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Increasing the efficiency of immobilization and chitin determination using copper oxide nanoparticles

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ABSTRACT

A novel polyurethane (PU) support with and without copper oxide nanoparticles (nCuO) was employed for immobilization of two chitinolytic enzymes, chitinase and N-acetyl β glucosaminidase (NAGase) to yield PU/nCuO/chitinase/NAGase and PU/chitinase/NAGase conjugates respectively. The surface morphologies, topologies and bonded interactions between different components of the immobilized enzyme conjugates were characterized using Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) and Fourier Transform Infrared Spectroscopy (FTIR) respectively. High conjugation yield of 0.811 ± 0.10 mg/cm^2 with $97.66 \pm 0.50\%$ retention of specific activity of enzymes on PU/nCuO support vis-a-vis a conjugation yield of 0.531 ± 0.50 mg/cm^2 with $75.23 \pm 0.60\%$ retention activity on PU support was achieved. Additionally, increased pH and temperature tolerance, better kinetic parameters and enhanced stabilities of PU/nCuO/chitinase/NAGase conjugates established their superiority over the PU/chitinase/NAGase conjugates. However, both the conjugates were successfully employed for the determination of chitin contents in the apparently healthy stored rice grains. The methods were tested on analytical parameters such as linearity, limit of detection, the coefficient of variation and reproducibility of the externally added chitin, which were again found to be superior for PU/nCuO/chitinase/NAGase conjugates.

1. Introduction

Chitinase (EC, 3.2.1.14) and N–acetyl β glucosaminidase (NAGase, EC 3.2.1.30) also known as N–acetyl β hexosaminidase (NAHase, EC, 3.2.1.52) are the main chitinolytic enzymes that act in synergistic and consecutive manner to hydrolyze the chitin (fungal cell wall) successfully into reducing sugar, N–acetyl glucosamine [1]. Production of N–acetyl glucosamine is helpful in detection of the level of fungal contamination in plants and plant products along with its other industrial applications. A number of chemical methods, extraction and separation techniques such as HPLC, GS–MS and IR Spectroscopy are available for quantification of N–acetyl glucosamine but enzymatic methods are considered superior due to their sensitivity, selectivity specificity and environmentally benign nature [2–6]. The problems associated with the separation of free enzymes from the reaction medium and their reusability has shifted the focus towards the use of immobilized enzymes. Previously, both chitinase and NAGase have been co-immobilized onto CM-Sephadex, SP-Sephadex, tannin-sepharose, AH-sepharose, CNBr-sepharose, AS-alumina and tannin-chitosan [7], while chitinase alone has been immobilized onto CGP/PVA film, hydroxyl propyl methyl cellulose acetate succinate (AS-L), phenyl sepharose, CM-sepharose, Amber lite IRC50, activated charcoal, DEAE cellulose, swollen crab shell chitin and chitosan beads [8–11]. Mostly, immobilization onto these matrices occurs via physical adsorption, weak ionic bonding, covalent linkage or entrapment, which causes either the leaching of immobilized enzymes from the polymers or greatly affects the activity and stability of the bound enzymes [12]. Polyurethane (PU), a versatile material with diverse structural forms (foam, glue, layer, microsphere, and membrane) has also been suitably used for immobilization of enzymes such as amyloglucosidase, alkaline phosphatase and lipase [13–15]. PU offers the advantage of having a large number of functional groups on its surface, which on one hand provides stability to the enzyme by multipoint covalent attachment and prevents leaching but on the other hand also limits the conformational flexibility of the enzymes [16,17].

Another class of material that is increasingly employed for enzyme immobilization is nanoparticles. Amongst the various nanoparticles available, metal and metal oxide nanoparticles are preferably used for this purpose since they are physically stable, resistant to microbial attacks, biocompatible and are very active electrochemically [18]. Besides this, high surface area to volume ratio substantially scales up the amount of loaded enzyme. CuO nanoparticles (nCuO) are well

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known for their high electrocatalytic activity, uniform quantum size, and good crystallinity, thus widely used for immobilization of enzymes such as glucose oxidase, cholesterol oxidase, horseradish peroxidase, uricase etc [19–21]. A hybrid support prepared by layering nCuO on the PU surface is expected to adsorb a significant fraction of enzyme onto the nCuO, thus shielding the enzyme from entering into too many covalent linkages with the PU. Hence, the enzyme will be both flexible and stable. Earlier, PU core-nano gold shell conjugates have been used for immobilization of pepsin and endoglucanase with increased thermal and pH stabilities [22].

Herein, suitability of polyurethane/nCuO as immobilization supports for chitinase/NAGase has been evaluated. To categorically evaluate the difference contributed by nCuO, chitinolytic enzymes were also immobilized onto the bare PU support. A comparative account of the two immobilized enzyme preparations with respect to their kinetic parameters, thermal, storage and operational stabilities has been included. Finally, both the enzyme preparations were employed for chitin determination in stored cereals and evaluated in terms of their respective analytical performance.

2. Materials and methods

2.1. Materials

Chitinase (EC 3.2.1.14, ≥63.54 units/mg protein) was purified from *Vigna mungo* seeds by a pre-established protocol [23]. N–acetyl β glucosaminidase (NAGase, EC: 3.2.1.52, ≥23 units/mg protein) from jack bean (*Canavalia ensiformis*) was purchased from Sigma-Aldrich Co. (St. Louis, USA). Chitin from shrimp shell, copper chloride ($CuCl_2$), glacial acetic acid, sodium hydroxide (NaOH), sodium acetate, sodium carbonate, sodium azide, sodium potassium tartrate, Tris base, and 3, 5-dinitro salicylic acid (DNS) was taken from Hi-Media, Mumbai, India. Polyurethane available under the trade name “Vetaseal” was purchased from the local market. All other chemicals were of analytical reagent grade.

2.2. Synthesis and characterization of nCuO

To prepare nCuO, a pre-standardized protocol was followed with slight modifications [24]. In a typical reaction, 0.9 M aqueous solution of NaOH and 0.45 M aqueous solution of $CuCl_2$ were mixed together in 1:1 ratio and kept at 55 °C for 60 min under high-speed magnetic stirring. A visual change in the color of the reaction mixture from blue to black confirmed the synthesis of nCuO wet precipitates. The preparation was cooled to room temperature and its pH neutralized with 1.0 N HCl solution. The precipitated nCuO were thoroughly cleaned with double distilled water and air dried at about 60 °C. The size of CuO nanoparticles was determined using Transmission Electron Microscopy (TEM, FEI Tecnai S Twin) at Sophisticated Advanced Instrumentation Facility (SAIF), Department of Anatomy, AIIMS, New Delhi. Powder XRD patterns were recorded on X – Ray diffractometer (XRD, Rigaku Ultima-IV), at Department of Chemistry, M.D.University, Rohtak.

2.3. Preparation of chitinase/NAGase bound supports and their characterization

‘Vetaseal’ comprising of a urethane prepolymer having polyisocyanate moieties (R-N=C=O) and polyol (R′-OH) groups was layered evenly onto a polyethylene sheet of size 4 cm×4 cm and allowed to polymerize for 2 min at 30 °C. During polymerization, isocyanate groups reacted with the hydroxyl group of polyol to yield the PU support. An emulsion of nCuO (2.0 ml, 10.0 mg/ml) was spread evenly on this PU layer and kept for 5 min at room temperature for drying. Finally, 2.0 ml of the enzyme solution having 1.0 ml each of purified chitinase (98.50 units and 3.10 mg protein) and NAGase (83.33 units and 2.10 mg protein) was poured over the above-prepared PU/nCuO support. The enzymes covered support was kept at 4 °C for 48 h with intermittent gentle shaking after every 8 h. Thereafter, the support was washed thrice with aliquots of sodium acetate buffer (0.02 M, pH 5.4). Each washing was collected separately and tested for the presence of protein by the method of Lowry et al. (1951) [25], using BSA as the standard protein. The amount of protein bound to the support was calculated by subtracting the protein content of washings from the amount that was initially added to the support. Chitinase and NAGase were immobilized onto the bare PU support in a similar fashion except for the addition of nCuO to the support. The immobilized enzyme preparations were stored in 0.02 M sodium acetate buffer pH 5.4, at 4 °C when not in use.

Bare PU, PU/nCuO, PU/chitinase/NAGase and PU/nCuO/chitinase/NAGase conjugates were characterized by Scanning Electron Microscopy (SEM) to know the differences in their surface morphologies and by Fourier Transform Infrared Spectroscopy (FTIR) to obtain the emission pattern corresponding to the presence of PU, nCuO and enzymes. SEM (LEO 435 VP 501 B-SEM, Zeiss) studies were carried out at Sophisticated Advanced Instrument Facility (SAIF), Department of Anatomy, AIIMS, New Delhi and FTIR (Alpha, Bruker, Germany) was done at Department of Genetics, Maharishi Dayanand University, Rohtak. The surface topology imaging of chitinase/NAGase immobilized onto bare and nCuO loaded PU was also done by NT -MDT Solver P47-Pro instrument separately in contact mode of atomic force microscopy (AFM) at CSIR – National Physical Laboratory, New Delhi. Freely available software Gwyddion 2.47 was used to visualize the three dimensional AFM images and also to compute their surface roughness parameters.

2.4. Assay of chitinase/NAGase

The chitinolytic activity of enzymes was determined by quantifying the amount of reducing sugar, N-acetyl amino-glucose produced from the digestion of chitin as per the method of Boller and Mauch (1988) with little modifications [26]. Colloidal solution of chitin of (100.0 mM, pH 5.4) was prepared as described by Sheng et al. (2002) [27] and 1.0 ml of it was added to the reaction mixture containing, 0.2 ml of sodium azide (3.3 mM), 0.8 ml of sodium acetate buffer (0.02 M, pH 5.4) and 1.0 ml of purified chitinase/NAGase solution (49.25 units of chitinase and 41.65 units of NAGase) in a total volume of 3.0 ml. After incubating the reaction mixture for 15 min at 50 °C, 2.0 ml of DNS reagent was added to stop the reaction. The mixture was heated in boiling water bath for 5 min and absorbance measured at 540 nm. The activity of immobilized enzymes was also determined in the same manner except that PU/chitinase/NAGase or PU/nCuO/chitinase/NAGase conjugates were used in place of soluble enzymes and the volume of the reaction buffer was raised by 1.0 ml in the reaction mixture. One unit of chitinase/NAGase activity was defined as the amount of enzyme that liberated 1.0 μg N-acetyl amino-glucose per min at pH 5.4 and 50 °C.

2.5. Optimization of immobilized enzymes

In order to maximize the activities of PU/chitinase/NAGase and PU/nCuO/chitinase/NAGase conjugates, pH, temperature and chitin concentrations of the assay mixture were varied. All experiments related to optimization of immobilized enzymes were carried out in triplicate and the results were expressed as mean ± S.D. Errors bars were used in the graphs to express the deviations from mean values.

To study the effect of pH on the activity of immobilized enzymes, pH of the assay buffer was varied from 4.0 to 9.0, using 0.02 M sodium acetate buffer in the pH range of 4.0–6.0, 0.01 M sodium phosphate buffer for pH between 6.0 to 8.0 and 0.01 M borate buffer in pH range of 8.0–9.0. Temperature dependent reaction rates were studied at optimum pH, by varying the incubation temperature from 20 to 80 °C