Journal of Molecular Catalysis B: Enzymatic 74 (2012) 132–137

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Polyaniline nanofiber as a novel immobilization matrix for the anti-leukemia enzyme L-asparaginase

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ARTICLE INFO

Article history:
Received 2 March 2011
Received in revised form 8 September 2011
Accepted 15 September 2011
Available online 22 September 2011

Keywords:
Nanostructures
Polyaniline
Polymerization
Enzyme immobilization

ABSTRACT

In this study, polyaniline nanofibers are used as a novel immobilization support and evaluated using L-asparaginase as a model protein enzyme for precise investigation of the immobilized state of enzymes as well as its functional activity on polyaniline nanofiber surfaces at different concentrations. The supported enzyme has been characterized by XPS, XRD, TEM and FTIR, and the effect of polymer nanofiber conformations on the enzymatic activity is discussed in detail. Immobilized L-asparaginase showed greater stability towards decomposition/denaturation at different temperatures and pH conditions compared to the free enzyme. The calculated K_m values are 1.809 and 3.705 mM L^{-1} for immobilized L-asparaginase, respectively, which are 2.05-fold lower in magnitude in comparison with the value obtained for free L-asparaginase; the V_{max} values for immobilized and free L-asparaginase are 90.57 and 48.04 μM min^{-1} mg^{-1} respectively.

1. Introduction

L-Asparaginase (L-asparagine amidohydrolase; EC 3.5.1.1) is a tetrameric protein with a molecular weight of 140 kDa that irreversibly catalyses the hydrolysis of L-asparagine to L-aspartic acid and ammonium ion under physiological conditions [1,2]. This enzyme gained importance in the Pharmaceutical industry as a choice for the treatment of acute lymphoblastic leukemia and lymphosarcoma, and in clinical experiments relating to tumour therapy, often in combination with chemotherapy. However, its potential application in anti-tumour treatment depends on the affinity of the enzyme towards a substrate, stability in various biological environments (pH, temperature and ionic strength), and the clearance rate by plasma. The major limiting factor in pharmaceutical application is its short half-life *in vivo* ($t_{1/2}$ = 1.2 days), necessitating multiple injections [3], which causes hypersensitivity (mild allergic reactions to anaphylaxis) and immunogenicity [4]. Consequently, attention is being given to prolong the half life, improve the stability in the body, decrease antigenicity and lower the affinity to natural inhibitors. One approach to improve the stability and functional property is by chemical modification via immobilization on biocompatible polymers.

In this context, many polymeric materials like polypyrrole and oxidases were studied as matrix materials for enzyme immobilization [5]. Among these, polyaniline (polymers, nanofibers and composites) is gaining importance in catalysis as energy storage devices, sensing materials [6] and as a biological mediator [7,8] because of its environmental stability (high stability to extremes of temperature and pH and resistance towards microorganisms), unique conduction mechanism (a direct electron transfer capability between an enzyme and a polymer), ease of preparation and high yield in synthesis. In addition, Liu et al. showed its biocompatibility nature in PC-12 cells [5,6,9–11]. In fact, conducting polymer nanofibers as immobilization matrices are preferred over other carrier materials (typical membranes) due to the smaller size (denoting large specific area) of the fiber, higher porosity (higher enzyme loading per unit mass with reduced diffusion resistance and the shortened path for substrate diffusion), higher conductivity and more facile fabrication, hence, they have great potential for enzyme immobilization with high efficiency, in situ formation of nanofiber reinforcement composites, biosensors, and biocatalysis/separation. Polyaniline-immobilized enzymes have also been applied in the fields of biosensing and biofuels [12–14]. Interestingly, little effort has been given to their medical applications and very few reports are available on L-asparaginase immobilization such as liposomes [15] and poly(D,L-lactide-co-glycolide) (PGLA) [4,16].

In the present study, L-asparaginase (known for its anti-leukemia properties) was chosen as a model protein enzyme to evaluate polyaniline nanofibers (PANI) as an immobilization

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1381-1177/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.molcatb.2011.09.009

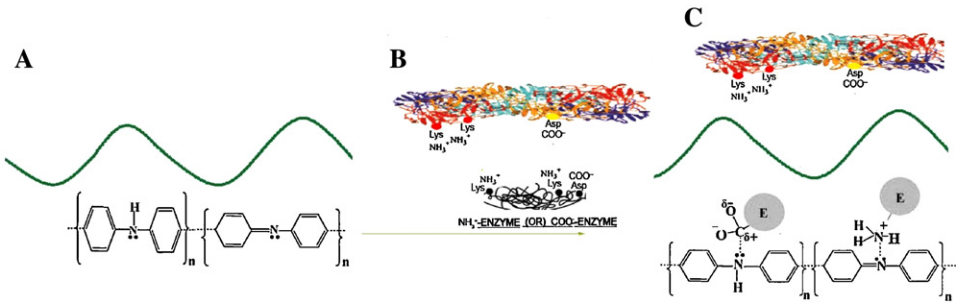

Fig. 1. Schematic representation of supporting L-asparaginase enzyme on polyaniline nanofiber. (A) Polyaniline nanofiber (PANI), (B) L-asparaginase enzyme (EI), and (C) L-asparaginase immobilized on polyaniline nanofiber (EI-PANI).

matrix, and the immobilized enzyme activity investigated. The results show enhanced stability and activity of the enzyme after immobilization and the effects of polymer nanofiber conformations on the enzymatic activity are also discussed in detail. A schematic representation of this study is shown in Fig. 1.

2. Experimental

2.1. Synthesis of polyaniline nanofibers (PANI)

All chemicals of analytical grade were used as received. Reactions were generally carried out in 20 mL vials. Polyaniline nanofibers (PANI) were synthesized following a literature method [17]. Typically, an aqueous solution of aniline (3.2 mmol) in 1 M hydrochloric acid (10 mL) and another solution of ammonium peroxydisulfate (0.8 mmol) in 1 M hydrochloric acid (10 mL) were prepared and mixed using a syringe pump. The feeding rates of one solution into another were set at 5 mL h^{-1} and were carried out at 0 °C. For rapid mixing of reactants, the two solutions were combined with vigorous stirring to ensure sufficient mixing before polymerization began. The onset of polymerization could be observed when the characteristic green color of polyaniline emeraldine salt began to appear. Reactions were repeated using increasing or decreasing concentrations of the reactants. In all cases, uniform nanofibers were obtained (confirmed TEM analysis). For reactions in ethanol and isopropanol, 1 M hydrochloric acid was prepared by diluting concentrated aqueous acid with the appropriate alcohol. The products were purified by centrifugation and washed with ethanol or isopropanol until the suspension pH was neutral.

The morphologies of the nanofibers were examined by TEM (JEOL 100CX) and SEM (JEOL 6700) [17]. For the morphological evolution experiments (Fig. 2), samples (0.1 mL) were extracted from the reaction at different times and diluted immediately in distilled water (0.5–2 mL). An appropriate amount of this suspension was then cast onto copper TEM grids (Formvar coated, 300 mesh, Ted-Pella Inc.). The grids were placed on filter paper to absorb any extra suspension and to facilitate rapid drying, thereby quenching the polymerization process. Samples for SEM experiments were made on conducting stages and observed without gold coatings (Fig. 3).

2.2. Preparation of enzyme impregnated PANI

L-Asparaginase derived from *Bacillus circulans* was used in this investigation and purified by a reported method [18]. Under nitrogen atmosphere, 100 mg of the enzyme was dissolved in 1 mL of distilled water followed by the addition of 100 mg of synthesized matrix particles (PANI). With constant mixing, the suspension was incubated at 30 °C for 30 min, then centrifuged at 10,000 rpm for 5 min at 30 °C. Unbound enzyme was removed by washing the solid with sterilized distilled water until the supernatant was free of protein and/or enzyme activity. Finally, the enzyme impregnated PANI (EI-PANI) was dried using a vacuum evaporator.

Characterization of PANI and EI-PANI: PANI and EI-PANI were investigated for their surface chemistry and morphology to understand the nature of physicochemical properties and enzyme binding using different analytical techniques (XRD, XPS, and FT-IR, etc.). Samples of PANI and EI-PANI were mounted onto quartz sybstrates and analyzed by X-ray diffraction (XRD) using D/8 BRUKER AXS operating at 40 kV with a current of 30 mA and Cu KR radiation. FT-IR studies were conducted using a Thermo Nicolet Nexus 670 spectrometer. Dried samples before and after enzyme binding (1–2 mg) were homogenized with 100 mg of dry KBr and made into pellets. These pellets were analyzed for transmittance in the range of 4000–400 cm^{-1}. X-ray photoelectron spectroscopy (XPS) measurements were obtained on a KRATOS-AXIS 165 instrument equipped with dual aluminum–magnesium anodes using Mg Kα radiation (the X-ray power supplied was 15 kV and 5 mA at 10^{-9} Torr). The peak positions were based on calibration with respect to the C 1s peak at 284.6 eV. The obtained XPS spectra were fitted using a nonlinear square method with the convolution of Lorentzian and Gaussian functions after the polynomial background subtraction from the raw data. For microscopic studies, different materials (PANI and EI-PANI) were fixed by incubating in 2% aqueous osmium tetraoxide for 2 h. The samples were then dehydrated using graded alcohol in series and dried to critical point by incubating in an Electron Microscopy Science CPD unit. Scanning electron microscopy (SEM; HITACHI) and transmission electron microscopy (TEM; TECH NAI-12, operated at 120 keV) were used to carry out the morphological studies.

2.3. Estimation of L-asparaginase activity

Following the method of Wriston and Yellin [19], L-asparaginase assays were measured by a colorimetric method at 436 nm and 37 °C using a CECIL UV-visible spectrophotometer. The ammonium ion produced during L-asparagine catalysis was determined using Nessler's reagent. The reaction mixture, consisting of 0.1 mL of 186 mM L-asparagine, 1.0 ml of 0.05 M tris buffer (pH 8.5) and 5 mg of EI-PANI, was incubated for 30 min at 37 °C then centrifuged to separate the EI-PANI from the solution. The reaction was stopped by the addition of 0.1 mL of 1.5 M trichloroacetic acid solution. The liberated ammonium ion was coupled with Nessler's reagent, quantitatively determined using a standard curve. The temperature effect on the enzyme was studied by performing the reaction at predetermined temperatures. Reusability studies were performed by washing the EI-PANI particles after every reaction with distilled water. One unit of the L-asparaginase (IU) is defined as that amount of enzyme capable of producing 1 μmol of ammonia per minute at 37 °C.

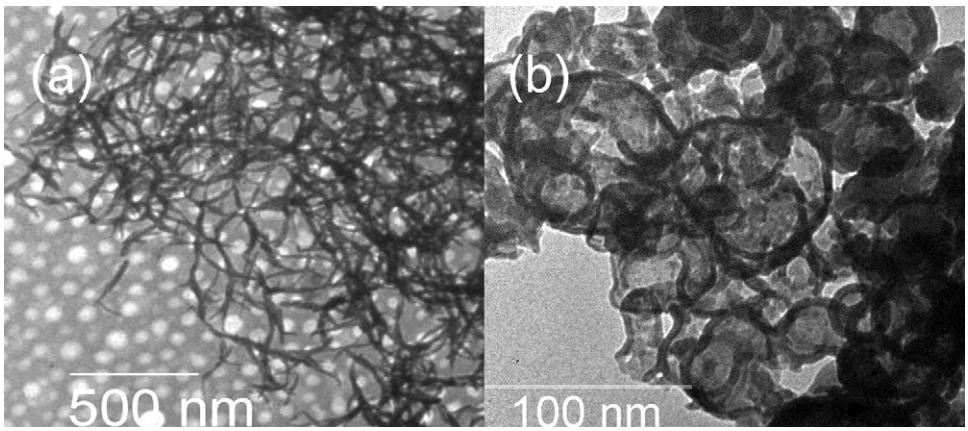

Fig. 2. TEM images of (a) neutral polyaniline nanofiber (PANI) and (b) L-asparaginase immobilized to polyaniline nanofiber (EI-PANI).

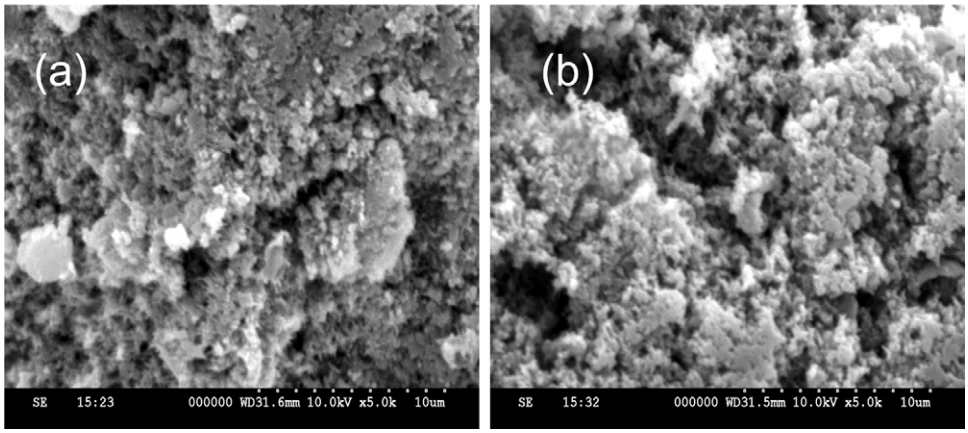

Fig. 3. SEM images of (a) L-asparaginase immobilized to polyaniline nanofiber (EI-PANI) and (b) neutral polyaniline nanofiber (PANI).

2.4. Calculation of K_m, V_{max}, activation energy values, and protein estimation

Reaction kinetics for EI-PANI and EI (free enzyme) were estimated by measuring the catalytic activity at different substrate concentrations ranging from 2 to 18 μmol with an interval of 2 μmol at 37 °C. K_m and V_{max} values were calculated by representing the enzyme activity values at different substrate concentrations in a Lineweaver–Burk equation. The protein content of the enzyme was estimated according to the method of Lowry et al. [20].

3. Results and discussion

3.1. Evaluation of enzyme binding

Conducting nanofibers as immobilization matrices are favored over other carrier materials due to their environmental stability (high stability to extremes of temperature, pH and resistance towards microorganisms), direct electron transfer capability between an enzyme and a polymer, ease of preparation, higher enzyme loading per unit mass with reduced diffusion resistance with the shortened path for substrate diffusing, higher conductivity and more facile fabrication. In view of the above, the present study assumes importance. Fig. 1 is a schematic representation for the formation of L-asparaginase impregnated PANI (EI-PANI) from polyaniline nanofiber (PANI). Figs. 2 and 3 show the TEM and SEM of PANI and EI-PANI. The TEM images of EI-PANI clearly show the change in morphology of the fibers, indicating the electronic interaction of the enzyme and PANI.

Fig. 4 shows the XRD pattern of neutral PANI and EI-PANI. It can be seen that the XRD pattern of EI-PANI with a low enzyme concentration is almost identical to that of neutral PANI. Furthermore, the intensity of the sharp diffraction peak of neutral PANI at $2\theta = 5.5^\circ$ slowly decreases and broadens as the enzyme concentration of the EI-PANI is increased. Moreover, two additional peaks

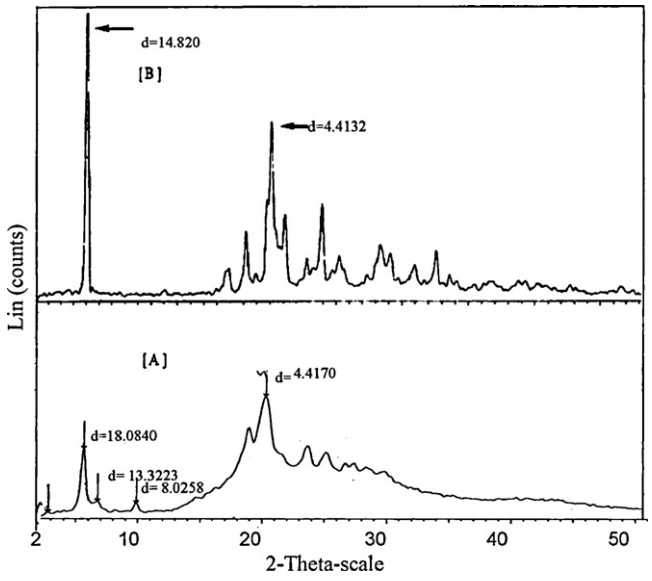

Fig. 4. XRD pattern of neutral PANI and EI-PANI.

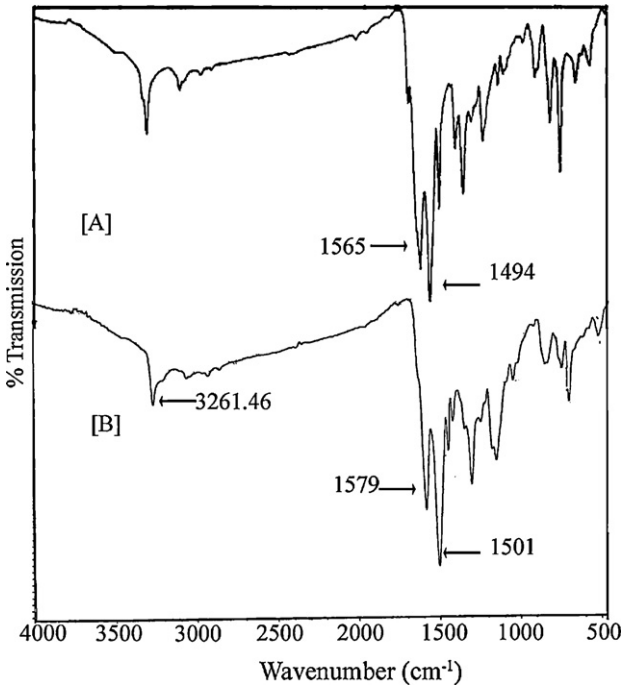

Fig. 5. FT-IR spectra of EI-PANI (immobilized L-asparaginase to polyaniline nanofiber) composite and PANI (polyaniline nanofiber).

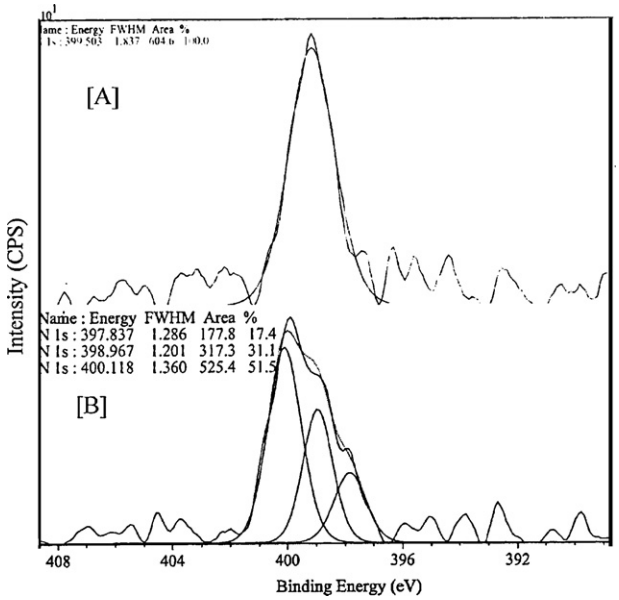

Fig. 6. Comparison of XPS of N 1s core-level spectrum of [A] neutral PANI (polyaniline nanofiber) fiber and [B] EI-PANI (immobilized L-asparaginase on polyaniline nanofiber).

at $2\theta = 7.0^\circ$ and $2\theta = 10.0^\circ$ are seen in EI-PANI, which indicates that the presence of enzyme in the PANI strongly affects the crystalline behavior of the polymer system. This is also shown in the calculated crystalline size of the polymer–enzyme composite based on the observed d-spacing values, which decrease with increasing enzyme concentration; the first peak is found to be more affected than the other two peaks. EI-PANI samples having 4:1, 8:1 and 20:1 ratios of PANI and enzyme show a crystalline size of 8.307 nm, 11.070 nm and 116.600 nm, respectively, for the first peak (Fig. 4). In Fig. 4, four well-resolved peaks are observed, suggesting that a highly ordered, hexagonally arranged pore system present. According to the Bragg equation [21], the strong, sharp peak is due to the (0 0 1) diffraction of the mesoporous film, and indicated a mesoscopic order with a d spacing of 5.8 nm.

Fig. 5 shows the FT-IR spectra of EI-PANI composite and PANI nanofibers. In the FT-IR spectra, the bands observed at the higher wave number region correspond to N–H stretching (3200–3500 cm^{-1}) and aromatic C–H stretching (2850–3000 cm^{-1}) vibrations. The group N═Q═N, where Q represents a quinoid ring, show bands in the region 1570–1600 cm^{-1} and N–B–B, where B represents a benzenoid ring, absorbs between 1480 and 1550 cm^{-1} [15]. Bands at 1297, 1250 and 1130 cm^{-1} are assigned to the C–N stretching vibration of the secondary aromatic amine, protonated C–N group, and aromatic C–H in-plane bending vibrations, respectively. The FT-IR spectra of EI-PANI composites are presented in Fig. 5. In the spectrum for the EI-PANI, a band at 3251 cm^{-1}, which is absent in the spectrum for PANI, is consistent with the presence of antisymmetric and symmetric methylene vibrations, presumably due to the presence of enzyme. This is further evidenced with an intense OH stretching band in the range of 3600–3400 cm^{-1}.

X-ray photoelectron spectroscopy, a sensitive surface specific analytical technique, was used to characterize the neutral PANI nanofiber and EI-PANI to determine the changes in the electronic environment arising from enzyme immobilization. The N 1s, C 1s and O 1s binding energies of the nanofibers differ slightly to those of the enzyme immobilized nanofibers. Peaks associated with C 1s are observed at 284.608, 286.145 and 287.999 eV for neutral PANI and shifted to 284.617, 285.834 and 286.950 eV in EI-PANI. Similarly, O1s peaks noticed at 530.815 and 531.923 eV for neutral PANI fiber are shifted to 531.331 and 533.093 eV upon enzyme binding. The binding energy of N 1s of PANI are deconvoluted into Gaussian component peaks corresponding to quinonoid imine (═N–), benzenoid amine (–NH–), polaron species (N+) and the peaks are centered at 397.837, 398.967, 400.118 eV, respectively. EI-PANI showed single N1 peak with a binding energy of 399.50 eV. This data further indicated that the binding energies observed for PANI nanofibers are lower with those of EI-PANI. Such a shift in binding energy of N 1s to a single peak is attributed to the binding of enzyme with that of polyaniline nanofiber similar to other reports [22]. The equal peak area of imine and amine N 1s indicated the conversion of amine to an imine form which acted as source of electrons for the binding of enzyme.

Fig. 6(A) and (B) show the wide scan XP spectra of neutral PANI fiber and EI-PANI. The quinoid/benzenoid ratio (–N/–NH–) for the PANI fiber was found to be 0.45, indicating the nitrogen is present mainly in the benzenoid amine form and therefore in the oxidation state of emeraldin, in agreement with the UV–vis spectrum of this sample. The signal at around 400.8 eV is associated with chain defects.

3.2. Evaluation of enzyme binding

The purified L-asparaginase enzyme isolated from *B. circulans* binding pattern is investigated with respect to solution pH by using a 4:1 ratio (PANI and enzyme) (w/w) at room temperature by measuring protein content (L-asparaginase enzyme) in reaction solution after separation of enzyme bound PANI particles by centrifugation. Reaction solution pH has a major influence on the enzyme loading. Maximum loading is noticed with PANI particles prepared using with mineral acid (HCl/H_2SO_4) than weak acid (acetic acid). It is observed that enzyme loading is higher at neutral pH [the maximum loading (48%) at pH 7.0] and further increase caused drastic reduction (3–4 times) in enzyme loading.

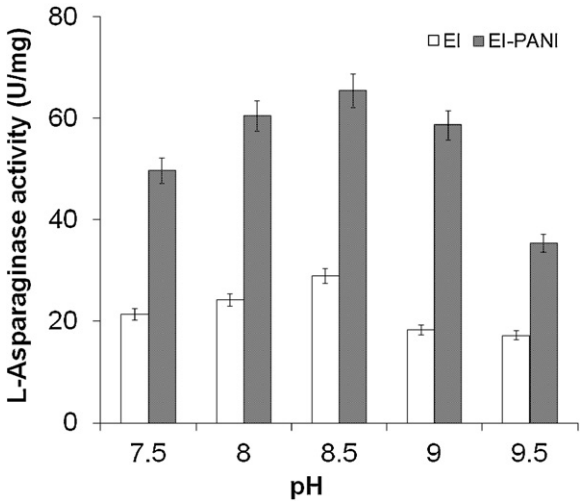

Fig. 7. Effect of different pH on free enzyme L-asparaginase (EI) and L-asparaginase immobilized to polyaniline nanofiber (EI-PANI).

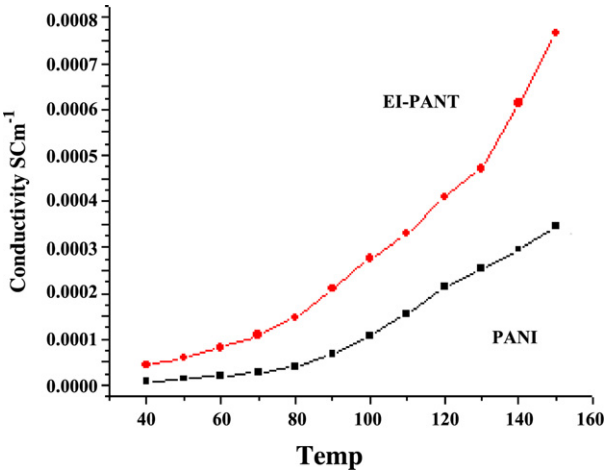

Fig. 8. Influence of different temperatures on conductivity of polyaniline nanofiber (PANI) and L-asparaginase enzyme immobilized to polyaniline nanofiber (EI-PANI).

3.3. Biocatalytic evaluation of EI-PANI

The biocatalytic properties of L-asparaginase enzyme adsorbed onto PANI (EI-PANI) was evaluated at different temperatures, substrate concentrations and pH by determining the amount of ammonium ion released during the catalytic reaction. Maximum enzyme activity (65 U/μg of protein) was observed at pH 8.5, and any variation in the medium pH drastically reduced the biocatalytic activity (Fig. 7). The enzyme activity inhibition for the free enzyme is greater than 35% when the pH is changed from 8.5 to 9.0 but is only 11% for the immobilized enzyme under similar conditions. This suggests immobilization mediated enzyme activity provides protection against variation in medium pH, which is dependent on the chemical and electrostatic nature of the immobilization matrix. This is consistent with the observation that a variation of one pH unit towards alkalinity revealed an almost similar inhibitory effect for free (40%) and PANI bound L-asparaginase activity (46%) compared to the enzyme activity at pH 8.5. Similarly, changing the medium pH by 0.5 pH units towards neutrality resulted in a reduction of enzyme activity of 17% for the free and 8% for the immobilized enzyme. A further decrease in pH from 7.5 to 7.0 resulted in enzyme activity inhibition of 12% and 18% for the free and immobilized enzyme, respectively (Fig. 7). However, a change of one pH unit from 8.5 to 7.5 showed an approximately 26% reduction in enzyme catalytic function irrespective of its physiological state. This data clearly suggest an enhanced activity (2.26 folds) of EI-PANI relative to that of the free L-asparaginase enzyme. This data indicate that upon immobilization of enzyme onto PANI, the electrostatic nature of the enzyme active site is varied, giving rise to variations in its catalytic behavior compared to that of the free enzyme. Such altered enzyme activity results have been noticed with enzyme activity when immobilized on thiolated gold-nanoparticles [23] or on composite temperature-sensitive membrane [24].

The pH dependent stabilization of enzyme is further confirmed by variations in the conductivity of the enzyme bound polyaniline nanofiber. An increase in conductivity is observed upon enzyme binding, which increases with increasing enzyme concentration (Fig. 8). Conformational changes of the enzyme upon ionic bonding with the polyaniline nanofiber may be responsible for the enhanced catalytic activity of EI-PANI.

Variation of external temperature also resulted in alteration of enzyme activity but, its influence is not as great as that of variations in pH (Fig. 9). The maximum enzyme activity of immobilized (66 U/μg protein) and free (29 U/μg protein) enzyme occurs at 37 °C, indicating >100% improvement in catalytic activity upon immobilization and suggests enzyme structural alterations are associated upon binding on the PANI. An increase in the reaction temperature from 37 to 40 °C did not have a significant effect on the catalytic activity of either the bound or free enzyme, however, increasing the temperature to 50 °C resulted in a reduction of enzyme activity. At this temperature, the enzyme activity was reduced to 17% and 26% for EI-PANI and free enzyme, respectively, compared to those obtained at 37 °C. Consequently, immobilized enzyme kinetics and reusability studies were studied at 37 °C and at pH 8.5. Under these conditions, the immobilized enzyme showed stable activity for more than 40 cycles with a variation of only 5% (results not shown).

3.4. Enzyme kinetics

To further understand at the interactive level, the activity of free and immobilized L-asparaginase enzyme were monitored using UV–vis spectroscopy. To carry out the enzyme assay, free and PANI nanoparticle-bound L-asparaginase enzymes (5 mg) were

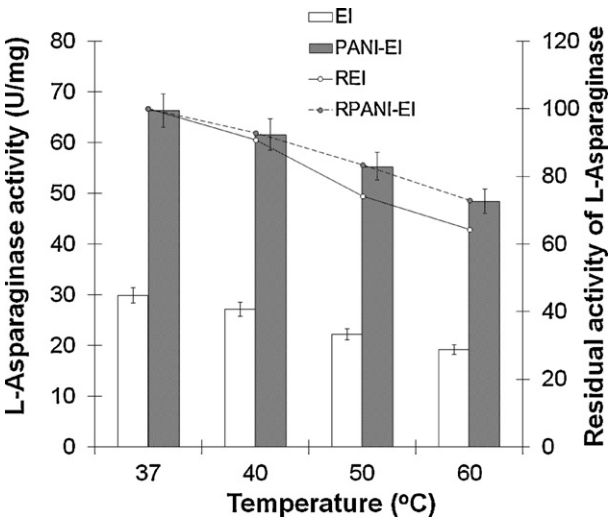

Fig. 9. Effect of different temperature on free enzyme L-asparaginase (EI) and immobilized L-asparaginase to polyaniline nanofiber (PANI-EI), residual activity of the L-asparaginase (REI) and residual activity of immobilized L-asparaginase on polyaniline nanofiber (RPANI-EI).

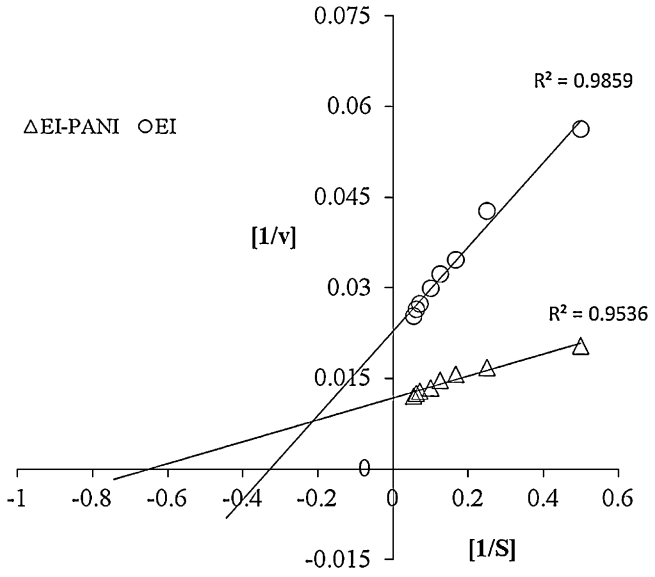

Fig. 10. Comparison of substrate kinetics for the free enzyme L-asparaginase (EI) and L-asparaginase immobilized to polyaniline nanofiber (EI-PANI), using Lineweaver–Burk plot.

mixed in 1 mL of tris-buffered solution of pH 8.5, 1 mL of distilled water and 0.1 mL of 186 mM of L-asparagine, a substrate, separately. The increased absorbance recorded at 436 nm indicated an enhanced activity of immobilized L-asparaginase on the PANI nanofibers. Additionally, the L-asparaginase activity increased with an increase in asparagine concentration up to 18 μmol mL^{-1}; any further increase in enzyme concentration did not show any significant change. The values of the kinetic parameters (K_m and V_{max}) were determined by an analysis of the Lineweaver–Burke plot (Fig. 10). The K_m value for an enzymatic reaction determines the affinity of the enzyme for the substrate, whereas the value of V_{max} provides the maximum rate of enzyme reaction when the enzyme is saturated by the substrate. The smaller value of K_m indicated an increased affinity of the enzyme for the substrate. The values of K_m in the present enzymatic assay were found to be 1.809 and 3.705 mM L^{-1} for the immobilized and free L-asparaginase, respectively. The value of V_{max} (90.57 μM min^{-1} mg^{-1}) obtained for the covalently immobilized L-asparaginase was one order of magnitude higher than that of free L-asparaginase (48.04 μM min^{-1} mg^{-1}). However, the value of K_m for the covalently immobilized L-asparaginase was 2.05 fold lower in magnitude in comparison with the value obtained for free L-asparaginase indicating more affinity towards substrate binding.

4. Conclusions

Enzyme–nanofiber composites provide numerous advantages over costly unbound enzymes in various fields of study and development, including food, pharmaceutical and bioremediation processes. In this study, we report greater activities of the enzyme L-asparaginase for catalysis of L-asparaginase when immobilized on polyaniline nano sized fibers compared to other supports like fibroin nanoparticles, carboxymethyl konjac glucomannan–chitosan nanocapsules, chitosan modified by poly(ethylene glycol) [25–27], activated carbon [28], polysaccharide levan [29], polyacrylamide gel [30] natural silk sericin protein [31] or polyethylene glycol hydrogel [32]. This report describes the enhanced activity, reusability and sustainability of the L-asparaginase enzyme with temperature and pH variations after immobilization on environmentally stable polyaniline nanofibers, which is a novel aspect of this work with respect to previous reports. The enhanced activity may be due to conformational changes of the enzyme upon ionic interactions with the polyaniline nanofiber. Conducting nanofibers as immobilization matrices show superior properties over other carrier materials, such as their environmental stability (high stability to extremes of temperature, pH and resistance towards microorganisms), direct electron transfer capability between an enzyme and a polymer, ease of preparation, higher enzyme loading per unit mass with reduced diffusion resistance with a shortened path for substrate diffusion, higher conductivity and more facile fabrication.

Using FT-IR, TEM, XPS and XRD analyses, this study has shown that the polyaniline nanofiber supported enzyme is more stable and more active than the free enzyme. After immobilization, the K_m value of the enzyme is significantly smaller than that of the free enzyme. The effects of reaction temperature and pH on the enzyme activity profile were also investigated and it was shown that changes in pH have a greater impact than temperature effects.

References

[1] J. Lubkowski, G.J. Palm, G.L. Gilliland, C. Derst, K.H. Rohm, A. Wlodawer, Eur. J. Biochem. 241 (1996) 201–207.
[2] R.S. Prakasham, Ch.S. Rao, R.S. Rao, G.S. Lakshmi, P.N. Sarma, J. Appl. Microbiol. 102 (2007) 1382–1391.
[3] H.J. Muller, J. Boos, Crit. Rev. Oncol. Hematol. 28 (1998) 97–113.
[4] M. Wolf, M. Wirth, F. Pittner, F. Gabor, Int. J. Pharm. 256 (2003) 141–152.
[5] M. Lu, X.-H. Li, H.-L. Li, Mater. Sci. Eng. A 334 (2002) 291–297.
[6] H. Devendrappa, U.V. Subba Rao, M.V.N. Ambika Prasad, J. Power Sources 155 (2006) 368–374.
[7] G. Liu, Y. Lin, V. Ostatna, J. Wang, Chem. Commun. (2005) 3481–3483.
[8] R.V. Parthasarathy, C.R. Martin, Nature 369 (1994) 298–301.
[9] P.S. Ghosh, A. Verma, V.M. Rotello, Chem. Commun. (2007) 2796–2798.
[10] S. Rauf, D. Zhou, C. Abell, D. Klenerman, D.-J. Kang, Chem. Commun. (2006) 1721–1723.
[11] S. Liu, J. Wang, D. Zhang, P. Zhang, J. Oua, B. Liu, S. Yang, Appl. Surf. Sci. 256 (2010) 3427–3431.
[12] L. Xu, Y. Zhu, L. Tang, X. Yang, C. Li, J. Appl. Polym. Sci. 109 (2008) 1802–1807.
[13] Z. Du, C. Li, L. Li, M. Zhang, S. Xu, T. Wang, Mater. Sci. Eng. C 29 (2009) 1794–1797.
[14] Y. Xian, Y. Hu, F. Liu, Y. Xian, H. Wang, L. Jin, Biosens. Bioelectron. 21 (2006) 1996–2000.
[15] M.E.M. Cruz, M.M. Gaspar, F. Lopes, J.S. Jorge, R. Perez-Soler, Int. J. Pharm. 96 (1993) 67–77.
[16] M.M. Gaspar, D. Blanco, M. Eugénia, M. Cruz, M.J. Alonso, J. Control. Release 52 (1998) 53–62.
[17] S. Bhadraa, D. Khastgir, N.K. Singha, J.H. Lee, Progr. Polym. Sci. 34 (2009) 783–810.
[18] R.S. Prakasham, M. Hymavathi, Ch. Subba Rao, S.K. Arepalli, J.V. Rao, P.K. Kennady, K. Nasaruddin, J.B. Vijayakumar, P.N. Sarma, Appl. Biochem. Biotechnol. 160 (2010) 72–80.
[19] J.C. Wriston, T.O. Yellin, Adv. Enzymol. 39 (1973) 185–248.
[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[21] W.L. Bragg, Proc. Cambr. Philos. Soc. 17 (1913) 43–57.
[22] M.I. Boyer, S. Quillard, E. Rebourt, G. Louarn, J.P. Buisson, A. Monkman, S.J. Lefrant, Phys. Chem. B 102 (1998) 7382–7392.
[23] P. Pandey, S.P. Singh, S.K. Arya, V. Gupta, M. Datta, S. Singh, B.D. Malhotra, Langmuir 23 (2007) 3333–3337.
[24] J.-P. Chen, Y.-M. Sun, D.-H. Chu, Biotechnol. Prog. 14 (1998) 473–478.
[25] Y.Q. Zhang, R.L. Xiang, H.B. Yan, X.X. Chen, Chem. J. Chin. Univ. 29 (2008) 628–633.
[26] R. Wang, B. Xia, B.J. Li, S.L. Peng, L.S. Ding, S. Zhang, Int. J. Pharm. 364 (2008) 102–107.
[27] H.G. Huo, B. Fang, T.Q. Jiang, J. East China Univ. Sci. Technol. 31 (2005) 13–17.
[28] M.E. Moharam, A.M.G. Eldeen, S.T. El-sayed, J. Am. Sci. 6 (2010) 157–161.
[29] I. Vına, A. Karsakevich, M. Bekers, J. Mol. Catal. B: Enzym. 11 (2001) 551–558.
[30] T. Mori, T. Tosa, I. Chibata, Cancer Res. 34 (1974) 3066–3068.
[31] Y.-Q. Zhang, M.-L. Tao, W.-D. Shen, Y.-Z. Zhou, Y. Ding, Y. Ma, W.-L. Zhou, Biomaterials 25 (2004) 3751–3759.
[32] J.J. François, E.M. D'Urso, G. Fortier, Biotechnol. Appl. Biochem. 26 (1997) 203–212.