGase conjugates were used for quantification of chitin, based on differences in the light absorption ability of reactants and products at 540 nm. The sample for chitin analysis consisted of undamaged and healthy looking grains of wheat, stored in the local grocery shops for about 6–8 months. The determined chitin contents were;1; presumed to be directly proportional to the amount of fungus mycelium and/or spores present in the stored grains. Chitin present in the collected samples was reduced to N-acetyl glucosamine by the synthesized nanobioconjugates. As shown in Eq. (2), 3,5-dinitrosalicylic acid reacted with the N-acetyl glucosamine at high temperatures and generated 3-amino, 5-nitro salicylic acid, which absorbed strongly at 540 nm.

$$\text{PU/nano ZnO/chitinase/NAGase 3, 5-dinitrosalicylic acid}$$
$$\text{3, 5-dinitrosalicylic acid} \rightarrow \text{N-acetylglucosamine} \rightarrow \text{3-amino, 5-}\textit{nitro salicylic acid}(\lambda_{max}540\ \text{nm}) \quad (2)$$

Stored grains of wheat were collected from the local grocery shops. 50 g of the grains were transferred to a beaker containing 25.0 ml of sodium acetate buffer (0.02 M, pH 5.4) and left undisturbed for 10 min. The buffer was then filtered using Whatman no. 42 filter paper and the filtrate used as a source of chitin. Chitin determination in the prepared samples was carried out by dipping the co-immobilized enzymes strip (PU/nano ZnO/chitinase/NAGase) in the reaction mixture consisting of 1.8 ml

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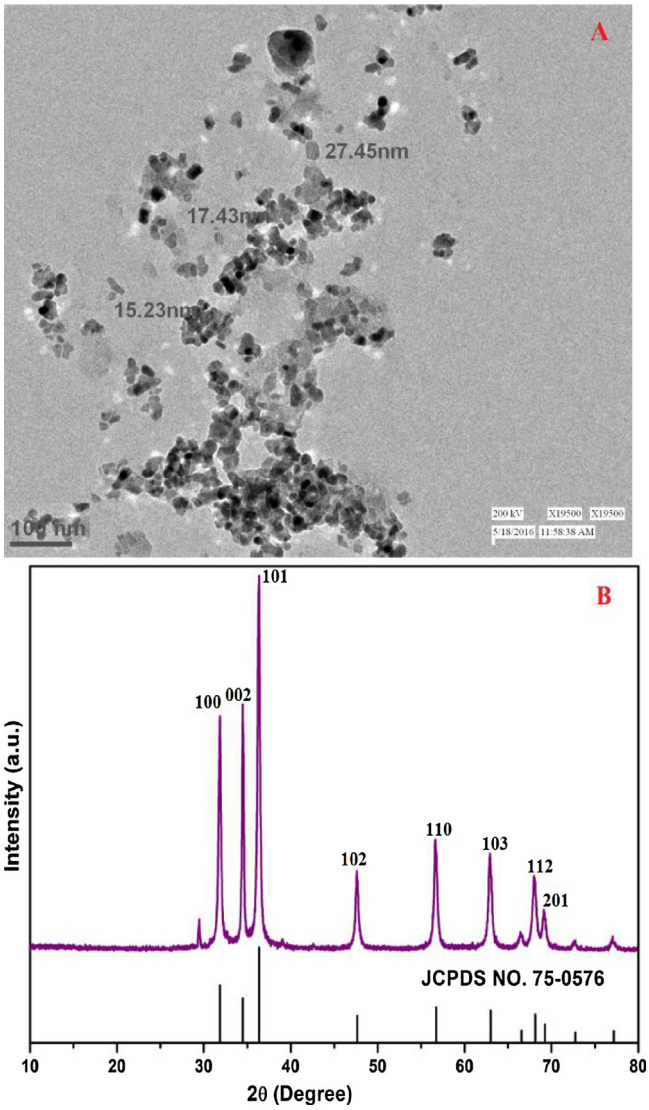

Fig. 1. TEM image (A) and XRD patterns of nano ZnO (B).

of sodium acetate buffer (0.02 M, pH 5.4), 1.0 ml of the filtrate prepared above and 0.2 ml of 3.3 mM sodium azide. The enzyme bound strip was taken out of the reaction mixture after incubating it at 50 °C for 15 min. Thereafter, 3,5-dinitrosalicylic acid reagent (2.0 ml) was added, mixture heated for 5 min in a boiling water bath and A_{540} was read. The nanobioconjugates were washed with 0.02 M sodium acetate buffer (pH 5.4) after every assay and stored in 0.02 M sodium acetate buffer pH 5.4 at 4 °C when not in use. Chitin content of the wheat samples was interpolated from a standard curve between colloidal chitin concentration (ranging from 0.01 to 15.0 mM) and A_{540} nm.

3. Results and discussion

3.1. Characterization of nano ZnO

TEM micrographs shown in Fig. 1A revealed that very fine nano ZnO in the size range of 15–30 nm were obtained. The nanoparticles were crystalline, polydispersed, polyhedral and showed a tendency to clump together. The structural pattern of the synthesized nano ZnO was characterized by XRD. As shown in Fig. 1B, all the characteristic diffraction peaks indexed at 100, 002, 101, 102, 110, 103, 112 and 201 were consistent with Joint Committee for Powder Diffraction Standards (JCPDS card No.75-0576). The XRD pattern clearly showed that nano ZnO have hexagonal wurtzite structure with lattice constants a = 0.325 nm and c = 0.521 nm [28]. Furthermore, no other peaks of impurity were apparent which proved high purity and good crystallinity of synthesized nano ZnO.

3.2. Immobilization of chitinase and NAGase

To fabricate the chitin sensor, purified chitinase from *Vigna mungo* and NAGase from *Canavalia ensiformis* were co-immobilized onto PU/nano ZnO composite support with a conjugation yield of 0.785 ± 0.01 mg/cm^2 and 96.19 ± 0.85% retention of initial activities. In order to study the impact of nano ZnO on immobilization parameters, both the enzymes were also co-immobilized onto bare PU support. A comparison of immobilization data on the two supports indicated substantial enhancement in the conjugation yield and% retention of activity after addition of nano ZnO to the PU layer (Table 1). On bare PU layer, enzyme is immobilized by multiple covalent interactions between the urethane groups and the amine and/or hydroxyl groups of the enzyme (Scheme 1), which may impose conformational restrictions on the movement of enzyme and hence might account for the reduced activity of the immobilized chitinase/NAGase. Conversely, aggregates of nano ZnO not only present large surface area but also large interfacial areas, which might have considerably scaled up the amount of adsorbed enzymes and higher retention of activity might be accredited to the hydrophilic and biocompatible nature of the added nanoparticles. Additionally, nano ZnO might have shielded a fraction of enzymes from coming in contact with the PU layer directly, resulting in less number of bonded interactions between the two, greater conformational flexibility and high retention of enzymes activities on PU/nano ZnO support. Overall, a comparison of parameters in Table 1 confirmed the usefulness of nano ZnO addition to the PU layer. Henceforth, further studies were carried out only with PU/nano ZnO bound enzymes including a comparison of their properties with that of free chitinase, NAGase and chitinase/NAGase (Table 2).

3.3. Characterization of the support conjugated chitinase/NAGase

SEM micrographs of the bare and modified PU are presented in Fig. 2. An uneven polymeric layer of PU shown in Fig. 2A appears highly irregular and coarse with conspicuous protuberances. The addition of nano ZnO suspension to PU layer resulted in marked shadowing of the cragginess but the surface still appeared coarse (Fig. 2B). Very evenly distributed enzymes, covering the entire support surface are well seen in Fig. 2C, presenting a visual confirmation of the high loading capacity of the PU/nano ZnO support. Surface topologies as studied by AFM (Fig. 3) also supported a distinct change in the surface of bare PU after modification with nano ZnO and enzymes. Highly rough and jagged surface of bare PU is visible in Fig. 3A. The addition of nano ZnO masked the roughness to a large extent but still, the surface was irregular and the large peaks could possibly be due to the aggregation of nano ZnO on the PU surface (Fig. 3B). A marked smoothening of the PU/nano ZnO surface occurred after addition of the chitinase/NAGase (Fig. 3C). The values for two of the most commonly used roughness parameters, root mean square roughness (Sq) and average peak height (Sy) as calculated from the software Gwyddion 2.47, were also found to decrease in the order of PU > PU/nano ZnO > PU/nano ZnO/chitinase/NAGase, the values for Sq and Sy being 82.09 and 364.15 nm for bare PU, 65.25 nm and 231.70 nm for PU/nano ZnO and 11.92 nm and 81.59 nm for enzymes modified PU/nano ZnO support respectively, corresponding to the successful layering of the bare PU support first with the nano ZnO and then with the enzymes. The decrease in roughness of the surface after addition of enzymes has also been

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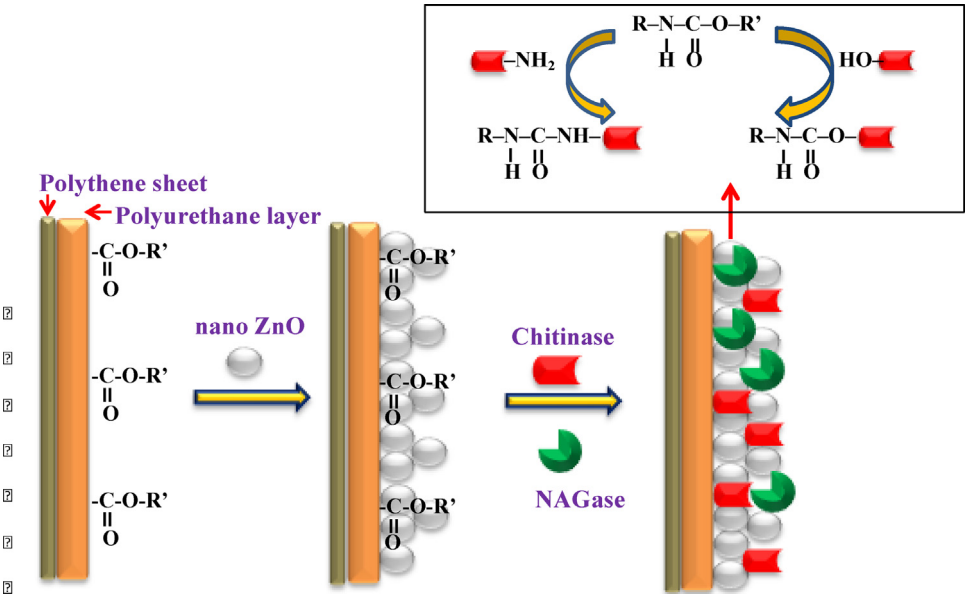
Table 1
Co-immobilization of chitinase and NAGase onto bare and nano ZnO annexed PU support. Data is presented as mean ± S.E. of three trials.

Support	Chitinase/NAG added to the membrane (mg/4 cm^2)	Chitinase/NAG coupled to the membrane (mg/4 cm^2)	Activity units* added	% Retention of activity	Conjugation yield (mg/cm^2)
Bare PU	5.20	2.11 ± 0.11	182.00	74.11 ± 0.08	0.527 ± 0.01
PU/nanoZnO	5.20	3.14 ± 0.21	182.00	96.19 ± 0.05	0.785 ± 0.02

*One unit of chitinase/NAG activity was defined as the amount of enzyme that liberated 1 μg N- acetyl amino-glucose per minute at pH 5.4 and 50 °C



Scheme 1. Co-immobilization of chitinase/NAGase onto PU/nano ZnO composite. Reaction between the urethane groups of PU and the amine and/or hydroxyl groups of the enzyme are shown in the box.

Table 2
Observed changes in the kinetic properties and stability of chitinase/NAGase after immobilization onto PU/nano ZnO support vis a vis free enzymes.

Properties studied	Free chitinase	Free NAGase	Free chitinase/NAGase	Co – immobilized chitinase/NAGase
Optimum pH	6.0	5.5	6.0	6.50
Temperature (°C)	55.0	50.0	55.0	60.0
Activation energy (E_a) (kJ/mol)	2.76	2.93	2.66	3.32
K_m (mM)	5.85	4.76	5.49	7.35
V_{max} μmol(min mg protein)$^{-1}$	988.57	1008.57	1063.82	980.39
k_{cat} (sec^{-1}) × 10^3	1.01	1.08	4.61	2.26
k_{cat}/K_m (M^{-1}sec^{-1})	2.46 × 10^4	2.27 × 10^4	8.41 × 10^5	3.16 × 10^5
Thermal stability at 75° C	25% activity retained	25% activity retained	25% activity retained	60% activity retained
Half-life of chitinase at 5 °C	25 days	45 days	30 days	90 days
Operational stability	–	–	–	50% retention after 55 reuses

reported for other immobilized enzymes such as chitinase/NAGase [29], glutamate dehydrogenase [30], glucose oxidase [31], cellulase [32] etc.

FTIR spectra in Fig. 4A showed characteristic peaks of PU between 2800 and 3200 cm^{-1} for C—H stretching vibrations, at, at 1725 cm^{-1} for urethane —C=O vibration, at 1630 cm^{-1} for carbonyl urethane stretching, at, at 1566 cm^{-1} for CHN vibration, at, at 1237 cm^{-1} for coupled C—N and N—H stretching and at 1162 cm^{-1} for C—O stretching [33]. The FTIR spectrum of PU/nano ZnO shown in Fig. 4B revealed the presence of a new peak at 712 cm^{-1}, characteristic for nano ZnO [34]. Rest all the characteristic peaks for PU were retained but shifted slightly either to a lower or higher wave number due to adsorption of nano ZnO over the polyurethane surface. Retention of PU peaks indicated that nano ZnO was only physically glued to the PU surface and no chemical bonding between the two took place. The IR spectra of PU/nano ZnO/chitinase/NAGase in Fig. 4C confirmed the presence of enzymes by appearance of peaks corresponding to amide I linkage at 1646 cm^{-1}, that results from the C=O stretching vibrations of the peptide bond, amide II and amide III linkages at 1575 and 1301 cm^{-1} respectively as a result of N—H bending vibration/C—N stretching vibration and amide V linkage at 800 cm^{-1} for out of plane N—H bending along with disappearance of characteristic PU peaks between 1000–2000 cm^{-1} [35]. The band at 2852 cm^{-1} was attributed to the stretching vibrations of the —CH and CH_2 groups present in the enzymes. The peak for nano ZnO shifted from 712 to 621 cm^{-1} probably due to adsorption of enzyme. Retention of peaks for nano ZnO and disappearance of characteristic PU peaks clearly indicated that the enzymes were simply absorbed onto the nano ZnO but covalently linked to the PU support [17,29].

3.4. Effect of pH

The effect of pH on the activity of free and immobilized chitinase/NAGase is shown in Fig. 5A. The optimum pH for free chitinase, NAGase and chitinase/NAGase was 6.0, 5.5 and 6.0 respectively,

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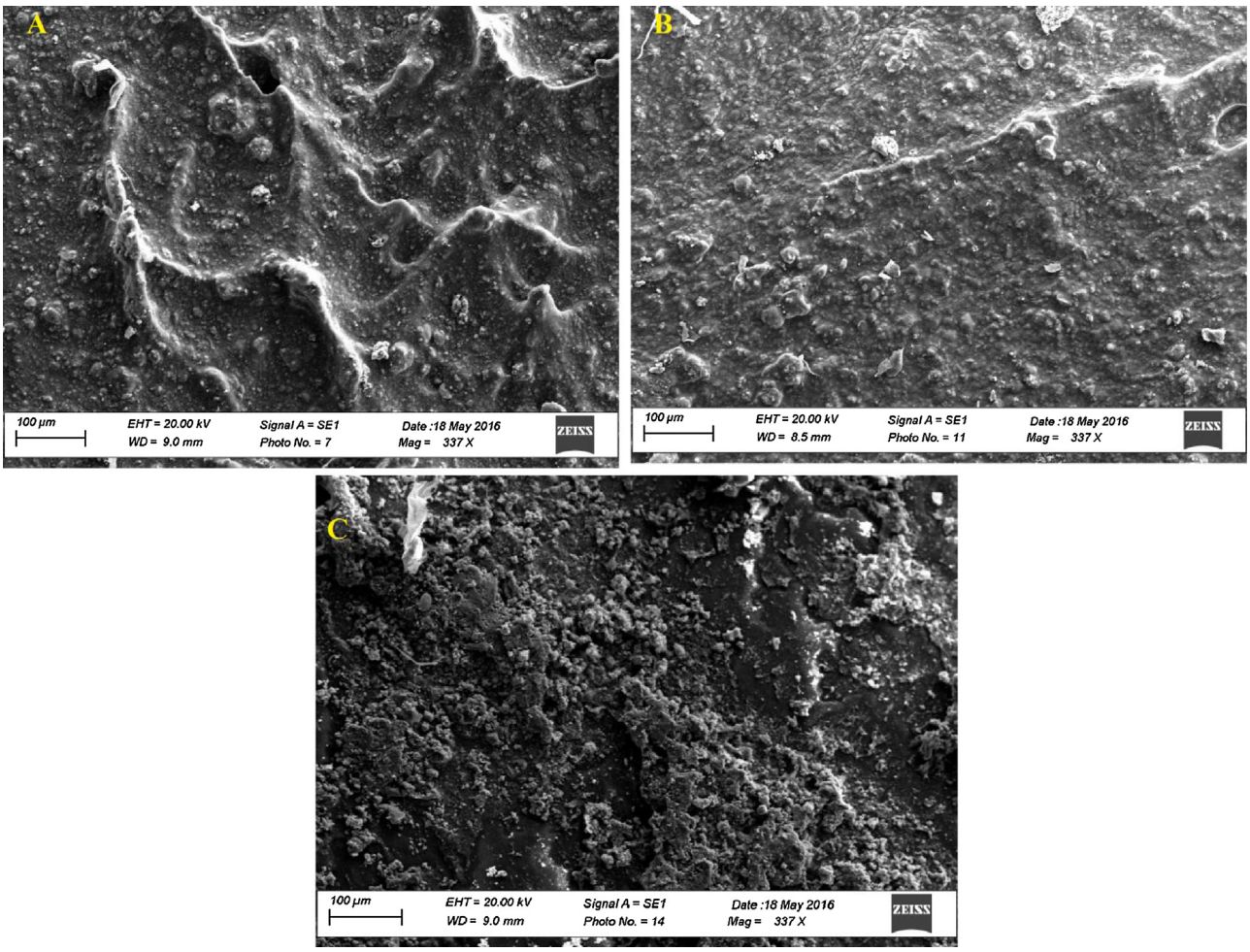

Fig. 2. SEM photographs of bare PU surface (A), PU/nano ZnO composite without enzyme (B) and with co-immobilized chitinase/NAGase (C).

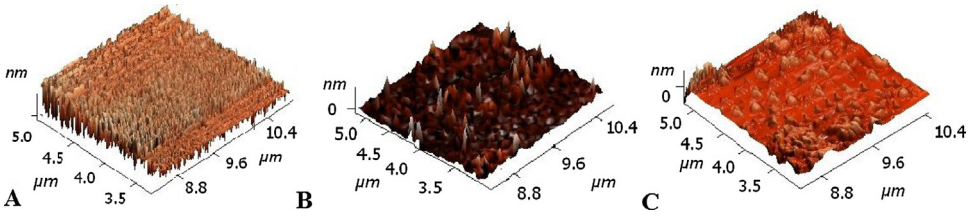

Fig. 3. Three dimensional images of the bare PU (A), PU/nano ZnO (B) and PU/nano ZnO/chitinase/NAGase (C) surfaces procured using atomic force microscopy.

while the co-immobilized enzymes were considerably active over a broader pH range of 6.0–7.0 with an optimum pH of 6.5 (Table 2). A broader range of pH for activity of the co-immobilized enzymes might be due to favorable changes in the microenvironment of the bound enzymes [36]. As reported earlier, chitinase immobilized onto hydroxyl propyl methyl cellulose acetate succinate (AS-L) showed maximum activity at a pH of 8.0 while phenyl sepharose bound chitinase had an optimum pH of 6.0 [37,38].

3.5. Effect of temperature

Temperature dependent activity profile of free and PU/nano ZnO bound enzymes was studied in the temperature range from 20 to 80 °C. Free NAGase showed an optimum temperature of 50 °C, which was lower by 5 °C to the optimum temperature required for maximum activity of both free chitinase and chitinase/NAGase (55 °C) and by 10 °C to the temperature at which co-immobilized chitinase/NAGase exhibited maximum activity (60 °C) (Table 2). Though the optimum temperature for PU/nano ZnO bound enzymes was 60 °C but it was almost fully active over a temperature range of 50–60 °C (Fig. 5B) and also showed higher activity than the free enzymes at each temperature, especially beyond 60 °C. The co-immobilized enzymes retained 50% of the initial activity even at 80 °C, which is quite appreciable compared to the activity retained by the free enzymes (12%) at the same temperature, clearly indicating the protective effect of the support. Supports such as PU, which are rich in reactive groups, may form additional linkages such as amide bonds with the proteins under mildly denaturing conditions [39]. The emergence of such bonds might have stabilized the structure of enzyme and allowed it to retain more activity than the free enzymes. ZnO nanoparticles are also known to impart structural stability to enzymes at higher temperatures [40]. Chitinase immobilized onto AS-L and phenyl sepharose also had an optimum temperature of 50 °C [37,38]. Acti-

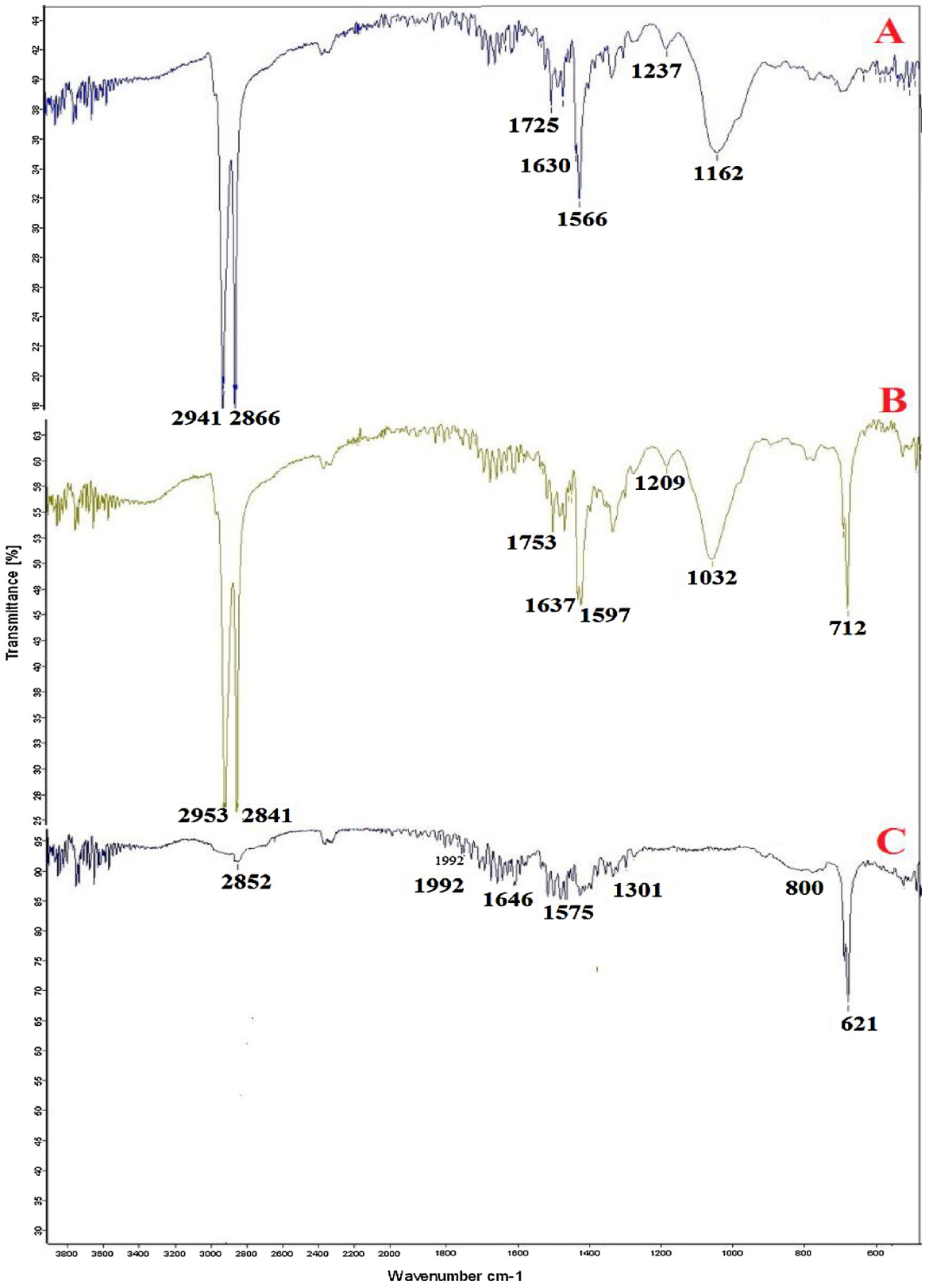

Fig. 4. FTIR image of bare PU (A), nano ZnO loaded PU (B) and nano ZnO/chitinase/NAGase loaded PU (C).

vation energies (E_a) for free chitinase, NAGase, chitinase/NAGase and co-immobilized enzymes were 2.76, 2.93, 2.66 and 3.32 kJ/mol respectively. Reduction in conformational flexibility after immobilization and differential gradients of substrate around the active site might account for a slightly higher E_a of bound enzymes.

3.6. Kinetic studies

Initial rates of substrate decomposition by free and co-immobilized enzymes were obtained for various concentrations of colloidal chitin. Free and co-immobilized chitinase/NAGase

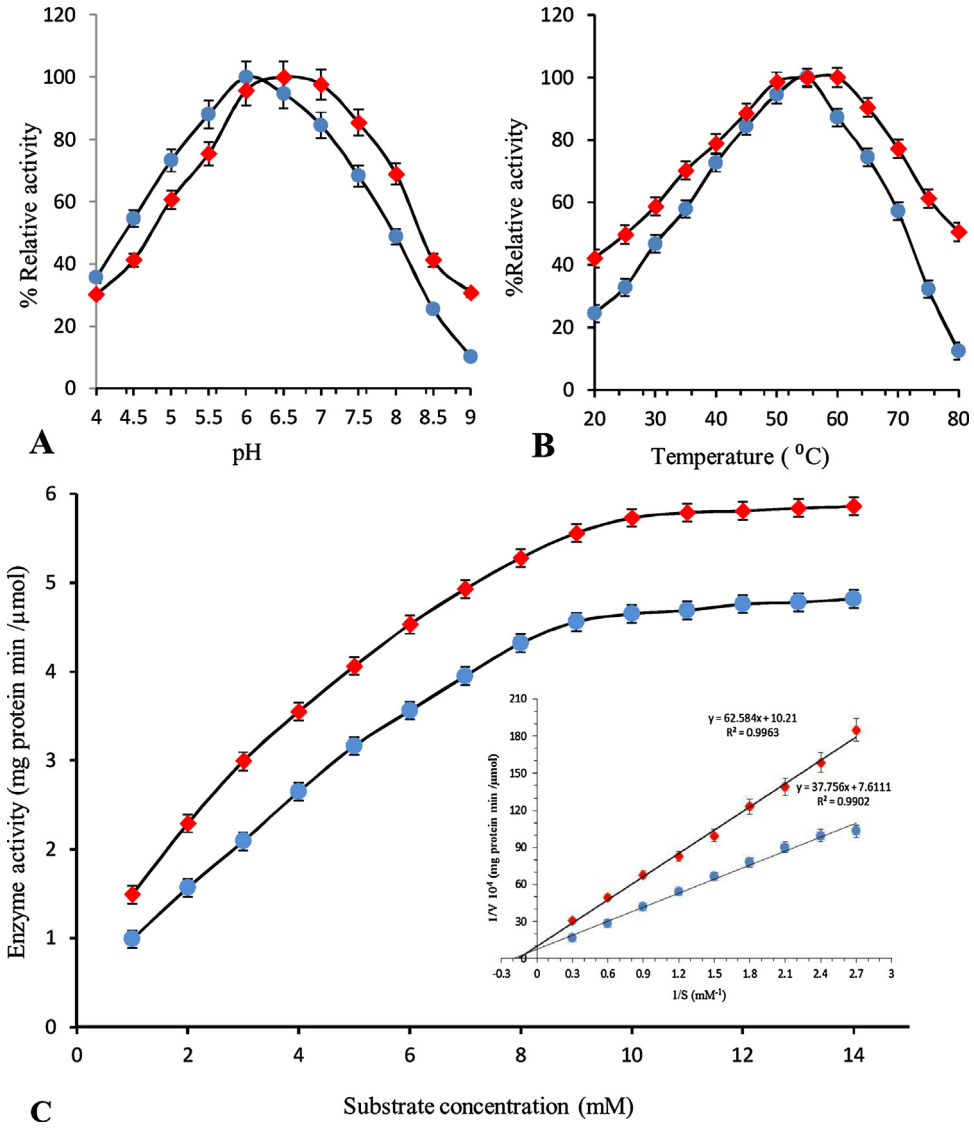

Fig. 5. Effect of pH (A), temperature (B) and substrate concentration (C) on activity of free (●) and co-immobilized chitinase/NAGase (◆). Lineweaver – Burk plot shown in the inset of Fig. 4C was used to calculate *K*m and *V*max values. Each assay was carried out using 3.3 mM sodium azide and 100 mM chitin in the reacion mixture, keeping the incubation time as 15 min. Effect of pH was studied at varying pH but at a constant temperature of 50 °C. For temperature studies, assays were carried out at the respective optimum pH for free (6.0) and co-immobilized enzymes (6.5), but at different temperatures ranging from 20 to 80 °C. Similarly, substrate concentrations were varied under optimum pH and temperature conditions to obtain the data for Fig. 4C.

exhibited saturation at 6.5 and 12.0 mM respectively (Fig. 5C), whereas the saturating concentrations of chitin for free chitinase and NAGase were found to be 7.0 and 6.5 mM respectively, which were comparable to the values obtained for free chitinase/NAGase but lower than the values obtained for co-immobilized enzymes. Lineweaver-Burk plots used for calculating the K_m and V_{max} values of free and co-immobilized chitinase/NAGase are shown in the inset of Fig. 5C. The value of K_m for PU/nano ZnO bound chitinase/NAGase was found to be 7.35 mM, which is higher compared to the *K*m of free chitinase/NAGase (5.49 mM), free chitinase (5.85 mM), free NAGase (4.76 mM) and the reported value of *K*m for phenyl sepharose bound chitinase (1.30 mM) [38]. The V_{max} value of 980.39 µmol (min mg protein)$^{-1}$ for PU/nano ZnO/chitinase/NAGase was lower than the V_{max} values obtained for either of the free enzyme preparation (Table 2). Reduced substrate availability due to altered conformation and orientation of the enzymes after immobilization may account for altered *V*max in the present study. Similar observations involving a lower V_{max} after immobilization have been reported by other researchers [41,42]. The turnover number (k_{cat}) for co-immobilized chitinase/NAGase ($2.26 \times 10^3\ s^{-1}$) was lower than the k_{cat} for free chitinase/NAGase ($4.61 \times 10^3\ s^{-1}$) but higher than the k_{cat} for free chitinase ($1.01 \times 10^3\ s^{-1}$) and free NAGase ($1.08 \times 10^3\ s^{-1}$). The catalytic efficiency of the enzymes measured by the ratio of k_{cat}/K_m, revealed that co-immobilized chitinase/NAGase was less efficient in catalyzing the conversion of substrate into products compared to the free form of chitinase/NAGase, but more efficient than either of the free chitinase or free NAGase (Table 2).

3.7. Thermal and storage stabilities

Thermal stability of both free and co-immobilized chitinase/NAGase preparations were determined after one-hour incubation at the respective temperature and then determining the residual activity under the optimum working conditions of pH, temperature and chitin concentration (100 mM). As shown in Fig. 6A, the activities of free and co-immobilized chitinase/NAGase decreased steadily with increase in temperature up to 70 °C, after

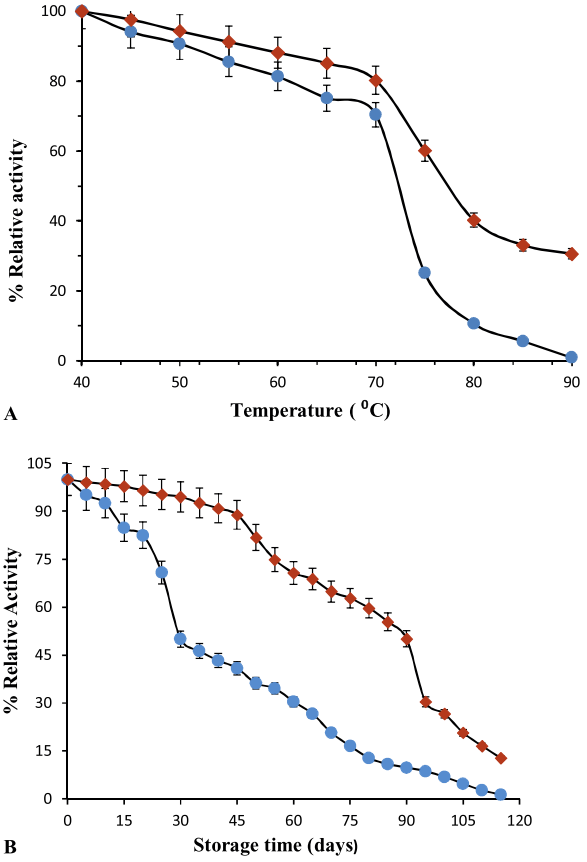

Fig. 6. (A) and storage stabilities (B) of free ( ) and co-immobilized chitinase/NAGase ( ). Residual activities after thermal treatment/storage were determined at 55 °C, pH 6.0 and at 60 °C, pH 6.5 for free and co-immobilized enzymes respectively. A chitin solution of 100 mM was used throughout.

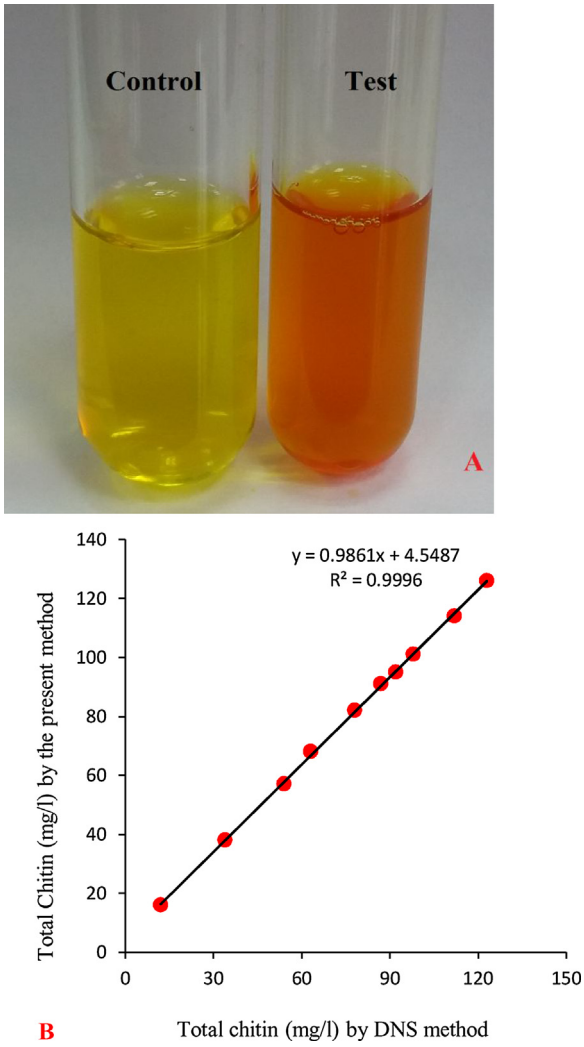

Fig. 7. A change in color after the colorimetric assay performed by the co-immobilized chitinase/NAGase on stored wheat grains as compared to control (A) and correlation between chitin values determined by DNS method (x axis) and by the present method (y axis) (B).

which the free enzymes denatured faster than the bound enzymes. Thermal treatment at 75 °C, induced an activity loss of about 75% for free chitinase, NAGase and chitinase/NAGase, whereas only 40% loss of activity for co-immobilized chitinase/NAGase was observed (Table 2). This difference in thermal stability could be attributed to the properties of hybrid support. ZnO nanoparticles themselves have high thermal stability and may enhance the heat absorption capacity of the hybrid support. Significant thermo stabilization of nano ZnO linked galactosidase [43] and nitrate reductase [44] has been reported in the literature. Moreover, as the reaction conditions change from being mild to harsh, formation of covalent linkages between the enzymes and the PU support gets optimized, enabling significant stabilization of the structure of enzymes [45]. The data obtained is comparable to the data for phenyl sepharose bound chitinase, which retained 45% activity after being exposed to 50 °C for more than 6 h [38].

Activities of free chitinase, NAGase, chitinase/NAGase and co-immobilized chitinase/NAGase preparations stored in sodium acetate buffer (0.02 M, pH 5.4) at 5 °C were checked on alternate days up to 4 months under the optimal assay conditions. Free and PU/nano ZnO conjugated chitinase/NAGase showed a gradual decline in activity during storage except for small regions of steep decline after 90 days in case of immobilized enzymes and after 40 days for free enzymes (Fig. 6B). The half-life of free chitinase, NAGase, chitinase/NAGase and co-immobilized chitinase/NAGase was around 25, 45, 30 and 90 days respectively, clearly suggesting the stabilizing effect of immobilization on the enzymes. This proves PU/nano ZnO to be a quite stable and suitable support for enzyme immobilization as the chitinase immobilized onto AS-L, phenyl sepharose and chitosan had a half-life of only 13, 15 days and 90 min respectively [35,36].

3.8. Detection and quantification of chitin in stored cereals

Colorimetric method for determination of chitin contents of the stored wheat grains was developed using PU/nano ZnO/chitinase/NAGase conjugates as sensing strips. The intensity of color change in the tested samples vis a vis control presented a visual confirmation of the assay (Fig. 7A), more specifically absorbance at 540 nm was recorded for exact quantification. The method was evaluated using various parameters included in Table 3. Linearity between chitin concentration and A_{540} nm was obtained from 0.1 to 10.0 mM with the lowest detection limit of 0.01 mM. Percent recoveries of added chitin (0.1 and 0.2 mM) in wheat samples were 95.03 ± 0.20 and 96.78 ± 0.35 respectively (n = 8, mean ± S.D.). Within and between-day variations in chitin measurements were employed to check the precision and reproducibility of the present method. The values for coefficients of variations for chitin determination in wheat samples (n = 8) were found to be 1.03% (within-day) and 1.78% (between-day), thus establishing the credibility of the method. The accuracy of the present method was evaluated by comparing chitin values of 20

Table 3
Evaluation of the method for chitin determination using PU/nano ZnO/chitinase/NAGase.

Sr. No.	Parameters Studied	Values
1.	Linearity (mM)	10.0
2.	Minimum Detection limit (mM)	0.01
3.	% Recoveries of added Chitin (Mean ± S.D, n = 8)	
	I. 0.1 mM	95.03 ± 0.20
	II. 0.2 mM	96.78 ± 0.35
4.	Coefficient of Variation (% n = 8)	
	I. Within Day	1.03%
	II. Between Day	1.78%
5.	Coefficient of determination R^2 (n = 20)	0.9996

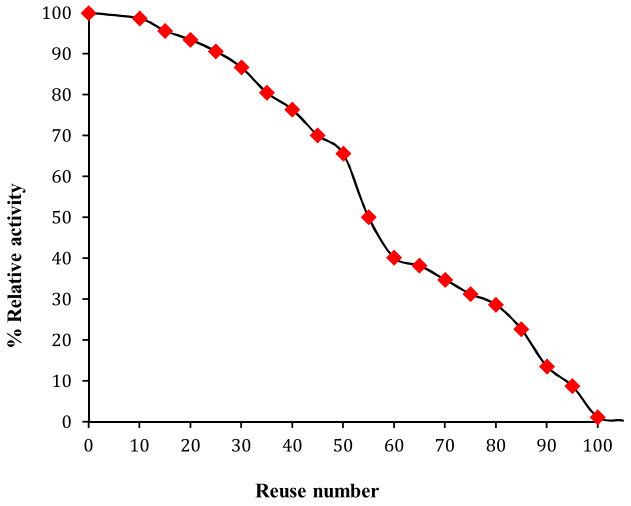

Fig. 8. Operational stability of co-immobilized chitinase/NAGase. Activity assays were performed on alternate days at pH 6.5, 60 °C and 100 mM chitin concentration.

stored wheat samples as determined by the present method with those determined by the popular 3,5-dinitrosalicylic acid method. The coefficient of determination (R^2 = 0.996) indicated that the regression line fitted the data pretty well and the chitin values obtained by the two methods showed good correlation (Fig. 7B). Though the wheat grains were apparently intact, not infested with insects and had no fouling but when tested with the present method showed presence of chitin, which is indicative of their contamination with dormant fungal spores. Hence, the method may prove useful for quality check of food grains on routine basis.

3.9. Operational stability

Reuse capacity of the co-immobilized chitinase/NAGase was determined at 60 °C and pH 6.5 at 100.0 mM concentration of chitin. Results presented in Fig. 8 showed that the co-immobilized enzymes retained 98.6% and 50% of the initial activities after 10 and 55 reuses respectively. Complete loss of activity was observed after 100 reuses. The results are comparable to that obtained for chitinase immobilized onto AS–L (70% activity retained after 10 batches of chitinolytic reactions) and on phenyl sepharose (45% retention of activity after 10 cycles and with tannin chitosan 100% retention of activity after 17 cycles) [37,38,46].

4. Conclusion

The two chitinolytic enzymes, chitinase and NAGase were co-immobilized onto PU/nano ZnO hybrid support (PU/nano ZnO/chitinase/NAGase) and used for developing an absorption based optical biosensor of determination of chitin contents in the stored wheat grains. The co-immobilized enzymes retained 96.19 ± 0.85% of the initial activity on the hybrid support and showed maximum activity when assayed at a pH of 6.5 and temperature 60 °C. Broader range of pH and temperature for activity, acceptable values for kinetic parameters and enhanced thermal and storage stabilities of the PU/nano ZnO/chitinase/NAGase conjugates in comparison to free enzymes also confirmed the suitability of the support. The effectiveness of the conjugates to sense the presence of chitin in stored wheat grains was proved by significant improvements in limit of detection and admissible values for analytical recovery, coefficient of variation and coefficient of determination. Overall, the results for chitin determination were consistent, reliable and reproducible. The method does not use any sophisticated instrument and may be aptly used for checking the quality of food grains/products on routine basis.

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