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Covalent Immobilization of β-galactosidase onto Electrospun Nanofibers of Poly (AN-*co*-MMA) Copolymer

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ABSTRACT: Immobilization of β-galactosidase in poly (acrylonitrile-*co*-methyl methacrylate) poly (AN-*co*-MMA) Nanofibers was studied by electrospinning, and a spacer-arm i.e., (Polyethyleneimine (PEI)) was covalently attached by the reaction of carbonyl groups of Poly (AN-*co*-MMA) nanofibers. β-galactosidase was then covalently immobilized through the spacer-arm of the Poly (AN-*co*-MMA) nanofibers by using glutaraldehyde (GA) as a coupling agent. Nanofibers mode of interaction was proven by FTIR and thermal gravimetric analysis and supported by morphological changes recognized through SEM examination. Factors affecting the modification process such as PEI concentration, reaction time, and reaction temperature have been studied. Its influence on the amount of coupled PEI was consequently correlated to the changes of the catalytic activity and the retained activity of immobilized enzyme, the main parameters judging the success of the immobilization process. Evidences of Poly (AN-*co*-MMA) nanofibers modification were extracted from morphological changes recognized through SEM examination. The maximum activity (V_{max}) and michaelis constant (K_m) of immobilized enzyme were found to be 8.8 μmole/min mg protein and 236.7 m*M*, respectively. Stabilities of the immobilized β-galactosidase were obviously improved. The optimum temperature for β-galactosidase immobilized on the spacer-arm attached nanofiber was 5°C higher than that of the free enzyme and was also significantly broader. The immobilized β-galactosidase had better resistance to temperature inactivation than did the free form. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: functionalization of polymer; surface modification; electrospinning; nanofiber

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INTRODUCTION

Electrospinning is a handy and cost-effective technique for producing nanowebs where the fiber diameters are in the range of a few hundred nanometers to a few microns. The technique is very promising and versatile since it facilitates the production of multifunctional nanofibers from various polymers, polymer blends, sol–gels, composites, ceramics, etc.[1–4] With this technique, a continuous filament is electrospun from polymer solutions[5] or polymer melts[6] under a very high electrical field, which resulted in the form of nonwovens consisting of nanofibers. Nanowebs produced by the electrospinning technique have several remarkable functional characteristics such as a very large surface area to volume ratio, pore size within the nano range, unique physical and mechanical properties along with the design flexibility for chemical/physical surface functionalization. It has been shown that the outstanding properties and multifunctionality of the nanowebs make them favorable candidates to be used in many areas including biotechnology (tissue engineering, controlled/sustained release systems),[7–9] membranes/filters,[9,10] textiles,[11] etc.

The diameter of electrospun nanofiber depends on a number of parameters that may be divided into two groups. Intrinsic parameters are intrinsic properties of the polymer solution (molecular weight, concentration, surface tension, viscosity, conductivity, etc.) and the environment (temperature, humidity, pressure, etc.) Control parameters, in contrast, are the parameters that may be manipulated easily in a manufacturing environment, which include applied voltage, flow rate, and distance between the nozzle and the collector. The nanofibers can be easily recovered from reaction media and be applied for continuous operations. Jia et al.[12] immobilized a-chymotrypsin on the surface of polystyrene nanofibers (120 nm) produced by

electrospinning and showed that the enzyme loading was 1.4% (wt/wt). Jiang et al.[13] studied the feasibility of lysozyme immobilization by electrospinning the enzyme-containing dextran solution into nanofibrous membranes (200–400 nm).

β-galactosidase (EC 3.2.1.23) from different sources is currently used in the production of lactose free milk products. Hydrolysis of lactose improves product sweetness, makes milk consumption by people who suffer from lactose intolerance possible, and increases product quality and process efficiency in the dairy industry. This hydrolysis reaction could also be applied to the upgrading of cheese whey, a product of cheese processing, disposal of which constitutes a problem.[14,15]

In this study poly (AN-*co*-MMA) polymer solution was used to produce nanofibers by electrospinning using a newly designed nanospider. The carbonyl groups of the poly (AN-*co*-MMA) nanofiber were converted into amino groups in the presence of spacer-arm (PEI). β-galactosidase was also immobilized onto spacer-arm-attached poly (AN-*co*-MMA) nanofibers using glutaraldehyde as coupling agent. The aminated poly (AN-*co*-MMA) nanofibers were used for the immobilization of β-galactosidase after activation with glutaraldehyde. This technique depends on the reaction between the available carbonyl group on poly (AN-*co*-MMA) nanofibers surface and the amine groups of PEI, glutaraldehyde (GA), which finally covalently binding with enzyme. The value of K_m for immobilized β-galactosidase was significant larger, indicating decreased affinity by the enzyme for its substrate, whereas V_{max} values were smaller for the immobilized β-galactosidase. The optimum pH and temperature for the free and immobilized enzymes were investigated.

MATERIALS AND METHODS

Materials

Methyl Methacrylate (MMA) was purchased from ACROS (USA), Acrylonitrile (AN), and Glutaraldehyde (GA) were obtained from BDH Chemical (England), β-galactosidase (E.C.3.2.1.23), Potassium persulfate ($K_2S_2O_8$), Brom Phenol Blue (BPB), and polyethylenimine (PEI) [low mol wt, 50% soln. in water, M_n 1.800 (GPC); Typical M_w 2.000] were supplied from the Sigma Chemical (St. Louis, MO), Tetrahydrofuran (THF), and Piperidin were obtained from Fluka AG (Switzerland), Lactose monohydrate GRG was obtained from WINLAB Chemical (England), All other chemicals were of analytical grade and were purchased from Sigma Chemical (St. Louis, MO). Tetrahydrofuran (THF, HPLC grade) was used for gel permeation chromatography (GPC) as received.

Preparation of (Poly (AN-*co*-MMA)) Copolymer

Poly (AN-*co*-MMA) copolymer in the particle form was prepared by solution polymerization.[16] The copolymer prepared by mixing with volume ratios of water: ethanol of 70: 30 as cosolvent, $K_2S_2O_8$ (0.01*M*) as polymerization initiator, mix the cosolvent with the initiator at the room temperature, after compiled dissolve the initiator in the cosolvent add AN and MMA (1 : 1), the total volume of comonomer to the cosolvent is 10%, The polymerization was carried out at 55°C for 4 h; the polymer was isolated by filtration and washed successively with heat distilled water to remove any impurities such as residual monomer and initiator. The product was then dried in an oven at 55°C for 24 h. A white powder was obtained as a product.

Nanospider Electrospinning of Poly (AN-*co*-MMA) Copolymer

Electrospinning Setup. The copolymer solutions were electrospun using needleless laboratory machine NS LAB 500S based on Nanospider™ technology developed by Elmarco s.r.o., Czech Republic. The NSLAB 500S consists of spinning head tub where rotating spinning electrode is wetted in solution under high voltage. Nanofibers are coating exchangeable substrate belt which is moving along the static collecting electrode. Internal control parameters of the process are electrode distance, high voltage, electrode speed, and substrate speed. External parameters used for control of electrospinning throughput and nanofiber quality are solution characteristics (viscosity, conductivity) and air properties (temperature, relative humidity). Nanospider setup was used to spin the poly (AN-*co*-MMA) copolymer. The procedure was typically as follows: poly (AN-*co*-MMA) copolymer was dissolved in aqueous Tetrahydrofuran (THF) in concentrations 10% (w/v). Poly (AN-*co*-MMA) copolymer aqueous Tetrahydrofuran (THF) solutions were fed into drum of NANOSPIDER (High Voltage Up to 80 kv DC, Elmarco, Czech Republic). A drum was used to feed polymer solution into the collector. Electrospinning parameter for electrospinning of (AN-*co*-MMA) copolymer nanofibers were as follows: Speed of drum = 5.3 rpm, voltage = 60.1 kV, Electrode distance= 19 mm, humidity = 31%, and temperature = 20°C), a positively charged jet of the (AN-*co*-MMA) copolymer aqueous ethanol solution formed from the drum and sprayed to a grounded drum. Tetrahydrofuran (THF) electrospun nanofibers were deposited and collected on the grounded drum.

Poly (AN-*co*-MMA) Electrospun Nanofibers Surface Modification

Poly (AN-*co*-MMA) nanofibers were aminated by treating it with excess of an aqueous solution of Polyethylenimine (PEI). The nanofibers were immersed in PEI solution at 70°C for 1 h with gentle shaking. The nanofibers were collected and washed successively with hot distilled water to remove the excess PEI. The aminated nanofibers were dried in an oven at 55°C for 24 h. The morphology of the aminated nanofiber was examined by SEM. The amine content of the modified nanofibers was tested by determination of amine group onto nanofiber. In the case where a quantitative evaluation of the functional end-group the UV–visible absorption spectroscopy was used for the determination of the functionalization yields.

Determination of Amine Groups onto the Copolymer Nanofibers

A stock solution of BPB was prepared by dissolving 10 mg/mL in DMF. Totally, 500 mL of the stock solution were diluted in 50 mL DMF. The nanofibers were incubated for 30 min in this solution and rinsed with ethanol. BPB forms a complex with amine groups.[17] This complex is stable against ethanol wash but can be removed by incubating the nanofibers in 20% piperidin in DMF for 10 min. The OD of the eluent was measured at 605 nm and used to quantify the amine groups on the sensors surface by using a modification of Lambert Beer's law [eq. (1)].

$$S_{NH_2} = \left(\frac{\text{nmol}}{\text{cm}^2}\right) = \frac{OD_{65} \times V \times 10^6}{\varepsilon_{605} \times A \times d} \quad (1)$$

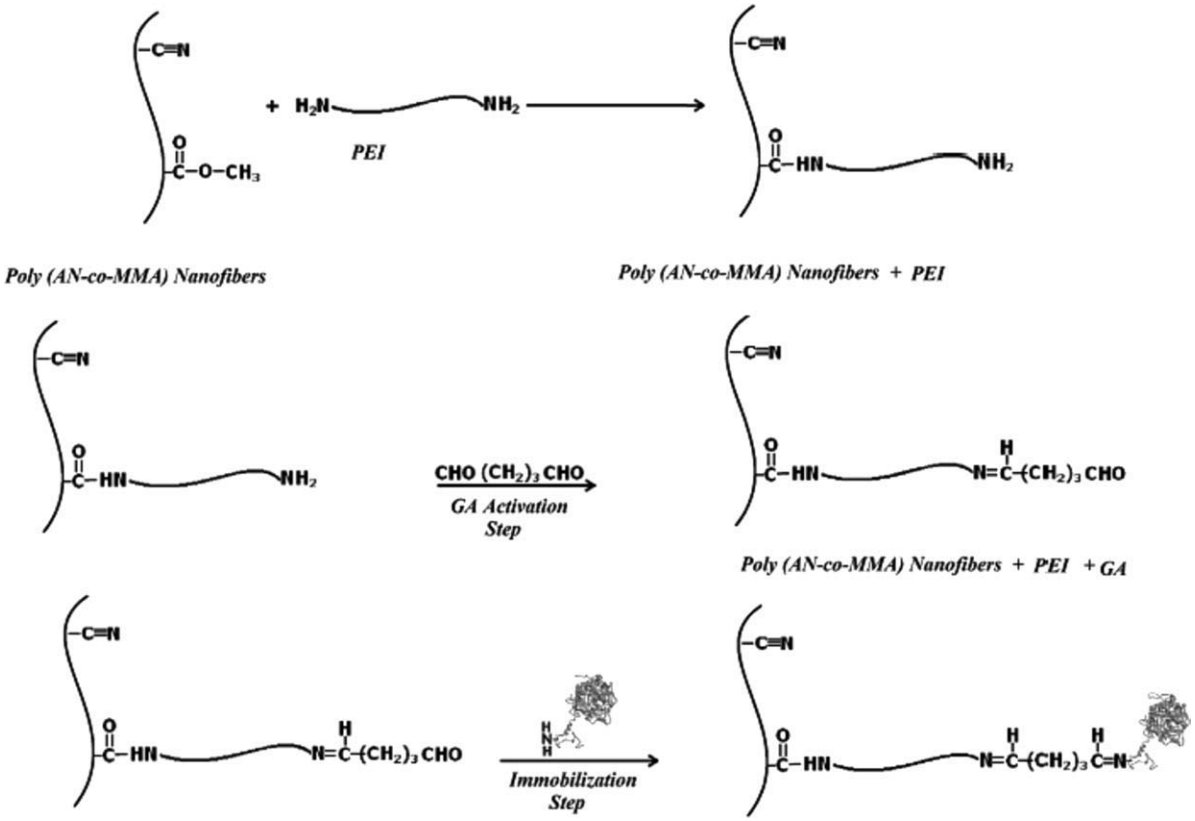

Figure 1. Schematic diagram of β-galactosidase immobilization onto electrospun nanofibers of poly (AN-*co*-MMA) copolymer.

The molar extinction coefficient (ε_{605}) for BPB is 91 800 per (mol/L) per cm.[16] (*V*) The volume of the piperidine solution in ml (cm^3). (*A*) Is the area of the slides (13.7 cm^2) and (d) the length of the optical path in the cuvette (1 cm). The term is multiplied by 10^6 in order to obtain the result in n mol per cm^2.

Immobilization of β-galactosidase in Poly (AN-*co*-MMA) Nanofibers

Poly (AN-*co*-MMA) modified nanofibers, was activated using 20 mL of glutaraldehyde (1%, pH 8.0 at 40°C for 60 min). After completion of the activation process, the poly (AN-*co*-MMA) activated nanofibers were taken out and rinsed with distilled water to remove unreacted glutaraldehyde. The activated poly (AN-*co*-MMA) nanofibers were then transferred to enzyme Phosphate-citrate buffer solution (Figure 1). β-galactosidase solution (5.0 mg/20 mL) was prepared by adding β-galactosidase powder to Phosphate-citrate buffer (pH 4.4). A designed weight of nanofibers was submerged in 20 mL of β-galactosidase solution in a vertical orientation and shaken gently in a water bath at 40°C for 1 h. Then, for 16 h at 4°C to complete the immobilization process, the nanofibers were taken out and rinsed with buffer until no soluble protein was detectable in washings.

Determination of the Activity for Free and Immobilized β-galactosidase

The activity of the immobilized β-galactosidase on the nanofibers in aqueous medium was determined according to the method reported previously.[18] The catalytic activity of the immobilized enzyme was determined by mixing 13.7 cm^2 of catalytic poly (AN-*co*-MMA) nanofibers with 50 mL of 100 m*M* lactose in phosphate-citrate solution of pH = 4.4 at 40°C in a vertical orientation and shaken gently in a water bath, for 30 min. Samples (0.1 mL each) were withdrawn every 5 min to assess the produced glucose using glucose Kit. The enzymatic activity was determined by the angular coefficient of the liner plot of the glucose production as a function of time.

Determination of the Kinetic Parameters Enzyme Preparation

To obtain the Michaelis–Menten kinetic models adequate for the description of the hydrolysis of lactose by the free and the immobilized enzyme, apparent K_m and V_{max} of free and immobilized β-galactosidase were determined for lactose using the Hanes–Woolf plot method, eq. (2)[19]:

$$\frac{[S]}{V_0} = \frac{1}{V_{max}} \times [S] + \frac{K_m}{V_{max}} \quad (2)$$

Where [*S*] is the substrate concentration (lactose), V_0 is the initial enzyme velocity, V_{max} is the maximum enzyme velocity, and K_m is the Michaelis constant and is defined only in experimental terms and equals the value of [*S*] at which V_0 equals 1/2 V_{max}. Experimentally, the K_m from the plot is equal to $-[S]$, whereas the V_{max} is equal to 1/slope. The kinetic constants were determined using lactose as substrate in the concentrations range (20–250 m*M*) using free and immobilized β-galactosidase as described above. The experiments were conducted under the

optimized assay conditions. The apparent K_m and V_{max} values for the free and immobilized β-galactosidases were calculated from Hanes–Woolf plot by using the initial rate of the enzymatic reaction.

Retention of Activity, R.A (%)

The success of any immobilization process is governed basically by keeping almost, if not all, of the enzyme activity after completion of the immobilization process, this factor known as retention of activity % eq. (3):

$$\text{Retention of activity (\%)} = \frac{\text{Measured activity of immobilized enzyme } (A)}{\text{Measured activity of free enzyme } (B)} \times 100 \quad (3)$$

where (B) is the measure activity of free enzyme for the same amount of immobilized one.

Characterization of Poly (AN-*co*-MMA) Copolymer

Morphological Characterization. The surface morphology of poly (AN-*co*-MMA) nanofiber and modified poly (AN-*co*-MMA) nanofiber observed with the help of a scanning electron microscopy (Joel JSM-6380 LA, Japan) at an accelerated voltage of 20 kV. The fracture surfaces were vacuum coated with gold for SEM. The average diameters of the electrospun poly (AN-*co*-MMA nanofibers were determined by measuring and averaging the diameter of $\sim$ 50 random nanofibers in each sample using scanning electron microscopy software (SMILE VIEW SOFTWARE developed by JOEL) after sputtering by gold.

Gel Permeation Chromatograph Analysis. Main characterization of the copolymers synthesized in this work was carried out by gel permeation chromatography (GPC) using Viscotek Model 350 HT with multidetection. Four detectors were coupled to the GPC technique: refractive index, viscometer, infrared, and multi-angle light scattering. And vortex Auto preparation/Auto sampler Module for automated sample preparation and delivery, Model 1122 Isocratic Pump for reproducible flow rates with an absence of pump pulsation, and Model 7510 Degasser for elimination of air and other solvent gases. All detectors reside within a temperature-controlled oven compartment (30–175°C). Molecular weight (M_n) calculations were based on a linear calibration curve obtained using eight narrow molecular weight polystyrene standards, the measurements were taken at 30°C with tetrahydrofuran as the mobile phase on one column, and analysis the data by *OmniSEC* GPC software for complete system control, data acquisition and processing.

FTIR Spectroscopy. FTIR spectra were recorded using FTIR spectrometer, Bruker, TENSOR Series FTIR Spectrometer, Germany, connected to a PC, and analysis the data by IR Solution software, analytical methods are standard in OPUS^{TM} software.

Thermal Characterization. The thermal degradation behaviors of the poly (AN-*co*-MMA) nanofiber, aminated poly (AN-*co*-MMA) nanofiber, and activated poly (AN-*co*-MMA) nanofiber were studied using Thermo Gravimetric Analyzer (TA Instruments, Q500 TGA, USA); instrument in the temperature range from 20 to 800°C under nitrogen at a flow rate of 40 mL/min and at a heating rate of 10°C/min.

RESULTS AND DISCUSSION

Preparation of the Poly (AN-*co*-MMA) Copolymer

Poly (AN-*co*-MMA) copolymer bearing an amino group at one end are key intermediates for various purposes because of the large nucleophilic character of the amino group, allowing reactions which are particularly useful in biological applications. The Poly (AN-*co*-MMA) copolymer was synthesized from the monomers acrylonitrile and methyl methacrylate in ethanol/water medium using 0.01M ($K_2S_2O_8$) as initiator.[16] The carbonyl groups of the poly (AN-*co*-MMA) nanofibers were converted into amino groups in the presence of PEI during the activation process.[20] Different amination and activation process for the nanofibers were investigated and its impact on catalytic activity, retention of activity of immobilized β-galactosidase, and concentration of the amine group in the nanofiber was also investigated.

GPC Behavior of Poly (AN-*co*-MMA) Copolymer

Gel permeation chromatography is the most popular tool for determining the molecular weights of polymers. Nevertheless, the molecular weights reported by GPC are actually those of the equivalent standard polystyrene hydrodynamic volume. In other words, information obtained from GPC depends on the hydrodynamic volume of polymers in the mobile phase rather than their molecular weights. Poly (AN-*co*-MMA) copolymer molecular weight (M_n 110,452 Daltons; M_w/M_n 1.588 determined by GPC) was carried out to obtain the poly (AN-*co*-MMA) copolymer sample in this study (Figure 2). Therefore with this technique it is possible to get complete information about copolymer distribution and conformation in solution of the copolymer chains. The SEM micrographs of poly (AN-*co*-MMA) copolymer sample in this study in the particle form was prepared by precipitation copolymerization are shown inside Figure 2.

Morphology of Poly (AN-*co*-MMA) Electrospun Nanofibers

Poly (AN-*co*-MMA) copolymer with a synthesized by precipitation copolymerization process and then electrospun into nanofibers, the electrospinning process was carried out using needles electrospinning setup called Nanospider which can afford higher area of electrospinning. It was found that the nanofiber diameter of the resulted fibrous membranes could be controlled by varying the concentration of poly (AN-*co*-MMA) copolymer solution. Almost homogenous networks (nanofibers) with fiber diameter ranging from 80 to 1300 nm were obtained. SEM micrographs of poly (AN-*co*-MMA) nanofiber fabricated by electrospinning are shown in Figure 3. Changes of chemical structure of poly (AN-*co*-MMA) nanofiber always reflect on its morphological characters. As shown in Figure 4, the morphology of the poly (AN-*co*-MMA), aminated poly (AN-*co*-MMA), activated poly (AN-*co*-MMA) with GA and immobilized poly (AN-*co*-MMA) nanofiber. In poly (AN-*co*-MMA), pure fibers of practically infinite length were obtained [Figure 3(A)]. The poly (AN-*co*-MMA) nanofibers are smooth and beadless with the average fiber diameter as 320 nm calculated by Image software. In the Figure [Figure 3(B)] show SEM pictures of aminated poly (AN-*co*-MMA) nanofiber, [Figure 3(C)] activated poly (AN-*co*-MMA) with GA, and [Figure 3(C)] immobilized poly (AN-*co*-

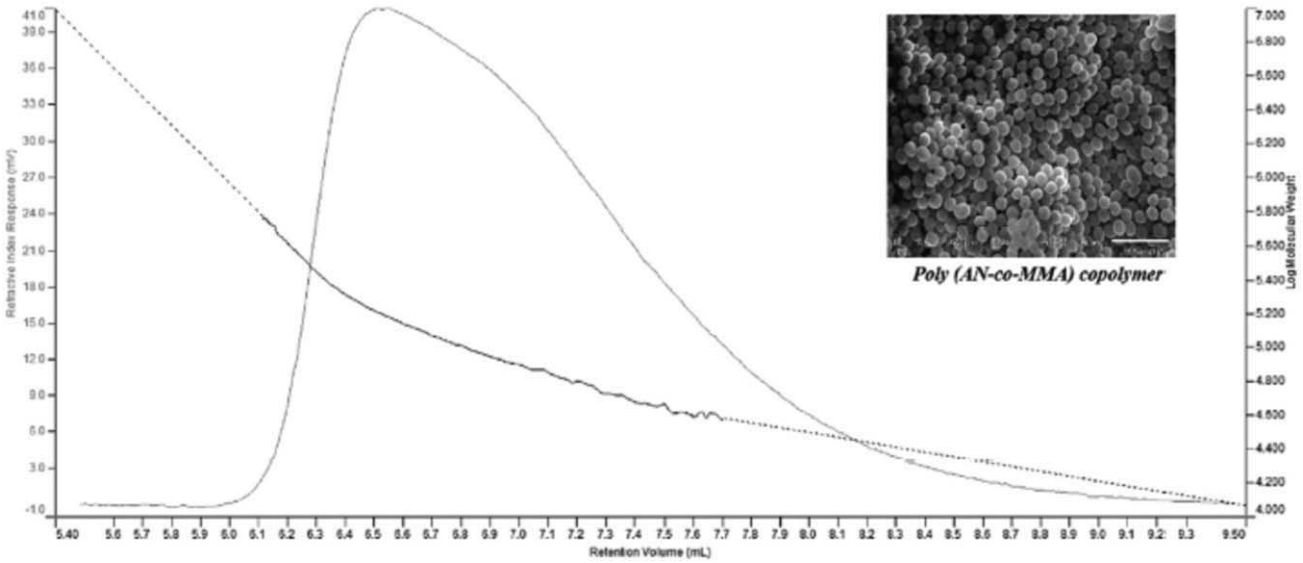

Figure 2. GPC chromatograms of poly (AN-*co*-MMA) copolymer and SEM micrograph.

MMA) nanofiber. The morphology of (AN-*co*-MMA) nanofiber changed from smooth nanofibers to porous, uniform nanofibers, rough, and thick because of the modification by the PEI, and GA. Its average diameter is 685 nm. This may be due to the crosslinking between PEI and the copolymer which consequently affect the nanofiber properties [Figure 3(B)]. It is clear

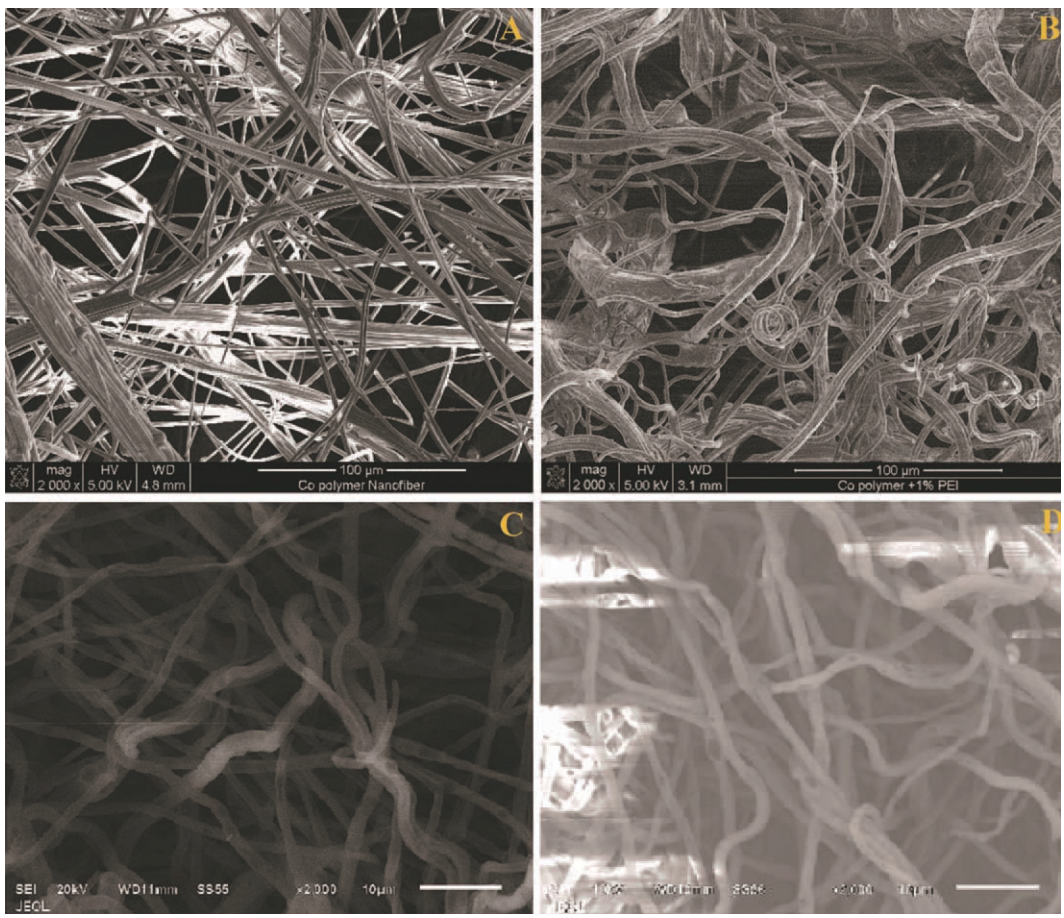

Figure 3. SEM photographs of poly (AN-*co*-MMA) nanofibers electrospun from THF solutions (A) poly (AN-*co*-MMA) nanofiber, (B) aminated poly (AN-*co*-MMA), (C) Activated poly (AN-*co*-MMA) with GA, and (D) Immobilized poly (AN-*co*-MMA) nanofiber.

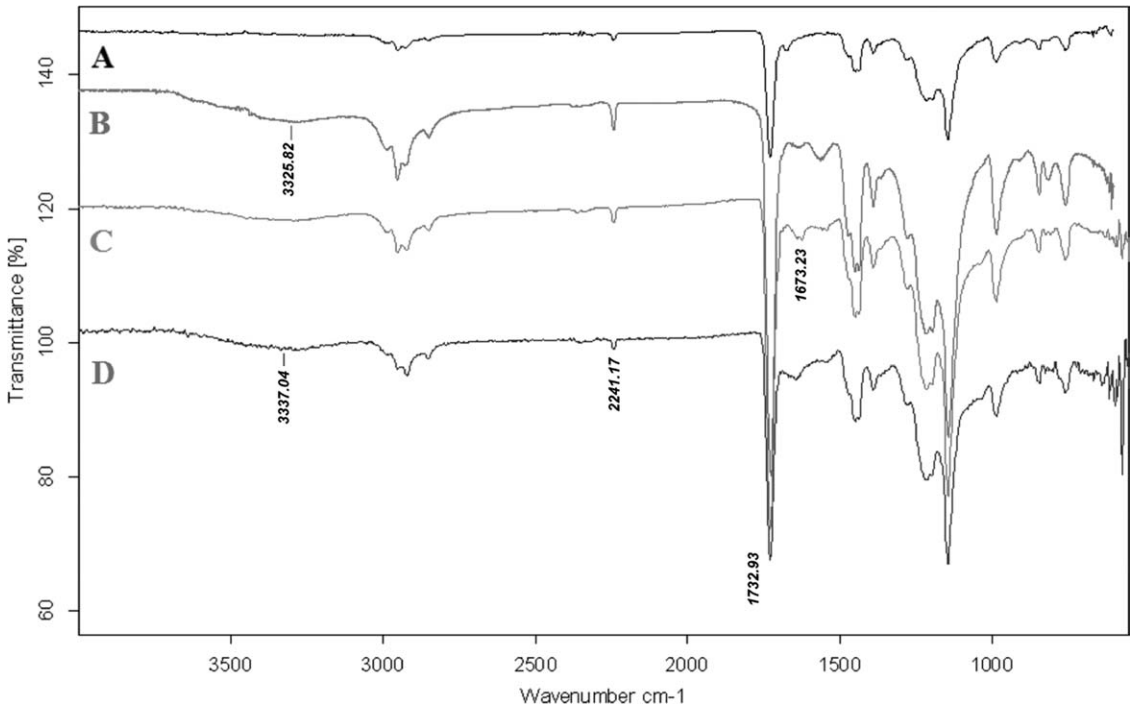

Figure 4. FTIR spectra for (A) poly (AN-*co*-MMA) nanofiber, (B) aminated poly (AN-*co*-MMA) nanofiber, (C) Activated poly (AN-*co*-MMA) with GA, and (D) Immobilized poly (AN-*co*-MMA) nanofiber in the range of 500–4000 cm^{-1}.

that the morphological structure of poly (AN-*co*-MMA) nanofiber differ from modified and activated poly (AN-*co*-MMA) nanofiber. The surface morphology of poly (AN-*co*-MMA) nanofiber after modification with PEI, GA, and enzyme shows a porous and uniform fibers structure and the resulting morphology was remarkably heterogeneous, showing large fibers. These images confirmed the successful immobilization of β-galactosidase on the poly (AN-*co*-MMA) nanofibers and also proved that the covalent treatment is an effective method to immobilize the β-galactosidase on the electrospun polymer nanofibers.

FTIR Spectroscopic Analysis

The IR spectra in the range 600–4000 cm^{-1} for poly (AN-*co*-MMA) nanofiber, aminated nanofiber, glutaraldehyde-activated nanofiber, and immobilized nanofibers are shown in Figure 4. The structure of the poly (AN-*co*-MMA) is characterized by typical absorption bands at around 1732 cm^{-1} for C═O and 2241 cm−1 forC≡N. The secondary structure of the glutaraldehyde-activated nanofiber; from the chart it is clear the appearance of characteristic band of CHO groups at 1637 cm^{-1}. Figure 4(C), the presence of such band proved the fictionalization of the surface with aldhyde groups as a result of modification with glutaraldehyde. The FTIR spectra of aminated nanofiber had absorption bands different from that of the poly (AN-*co*-MMA) nanofiber [Figure 4(A)] at 3325.82 and 1633 cm^{-1} corresponds to the —NH_2 stretching vibration and N—H deformation, respectively, are due to the incorporation of the amine groups on the nanofiber structure [Figure 4(B)].

Thermal Analysis

Figure 5 shows the thermogram of electrospun poly (AN-*co*-MMA) nanofiber, PEI aminated nanofiber, and glutaraldehydeactivated nanofiber. It is clear that attaching of glutaraldehyde molecules onto the surface of the nanofibers backbone enhances the thermal stability of the poly (AN-*co*-MMA) nanofiber. This was reflected on the shift of half weight loss temperature (T_{50}) of poly (AN-*co*-MMA) nanofiber from 360 to 379°C of glutaraldehydeactivated nanofibers. Also in the weight loss, percent at temperature ranged from 100 to 400°C. Such behavior confirms the formation of new chemical structure different from the native Poly (AN-*co*-MMA) nanofiber structure. Possible cross-linking is also expected.

Immobilization of β-Galactosidase

Enzyme immobilization has been a popular strategy for most large-scale applications due to the ease in catalyst recycling, continuous operation, and product purification. Poor biocatalytic efficiency of immobilized enzymes, however, often limits the development of large-scale bioprocessing to compete with traditional chemical processes. Improvements of biocatalytic efficiency can be achieved by manipulating the structure of carrier materials for enzyme immobilization. Nanofibers materials, to which enzymes are attached to the surfaces are subjected to minimum diffusion limitation while enzyme loading per unit mass of support is usually low.[21–24] Reduction in the size of enzyme-carrier materials can generally improve the efficiency of immobilized enzymes. Electrospun nanofibers provide a large surface area for the attachment or entrapment of enzymes and the enzyme reaction. The conditions affecting the activation process such as PEI concentration, incubation time, and incubation temperature were investigated. The impact of variation conditions on the amount of attached PEI molecules and consequently on the activity and retention of activity for the

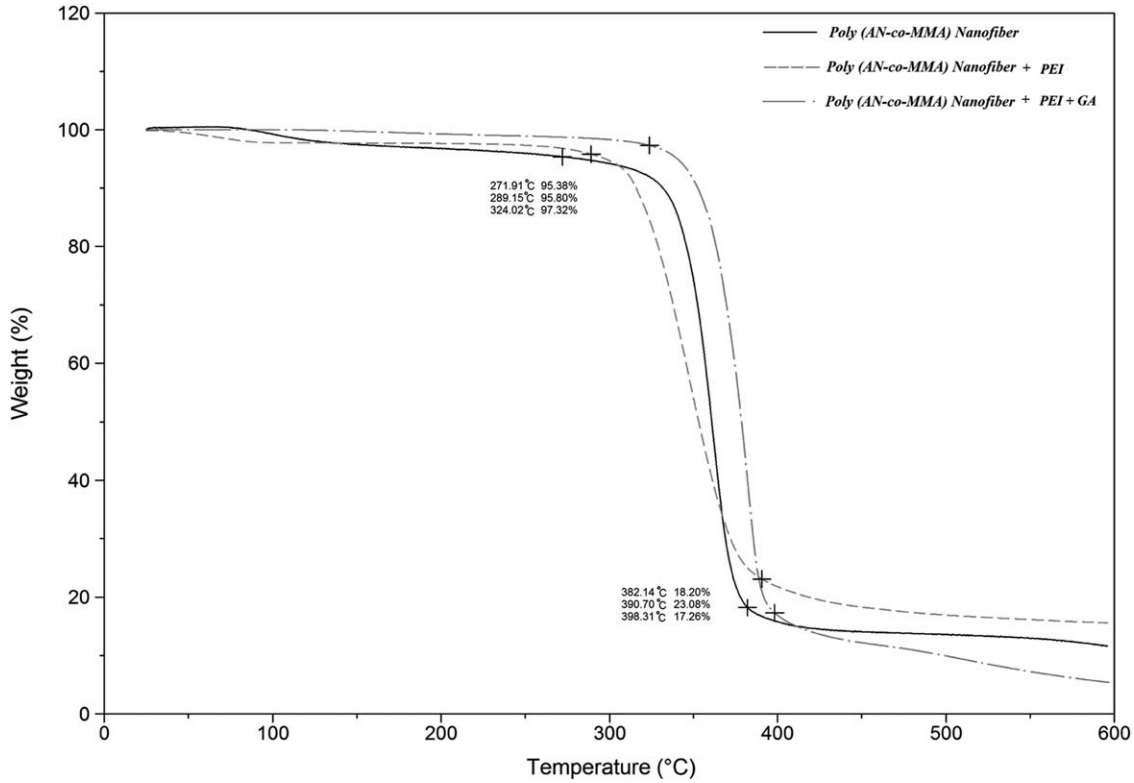

Figure 5. TGA analysis: for (A) poly (AN-*co*-MMA) nanofiber, (B) aminated poly (AN-*co*-MMA) nanofiber, (C) Activated poly (AN-*co*-MMA) with GA.

immobilized β-galactosidase was followed. And study the kinetics parameters; optimum pH and temperature for the free and immobilized enzymes were investigated. The obtained results are discussed in the following.

Effect of PEI Concentration on Coupling of Amine Groups and Catalytic Activity

Figure 6 shows the effect of variation PEI concentration on the attached amount of PEI, catalytic activity, and retention of

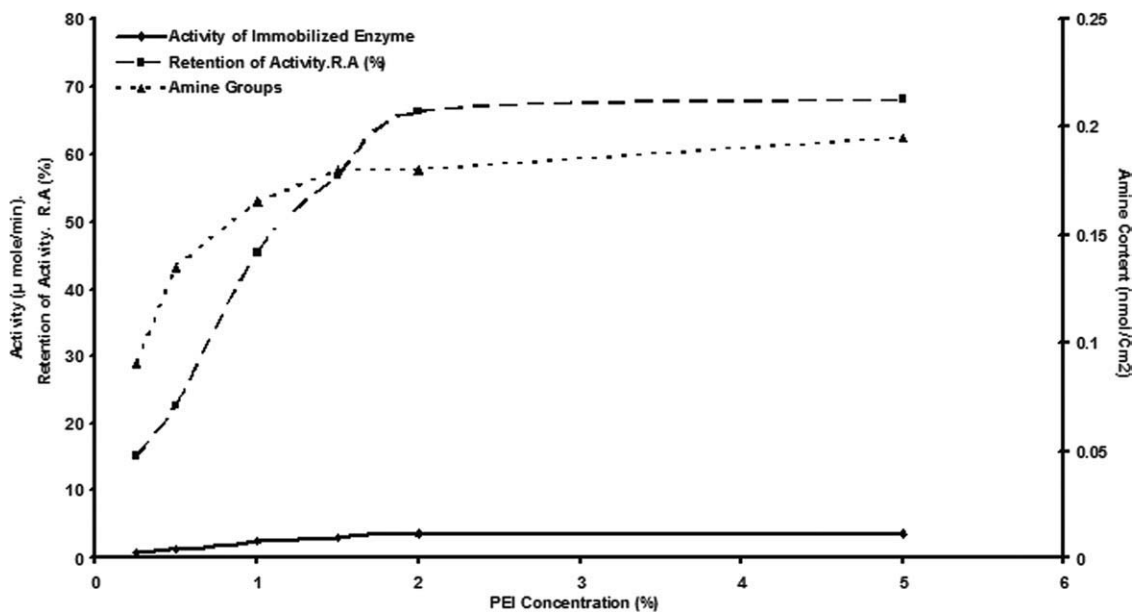

Figure 6. Effect of PEI concentrations on immobilized enzyme catalytic activity, retention of activity, and amine content.

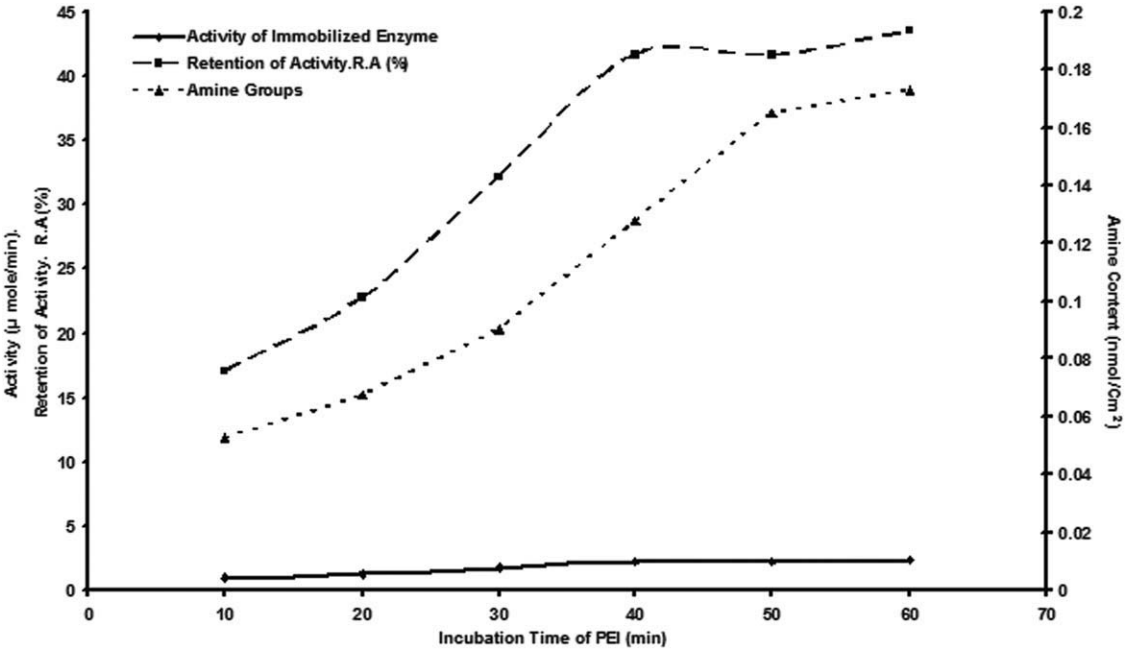

Figure 7. Effect of incubation time with PEI on immobilized enzyme catalytic activity, retention of activity, and amine content.

activity of immobilized β-galactosidase. From the figure, it is clear that increasing the PEI concentration, increase all the above mentioned parameters. The obtained values of amine concentration were in the range of 0.09–0.3 nmol/cm^2. The influences of variation of PEI concentration on the amine content is shown in Figure 6, which showed an increase in the amine content of the nanofiber with the increase in the concentration of PEI up to 1%. This was expected since the amount of amine is increased, there is a liner increase of the amine content. About 38.5% has been observed with increase of PEI concentration from 0.25% to 1%. Further increase in the concentration to 2 and 3%, was followed by increase of the amount of amine in the nanofiber. The behavior of increase of amine content with increase the PEI concentration could is expected due to availability of the amine groups to react with the free terminal carbonyl groups on the Poly (AN-*co*-MMA) leading to the formation of more bonds between amine and the polymer functional groups. On the other hand, both catalytic activity and retention of activity of immobilized enzyme were found increased with the attached PEI amount. This behavior of increasing the activity could be explained in the light of increased the free terminal amine groups on the surface which increases the possibility of more than aldehyde ends of glutaraldehyde to react with two amine terminal groups of enzymes leading to the formation of multiattachment bonds. This leads consequently to increase the "response" of immobilized enzyme and directly the catalytic activity of poly (AN-*co*-MMA) nanofibers.

Effect of Incubation Time with *PEI* on Coupling of Amine Groups and Catalytic Activity

The effect of reaction time of the copolymer with PEI was investigated. The results clearly indicated that the reaction time affected the yield of coupling of amine groups (Figure 7). Linear increment of amine groups has been observed with increasing reaction time up to 30 min after which, exponential increment obtained with reaction time increase up to 1 h. This could be attributed to the reason that at higher reaction time with PEI allow higher amount of amine to react with functional groups either on the surface or on the bulk of the material.[25] As expected increasing the reaction time, increases the chance for the diffusion of the amine to the bulk functional groups. Further increase of the reaction time of PEI with poly (AN-*co*-MMA) nanofibers up to 40 min did not lead to a significant change in the activity of immobilized β-galactosidase. The activity at 60 min reaction time still higher than the activity at both 40 and 50 min reaction time. On the other hand, with increasing activation time from 10 to 60 min, the number of covalently immobilized β-galactosidase molecules increases relative to the total immobilized molecules. This leads directly to increase the retained activity of immobilized enzyme.

Effect of Incubation Temperature with *PEI* on Coupling of Amine Groups and Catalytic Activity

Figure 8 shows the effect of variation reaction temperature with PEI on the attached amount of amine, catalytic activity, and retention of activity of immobilized β-galactosidase. From the figure, it is clear that increasing the PEI concentration, increase all the above mentioned parameters but with different rates. The attached amount of PEI increased exponentially up to about 0.15 nmol/cm^2 with 60°C then slightly decreased, and the catalytic activity of poly (AN-*co*-MMA) nanofibers. From the figure it is clear that increases the temperature, within studied range (40–60°C), has liner positive effect on the catalytic activity and the retention of activity, the explanations could be given to interpretation the obtained results. The probability of formation multiattached covalent bonds with enzyme molecules leads to improvement of the activity and the retention of

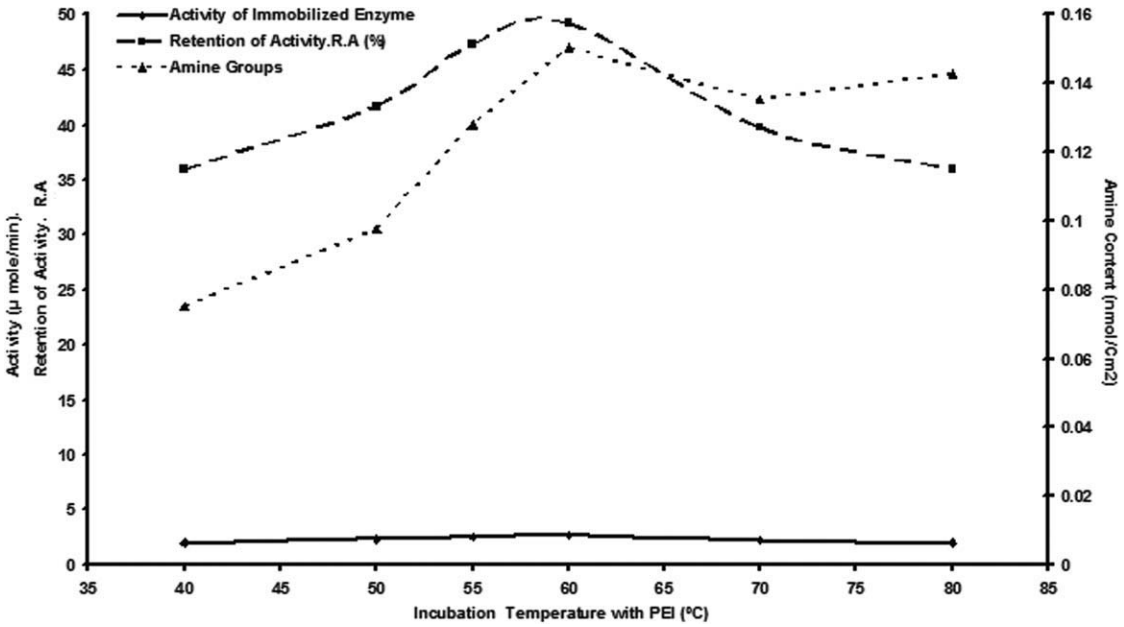

Figure 8. Effect of incubation temperature with PEI on immobilized enzyme catalytic activity, retention of activity, and amine content.

activity, after that the activity slightly decreased at 70–80°C. The explanation which focuses on the formation of multi covalent attachment bonds between the enzyme molecules and active aldehyde end groups, on the nanofiber surface. Increasing the number of attachment points leads to two things. The first is "freezing" of the conformational structures of enzyme molecules which make its active center not free succible to the substrate. The second is creating of "protein–protein" interaction which causes substrate diffusion limitation. Both causes lead finally to reduction of the catalytic activity.

Biochemical Properties of Free and Immobilized β-galactosidase

Effect of pH on the Free and Immobilized β-galactosidase Activity. The pH is one of the important parameters capable of altering enzymatic activities in aqueous solution. The effect of pH on the activity of the free and immobilized preparations for lactose hydrolysis was examined in the pH range from 2.0 to 7.0 at 40°C and the results are presented in Figure 9. The pH value for optimum activity for the free β-galactosidase was found to be 4.4. On the other hand, The pH profile of the immobilized enzyme was much broader with respect to the free enzyme, the optimal pH for the immobilized β-galactosidase is found to have shifted to pH range 4.0–5.2. The shift to neutral region for the immobilized enzyme can be due to the basic nature of the amino functionalized surface of the poly (AN-*co*-MMA) nanofibers; these observations suggested a significant alteration of enzyme environment upon immobilization on the nanofibers and consisted with documented literature.[26] It is known that polyionic matrices cause the partitioning of protons between the bulk phase and the enzyme microenvironment causing a shift in the optimum pH value. The shift depends on the method of immobilization as well as on the structure and charge of the matrix.[20] The pH profiles of the immobilized β-galactosidase display an improved stability on both sides of the optimum pH value, in comparison to that of the free form, which means that the immobilization method preserved the enzyme activity in a wider acidic range. The pH stability of the immobilized β-galactosidase was found to be higher than that of native β-galactosidase. The difference was significant in the acidic range (Figure 9).

Effect of Temperature on the Free and Immobilized β-galactosidase Activity. The effect of temperature on the free and immobilized enzyme activity was investigated in phosphate-citrate buffer (pH 4.4) in the temperature range 25–70°C. The apparent temperature optimum for free enzyme was about 45°C, while that for the immobilized enzyme and the form stabilized by immobilization was about 50°C (Figure 10). In the literature, most immobilized β-galactosidase exhibited higher optimum temperature values than their free counterpart.[20] The increase in optimum temperature was caused by the changing physical and chemical properties of the enzyme. The multipoint covalent attachment of the β-galactosidase molecule on the poly (AN-*co*-MMA) nanofibers surface via PEI and glutaraldehyde coupling could reduce the conformational flexibility of the enzyme and might result in higher activation energy for the molecule to reorganization the proper conformation for the binding to its large substrate, in the temperature range from 25°C to optimum appear linear and activation energies were found to be 9.5 and 7.4 kcal mole^{-1} for free and immobilized.[27] Thus, the immobilized β-galactosidase showed its catalytic activity at a higher reaction temperature compared with that of the free counterpart.

Kinetic Parameters

The kinetic parameters of the free and immobilized β-galactosidase, V_{max} and K_m, were assayed using various initial substrate concentrations from 20 to 250 m*M*, as shown in Figure 11. V_{max}, the maximum reaction rate, reflects the intrinsic characteristics of the enzyme. K_m indicates the effective diffusion

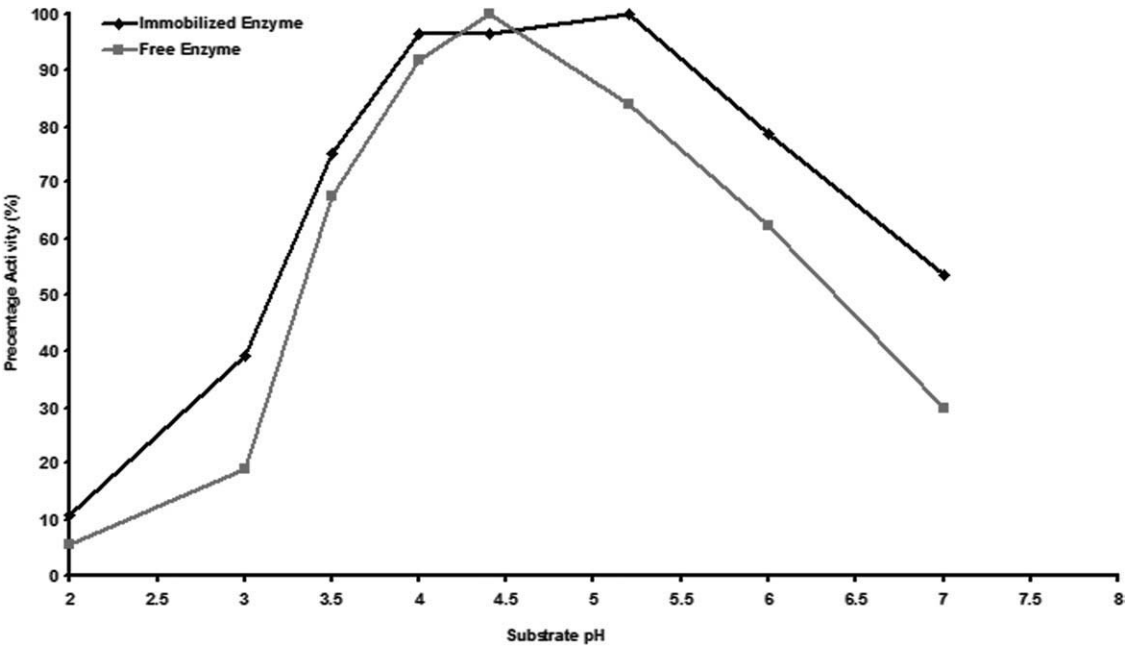

Figure 9. pH profiles of the free and immobilized β-galactosidase preparations.

characteristics of the enzyme. V_{max} and K_m are the kinetic parameters used for determining the enzymatic reaction and diffusion effects.[23] The kinetic parameters K_m and V_{max} of the free and immobilized enzymes were determined by using lactose as substrate. The activities of free and immobilized β-galactosidase for various concentrations of the substrate were plotted in the form of Hanes-Woolf plots, as shown in Figure 11, and K_m and V_{max} values were calculated from the intercepts on *x*- and *y*-axes, respectively. K_m values were estimated as 146.8 and 236.7 m*M* for the free and immobilized β-galactosidase, respectively. The apparent K_m value of the immobilized β-galactosidase was one times higher than that of the free enzyme. The V_{max} value of the immobilized enzyme increased about 0.5 μmole/min mg protein, compared with the free enzyme, the V_{max} value for the immobilized β-galactosidase is (8.8 μmole^{-1}min mg protein), which is similar to (8.3 μmole^{-1}min mg protein) for free β-galactosidase. This indicates that the present immobilization method shows tighter binding between the enzyme and

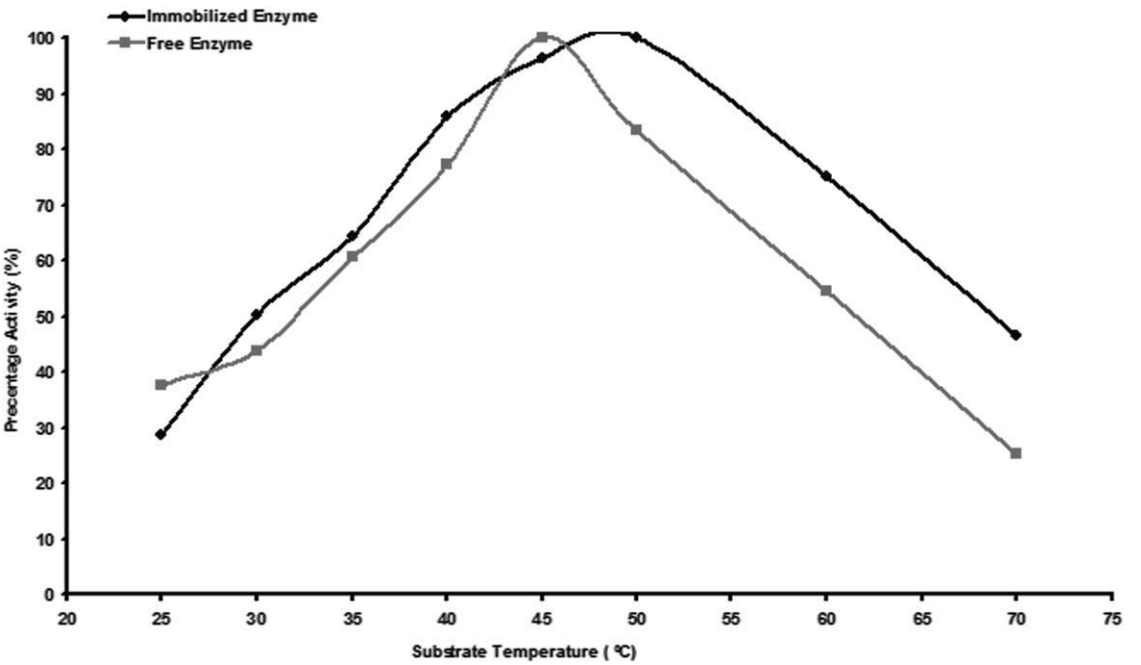

Figure 10. Temperature profiles of the free and immobilized β-galactosidase preparations.

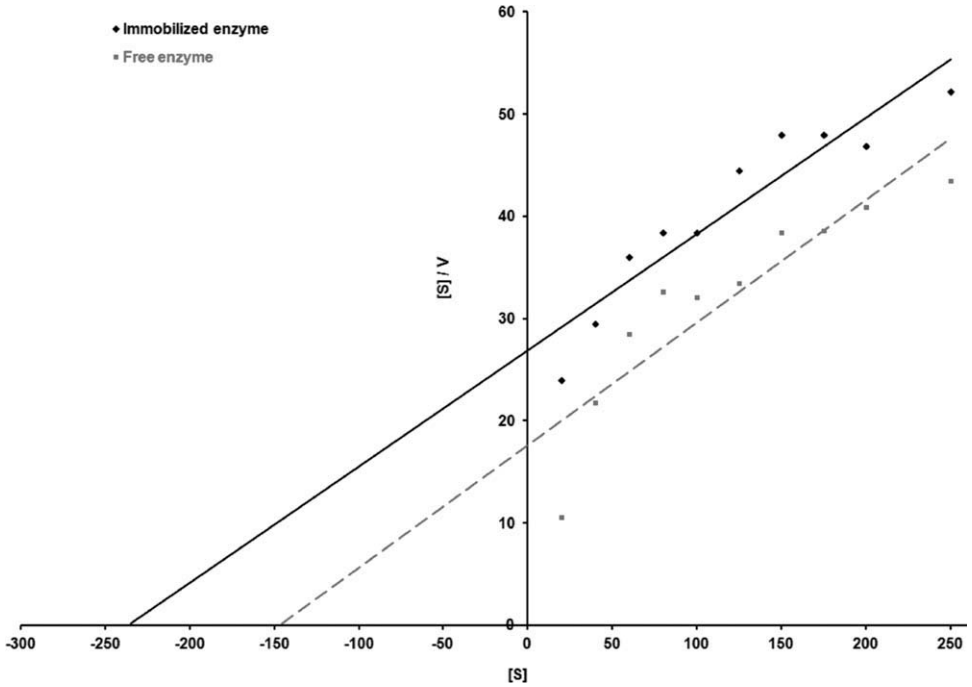

Figure 11. Hanes-Woolf plot curve for free β-galactosidase, and immobilized β-galactosidase on the poly (AN-*co*-MMA) nanofibers.

substrate. The large K_m value after enzyme immobilization, caused by the conformational changes of the enzyme, resulted in a lower affinity for substrate contact. This also may be caused by the lower accessibility of the substrate to the active site of the immobilized enzyme by the increased diffusion limitations.[28] When β-galactosidase is immobilized on the surface of the poly (AN-*co*-MMA) nanofibers, the exposed β-galactosidase active sites can easily make contact with the substrate and then efficiently transfers from the substrate to the active site.

CONCLUSIONS

In summary, the work introduced a novel support; one of the most important aims of enzyme technology is to enhance the conformational stability of the enzyme. The extent of stabilization depends on the enzyme structure, the immobilization methods, and type of support. Poly (AN-*co*-MMA) nanofibers could be fabricated by electrospinning and the enzyme molecules could be covalently bound to the nanofiber with PEI and GA. With the huge specific surface area provided by the nanofiber, Poly (AN-*co*-MMA) nanofibers were successfully modified with spacer-arm (PEI) and as a result its surface has been functionalized with amine groups. Immobilized β-galactosidase retains 68% of its initial activity. After immobilization, maximum PEI concentration and medium reaction time with PEI gave the best results of catalytic activity of immobilized enzyme; 5.0% PEI and 60 min respectively. Reaction temperature with PEI for 60°C was found as optimum temperature needed for reach to the best catalytic activity. Surface aminated poly (AN-*co*-MMA) nanofibers were activated using glutaraldehyde as a coupling agent. There was a 0.9-fold increase in K_m value for immobilized enzyme and in addition, immobilized β-galactosidase can retain most of the activity at lower pH values when compared with the free enzyme. This immobilized system may have industrial application for milk hydrolysis

REFERENCES

1. Greiner, A.; Wendorff, J. *J. Angewandte Chemie Int. Ed.* **2007,** *46*, 5670.
2. Li, D.; Xia Y. *J. Adv. Mater.* **2004,** *16*, 1151.
3. Chronakis, I. S. *J. Mater. Proc. Technol.* **2005,** *167*, 283.
4. Huang, Z.; Zhang, Y.; Kotaki, M.; Ramakrishna, S. *J. Compos. Sci. Technol.* **2003,** *63*, 2223.
5. Reneker, D.; Chun, I. *J. Nanotechnol.* **1996,** *7*, 216.
6. Dalton, P.; Grafahrend, D.; Klinkhammer, K.; Klee, D.; Moller, M. *J. Polym.* **2007,** *48*, 6823.
7. Pham, Q.; Sharma, U.; Mikos, A. *J. Tissue Eng.* **2006,** *12*, 1197.
8. Schiffman, J.; Schauer, C. *J. Polym. Rev.* **2008,** *48*, 317.
9. Burger, C.; Hsiao, B.; Chu, B. *J. Ann. Rev. Mater. Sci.* **2006,** *36*, 333.
10. Barhate, R.; Ramakrishna, S. *J. Membr. Sci.* **2007,** *296* (1–2), 1.
11. Lee, S.; Obendorf, S. *J. Appl. Polym. Sci.* **2006,** *102*, 3430.
12. Jia, H.; Zhu, G.; Vugrinovich, B.; Kataphinan, W.; Reneker, D. H.; Wang, P. *J. Biotechnol Prog.* **2002,** *18*, 1027.

13. Jiang, H. L.; Fang, D. F.; Hsiao, B. S.; Chu, B.; Chen, W. *J. Biomacromol.* **2004,** *5*, 326.
14. Prashanth, S.; Mulimani, V. *J. Proc. Biochem.* **2005,** *40*, 1199.
15. Zhou, Q.; Chen, X.; Xuemeli, L. *J. Biotechnol. Bioeng.* **2003,** *81*, 127.
16. Hafez, E.; El-Aassar, M. R.; Khalil, K.; Al-Deyab, S. S.; Taha, H. T. *African J. Biotechnol.* **2011,** *10*, 19658.
17. Krchnak, V.; Vagner, J.; Lebl, M. *Int. J. Peptide Protein Res.* **1988,** *32*, 415.
18. Mohy Eldin, M.; Hassan, E.; Elaassar, M. R. *Deutsche Lebensmittel-Rundschau. 101. Jahrgang, Heft* **2005,** *6*, 255.
19. El-Masry, M.; De Maioa, B.; Martelli, C.; Casadio, C.; Moustafa, A.; Rossi, A. *J. Mol. Catalysis. B, Enzymatic* **2001,** *16*, 175.
20. Arica, M.; Yavuz, H.; Patir, S.; Denizli, A. *J. Mol. Catalysis B: Enzymatic* **2000,** *11*, 127.
21. Elnashar, M.; Yassin, A. *J. Appl. Biochem. Biotechnol.* **2009,** *159*, 426.
22. Arica, M.; Hasirci, V.; Alaeddinoğlu, N. *J. Biomater.* **1995,** *16*, 761.
23. Aksoy, S.; Tümtürk, H.; Hasirci, N. *J. Biotechnol.* **1998,** *60*, 37.
24. Mohy Eldin M. S.; Seuror, E. I.; Nasr, M. A; El-Aassar, M. R.; Tieama, H. A. *J. Appl. Biochem. Biotechnol.* **2011,** *164*, 10.
25. Akkoyun, A.; Bilitewski, U. *J. Biosens. Bioelectron.* **2002,** *17*, 655.
26. Bayramoglu, G.; Tunali, Y.; Arica, M. *J. Catalysis Commun.* **2007,** *8*, 1094.
27. Ye, P.; Xu, Z.; Che, A.; Wu, J.; Seta, P. *J. Biomater.* **2005,** *26*, 6394.
28. Li, S.; Chen, J.; Wu, W. *J. Mol. Catalysis B: Enzymatic.* **2007,** *47*, 117.