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Immobilizing cholesterol oxidase in chitosan–alginic acid network

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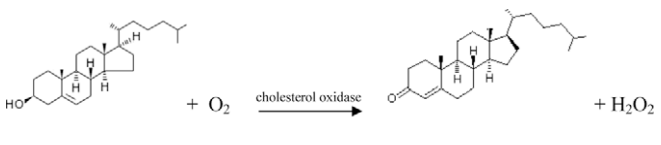
ABSTRACT

Proton conducting biopolymer networks have potential use for bio-sensors. The cost-effective, non-hazardous and environmentally safe biopolymer, such as chitosan, is an attractive feature for bio-sensors. Cholesterol oxidase was immobilized in conducting network via complexation of chitosan with alginic acid. A method for the preparation of the complex along with characterization by elemental analysis, FTIR spectroscopy, TGA and DSC were reported. The proton conductivity chitosan–alginic acid network was studied via impedance spectroscopy under humidified condition. The complex polymer electrolyte with $x = 1$ exhibited maximum proton conductivity of 1.4×10^{-3} S/cm at RT, RH ~ 50%. The potential use of this network in enzyme immobilization was studied by manufacturing cholesterol oxidase entrapped polymer networks. Additionally, the maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) were investigated for the immobilized cholesterol oxidase. Also, temperature and pH optimization studies were performed, and operational stability and shelf life of the polymer network were examined.

1. Introduction

Enzyme bio-sensors have rapidly increasing importance in clinical diagnostics, food industries and environmental control (Singh, Singhal, & Malhotra, 2007). Immobilization of enzymes to the desired bio-sensor surface and maintaining their activity during a desired application are important factors for the success of enzyme bio-sensors. Enzymes are expensive, hence, it is cost-effective to use them more than once. However, it is difficult and costly to separate them from the reaction mixture. On the other hand, immobilized enzymes have advantages of repeated use, easy separation from product environment, enhanced stability, and reduction in the cost of operation.

Cholesterol determination is important for the diagnosis of coronary heart diseases, arteriosclerosis, and cerebral trombosis (Motonaka & Faulkner, 1995). Cholesterol oxidase catalyzes the oxidation of cholesterol into cholest-4-en-3-one and hydrogen peroxide as given by the following chemical reactions:



Cholesterol — Cholest-4-en-3-one

$$H_2O_2 + \text{Phenol} + 4 - \text{aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinonemine dye} + 4H_2O$$

Biological molecules, enzymes, can be immobilized in a biosensor surface using different methods. These methods are cross-linking, covalent attachments, adsorption, and entrapment/encapsulation of the biological molecules within polymeric gels or carbon paste (Andres, Kuswandi, & Narayanaswamy, 2001; Avnir, Braun, Lev, & Ottolenghi, 1994; Glezer & Lev, 1993; Rosen-Margalit & Rishpon, 1993).

Chitosan–alginic acid network is used for the first time in the immobilization of enzymes. In this network, an enzyme can be entrapped within a polymeric network of porous gel. Compared to other immobilization matrices, in this case, enzyme molecules become entrapped in a covalent network rather than being chemically bound to an organic matrix which may inactivate the enzyme.

Chitin is a monopolysaccharide which is present in the cell wall of fungi and in the outside skeleton of crustaceans and insects. Chitosan, deacetylated form of chitin, is a low-cost, biocompatible, biodegradable, nontoxic, and natural biopolymer. It has properties of wound healing, antibacterial activity, chemical and thermal stability, and ability of binding to tissue (Majeti & Kumar, 2000). Chitosan (Scheme 1), is insoluble in water at a pH near neutrality, in concentrated acids with the exception of sulphuric acid, in bases and organic solvents. Their acid base properties allow easy dissolution. Therefore, chitosan is soluble in dilute HCl, HBr, HI, HNO_3, H_3CCOOH, CCl_3COOH and $HClO_4$. Its solubility in dilute organic acids, which account of a protonation of free amine groups, allows for gel formation in various configurations (Mukoma, Jooste,

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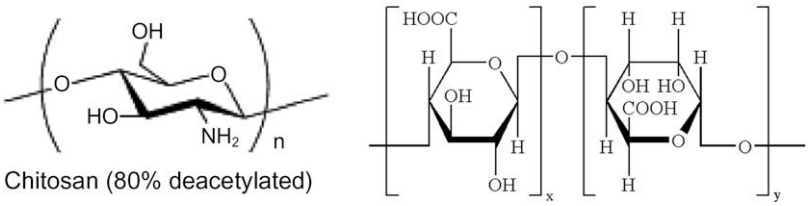


Scheme 1. Structures of chitosan and alginic acid.

& Vosloo, 2004). Chitosan can be used in the preparation of solid polymer electrolytes (Khiar, Puteh, & Arof, 2006) since it has a pair of lone pair electrons which enables the chelation of a proton donor hydroxyl and amine functional groups (Mohamed, Subban, & Arof, 1995).

Cholesterol oxidase enzyme has been immobilized/co-immobilized via several methods (Huang, Juan, & Guilabult, 1977; Karube, Hara, Mastsuoka, & Suzuki, 1982; Kumar, Kumar, Kumar, Jyotirami, & Tulsani, 1999; Kumar, Malhotra, Malhotra, & Grover, 2000; Mattiasson, Danielsson, & Mosbach, 1978; Tabata, Endo, & Murachi, 1981). It was immobilized in sol-gel films (Kumar et al., 2000), polyaniline, polypyrrole (Trettnak, Lionti, & Mascini, 1993; Vidal, Garcia, & Castillo, 1999a, 1999b, 2000) membranes (Trettnak & Wolfbeis, 1990), and in copolymers of methyl and glycidal methacrylates (Hall, Datta, & Hall, 1996). In this work, chitosan was used as the polymer with alkaline character. Proton conducting polymer networks were obtained by mixing chitosan and alginic acid at several molar ratios with respect to repeating units, and used for immobilization of cholesterol oxidase. Proton conductivity of humidified samples with $x = 1$ was measured. Additionally, the maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) were investigated for the immobilized cholesterol oxidase. Also, temperature and pH optimization, operational stability and shelf life of the polymer network were examined.

2. Experimental

2.1. Materials

Cholesterol oxidase (COD) (E.C. 1.1.3.6) with a specific activity of 26.4 U mg^{-1} solid and horseradish peroxidase (HRP) (E.C. 1.11.1.7) with a specific activity of 181 U mg^{-1} solid were procured from Sigma. Cholesterol, alginic acid (AA), isopropanol (99%), triton X-100 (*t*-octylphenoxypolyethoxyethanol), and 4-aminoantipyrine were purchased from Sigma–Aldrich. Low molecular weight chitosan (80% deacetylated) was purchased from Fluka. This polymer was used for the synthesis of proton conducting membranes (Goktepe, Celik, & Bozkurt, 2008). Phenol was purchased from Carlo Erba. Phosphate buffer (pH 7.0) was prepared using sodium monobasic and sodium dibasic in distilled water. Glycial acetic acid (GAA) was purchased from Aldrich.

2.2. Instrumentation

Shimadzu UV-1601 UV–visible spectrophotometer was used to determine the activities of immobilized enzyme. A Scimitar Series Varian 1000 FTIR was used to obtain the FTIR spectra of proton conducting polymer network as dispersed in KBr pellets. Elemental analyses of the networks were performed using LECO, CHNS-932. Thermogravimetry and differential thermal analysis were done in temperature range of 25–300 °C using Setaram Labsys TGA/DTA. The proton conductivity studies of the samples were performed using a Novocontrol dielectric impedance analyzer. The films were sandwiched between platinum blocking electrodes and the conductivities were measured in the frequency range 0.1 Hz–3 MHz at 10 °C intervals.

2.3. Method

2.3.1. Preparation of cholesterol solution

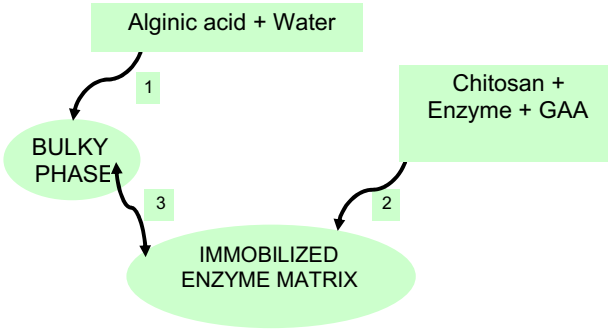
Cholesterol is soluble in alcohol and also in water in the presence of surfactants (Charpentier & Murr, 1995; Kumar et al., 2000; Masoom & Townshend, 1985; Trettnak & Wolfbeis, 1990). Solutions were prepared daily by dissolving cholesterol in isopropanol, Triton X-100, and the phosphate buffer (pH 7.0). The isopropanol, Triton X-100, phosphate buffer ratio is 10:4:86 by weight.

2.3.2. Preparation of chitosan–alginic acid network and entrapment of cholesterol oxidase

First the most suitable chitosan/AA ratio was determined by mixing 0.10 grams of chitosan with different amounts of AA. Chitosan was mixed with water containing cholesterol oxidase, and alginic acid was mixed with 1% GAA (Scheme 2). Then the two phases were put together to get enzyme entrapped polymer network (EEPN). We expected that the most suitable matrix will be with the ratio of 1, where protonation is maximum. AA is a dibasic acid hence, we used $x = 2$ (x is the number of moles of chitosan per moles of –COOH units in AA). However, the elemental analysis and the FTIR results showed that the most suitable matrix was with $x = 1$. Therefore, throughout the study, AA/chitosan matrices were prepared with $x = 1$. During complexation, 1.5 mg of cholesterol oxidase enzyme was entrapped in 1 mL of AA and 1 mL of chitosan mixture, and the maximum water absorbing capacity was found to be 1.0 mL in 2.0 mL of AA (0.1 g/mL) and chitosan (0.1 g/mL). This EEPN was used for activity determinations.

2.3.3. Enzyme activity measurements

The activity of cholesterol oxidase was determined spectrophotometrically using Kumar method (Kumar et al., 2000). One unit converts 1.0 μmol of cholesterol to 4-cholesten-3-one per min at pH 7.0 at 25 °C. Different concentrations of cholesterol solutions were prepared, and then a 2.5 mL of cholesterol solution was contacted with the cholesterol oxidase immobilized chitosan–alginic acid network. Different incubation times (2, 4 and 6 min) were applied. After these incubation times, 2.5 mL of a solution containing 4-aminoantipyrine, phenol, peroxidase were added and waited for



Scheme 2. Preparation of chitosan-AA network.

10 min to complete quinoneimine dye formation. The spectrophotometric determination of cholesterol is based on the color of quinoneimine dye produced by coupling of peroxide with 4-aminoantipyrine and phenol in the presence of peroxidase (HRP) (Braco, Daros, & Guradia, 1992; Deeg & Ziegenhorn, 1983; Kumar et al., 2000). The product was determined spectrophotometrically at 500 nm.

2.3.4. Determination of kinetic parameters

To determine the V_{max} and K_m values, the activity assay was applied to different concentrations (0.1, 0.01, 0.03, 0.05, 0.005 mM) of cholesterol solutions prepared in phosphate buffer (pH 7.0). V_{max} and K_m were calculated from Lineweaver–Burk plots (Palmer, 1995, chap. 7).

2.3.5. Determination of optimum temperature and pH

Optimum temperature and pH were determined by keeping the substrate concentration at 10 K_m. To find optimum temperature, activity assay was applied by changing the incubation temperature between 10 °C and 50 °C. To determine optimum pH, an assay was applied by changing the pH between 2 and 10 at a constant temperature (25 °C).

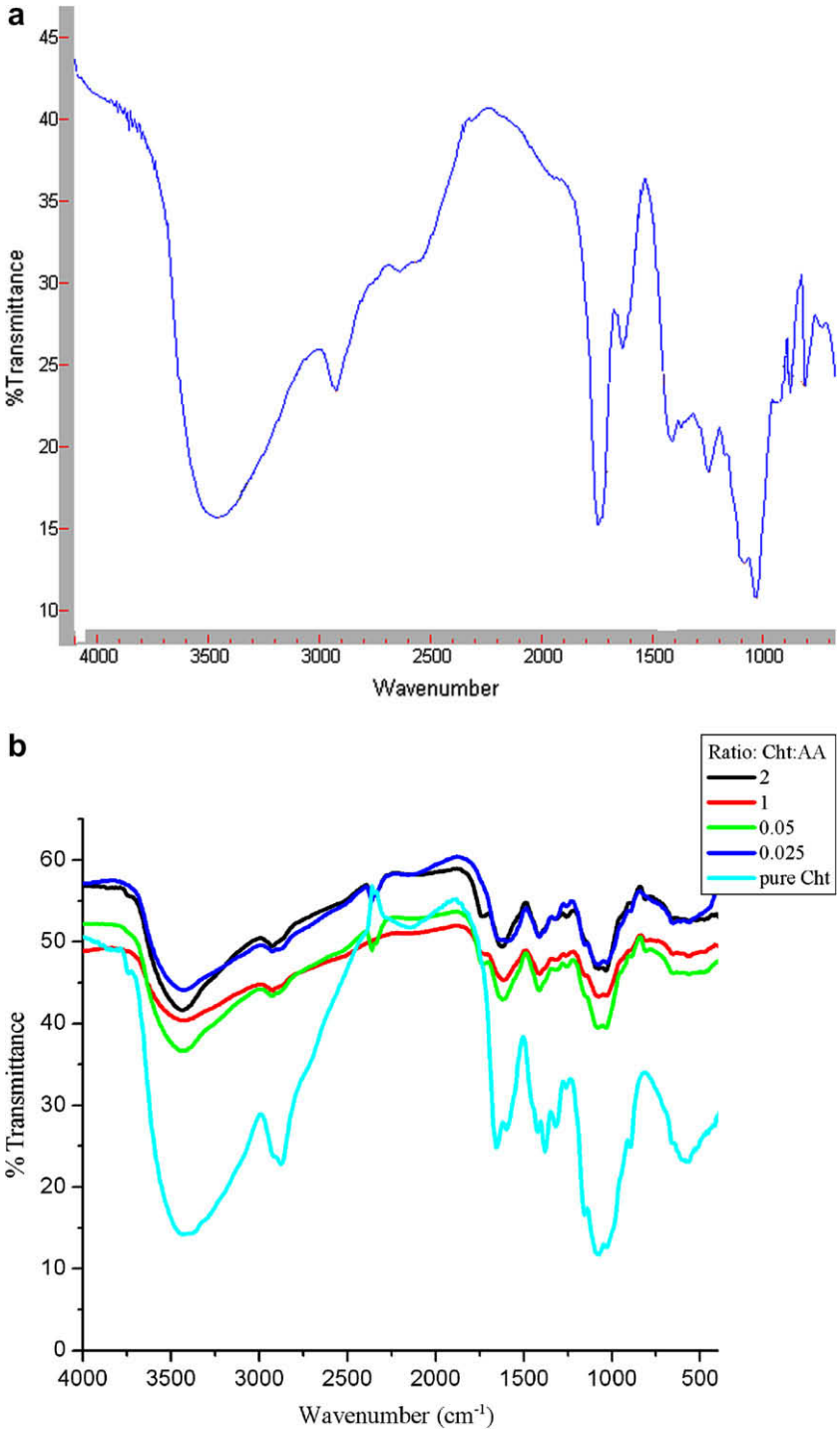


Fig. 1. FTIR spectra of (a) AA; (b) pure chitosan and chitosan/AA networks of different weight ratios.

2.3.6. Operational stability and shelf life

The enzyme bioreactor, at optimum activity conditions, was used in 47 activity assays in one day to determine the operational stability. The shelf life of the enzyme bioreactor was determined by performing activity assays within 15 days.

2.4. Characterization of chitosan–alginic acid network

The thermal stability of the chitosan/AA network was determined by thermogravimetry and differential thermal analysis (TGA/DTA) in the temperature range between 25 and 300 °C. Measurements were under dry-nitrogen flow at a heating rate of 10 °C min^{-1}. The samples were subjected to TGA both before and after blending to determine the thermal stability and decomposition characteristics. The infrared (IR) spectra of the samples were taken as dispersed in KBr pellets before and after blending.

3. Results and discussion

3.1. FTIR results

Fig. 1b shows the FTIR spectra of chitosan/AA complexes. The band at 1597 cm^{-1} was attributed to the bending vibration of the amino group in chitosan (Silverstein & Webster, 1998), which disappeared upon complexation with AA. The bands at 1595 and 1622 cm^{-1} (Silverstein & Webster, 1998), are the symmetric and asymmetric bending vibrations of $-NH_3^+$, respectively. The disappearance of $-NH_2$ and appearance of $-NH_3^+$ indicated the protonation of the amino group in the chitosan with the addition of AA. Also, the absorption band at 1641 cm^{-1}, related to the C=O stretching peak of the chitin form, was hidden by the $-NH_3^+$ band. Similarly, the intensity of strong C=O stretching of AA at 1731 cm^{-1} is decreased (Fig. 1a) and new broad peak is assigned to asymmet-

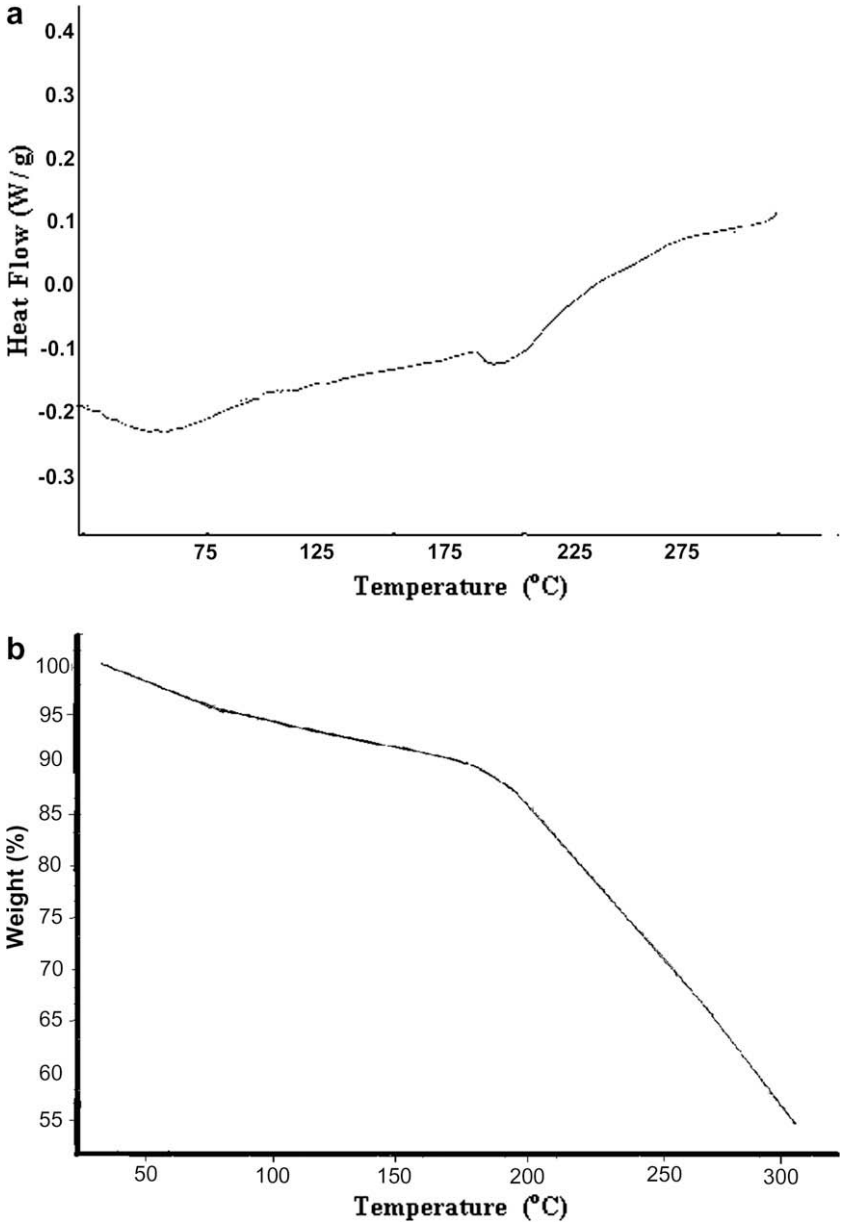


Fig. 2. (a) DSC; (b) TGA of the polymer network.

ric stretching of $-CO_2^-$ unit (Mukoma et al., 2004). These results demonstrated the proton exchange reaction between chitosan and AA leading to polymer complexation.

3.2. DSC and TGA analysis

The DSC thermogram reveal the formation of two endothermic transition at around 60 °C and 180 °C, which resulted respectively from the elimination of water and the formation of amide linkages in the complex (Smitha, Sridhar, & Khan, 2005). The DSC result is in accordance with TGA data of the complex and shows that with increasing temperature the weight of the sample decreases gradually up to 180 °C and faster at temperatures ranging from 180 °C to 300 °C (Fig. 2a and b.)

3.3. Elemental analysis

Chitosan and AA contents in the feed and in the complex were determined by elemental analysis. Different mixing ratio of chitosan/AA complexes were prepared and dried for two days under vacuum for elemental analysis. In Table 1, results reveal that the chitosan content varies between 33% and 80% in the feed, whereas it varies between 44% and 64% in the complex. According to the results, the feed ratio is expressed with $x = 1$, since it refers to higher chitosan content in chitosan/AA complex. Chitosan enhances the stability of the complex, making it more stable than the products obtained with other mixing ratios.

3.4. Proton conductivity

Frequency dependent proton conductivity of the samples was measured by impedance method as a function of temperature. Alternating current (AC) conductivity, σ_{ac} versus frequency curves are plotted and the direct current (DC) conductivity, σ_{dc} of the samples was derived from the σ_{ac} data, as described in previously (Mukoma et al., 2004). DC conductivities at several temperatures of the chitosan-AA ($x = 1$; RH = 50%) are compiled in Table 2. Clearly the conductivity of the samples depends on the temperature. At higher temperatures the decrease in conductivity can be explained by the loss of humidity. Chitosan has very low electrical conductivity due to non-ionizable units in the structure (Mohamed et al., 1995). However, it gains some mobilized ions, i.e., H_3O^+ after dissolving with AA acid under humidified conditions. Also, protonation of $-NH_2$ groups result in the formation of chitosan based polyelectrolyte and proton conductivity may also occur over the protonated and unprotonated units in the complex matrix.

Table 1
Results of elemental analysis.

Sample (chi:AA)	Chi in the feed (mol%)	Chi in the complex (mol%)
1:2	33	44
1:1	50	64
1:0.5	67	62
1:0.25	80	62

Table 2
Results of proton conductivity measurements.

Temperature (K)	Proton conductivity (S/cm)
353	1.4×10^{-3}
333	1.9×10^{-3}
313	2.5×10^{-3}
293	2.7×10^{-3}

3.5. Kinetic parameters

For the most suitable matrix ($x = 1$) preparation, 1.5 mg (39.6 U) of cholesterol oxidase were immobilized in this polymer network. Then, this EEPN was used for the activity assay while varying the substrate concentration at constant temperature and pH. Kinetic parameters were calculated using Lineweaver–Burk plots. K_m is the Michaelis–Menten constant and shows the affinity of enzyme to its substrate. V_{max} is the maximum rate for enzymatic reaction. Thus, as given in the Table 3, K_m of the immobilized cholesterol oxidase decreased compared to that of the free enzyme. The low K_m value indicates that the immobilized enzyme interacts with its substrate for a longer time (compared to free enzyme) and thus, the reaction gives lower amount of product than that of free enzyme. As a result, V_{max} of this long time interaction between immobilized enzyme and its substrate reveals smaller V_{max} (Table 3).

3.6. Temperature and pH influence on enzyme entrapped polymer

Thermal stability of the EEPN was studied by measuring the absorbance at different temperatures (10 °C–50 °C). The effect of temperature on the immobilized enzyme activity is shown in Fig. 3. Immobilized enzyme shows appreciable activity between 10 °C and 50 °C, with a maximum activity at 40 °C. The reason of increasing value of absorbance with increasing temperature may be due to the minimized protein denaturation through the protection by chitosan/AA matrix. However, after 40 °C, there is a sudden decrease in the absorbance due to the denaturation of enzyme.

Fig. 4 shows the response of the EEPN as a function of pH. Immobilized cholesterol oxidase enzyme showed maximum activity at pH 7.5, with a reasonable activity between pH 6 and 8. Decrease in activity at pH 7 is due to the isoelectric point of the enzyme. At this point molecule carries no net electrical charge, hence the activity decreased rapidly due to the difficulty in substrate binding. At extreme pH values the enzyme was irreversibly denaturated. As a result, chitosan/AA matrix provides enzyme broader ranges of temperature and pH.

3.7. Operational stability and shelf life of the enzyme bioreactor

The operational stability was studied by applying activity assay (under optimum conditions) for 44 times in the same day at constant temperature, pH and substrate concentration. At the end of the 44 measurements, the bioreactor lost 37% of its initial activity (Fig. 5). This shows that immobilized enzyme has advantages of repeated usage over free enzyme. The activity assay was also applied for every 5 days within 15 days to display the shelf life of immobilized enzyme. As given in Fig. 6, EEPN exhibits 20% activity after five days.

4. Conclusion

In this work, cholesterol oxidase was immobilized in a chitosan/AA polymer matrix successfully. This polymer matrix was characterized by FTIR spectroscopy, and found that the maximum protonation occurs with $x = 1$. Also, complex content include more chitosan and so more stable than other when $x = 1$ is observed by elemental analysis. Proton conductivity of this systems is

Table 3
Kinetic parameters for free and immobilized cholesterol.

K_m (M)	V_{max}
Free CHOX 2.6×10^{-1}	3.0×10^{-1}(μmol/min ml)
Immobilized CHOX 2.05×10^{-5}	5.3×10^{-6}(μmol/min mg complex)

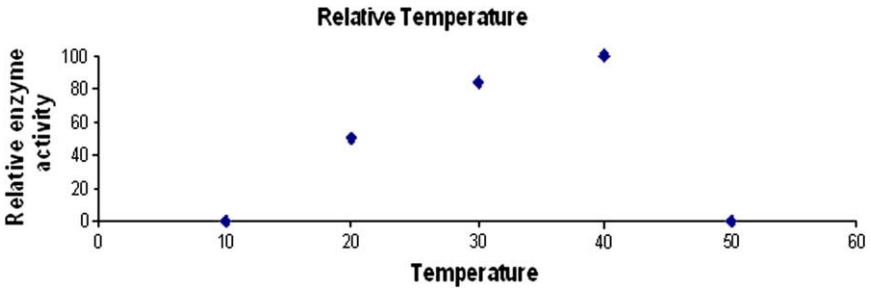


Fig. 3. Optimum temperature (°C) of enzyme bio-sensor.

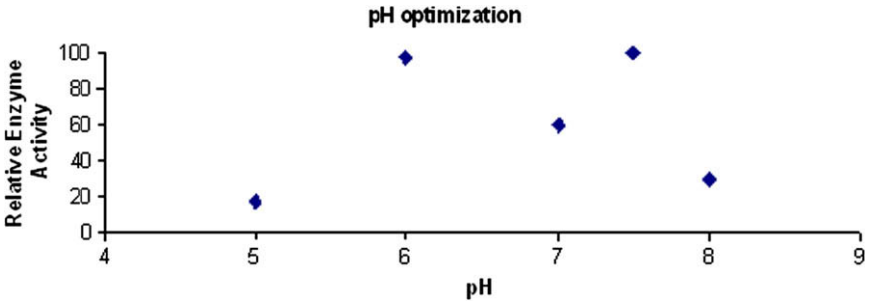


Fig. 4. Optimum pH of enzyme bio-sensor.

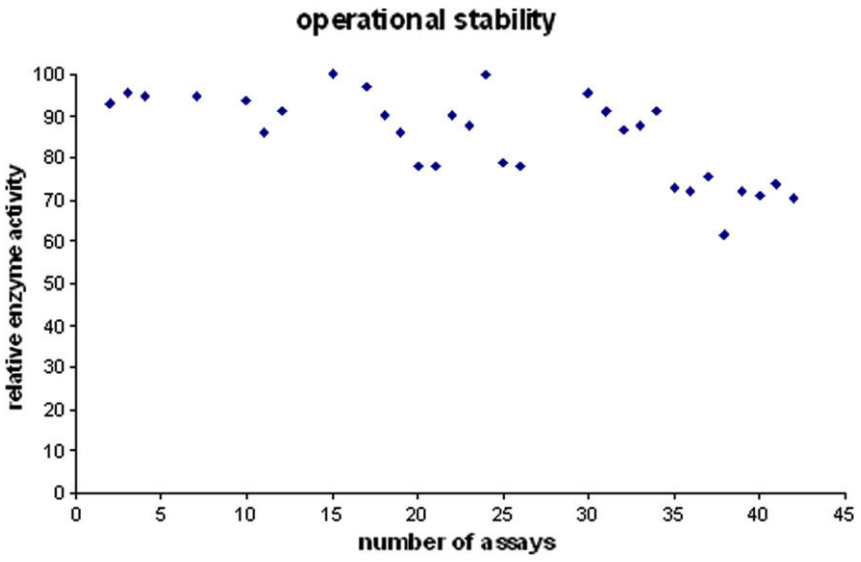


Fig. 5. Operational stability of enzyme bio-sensor.

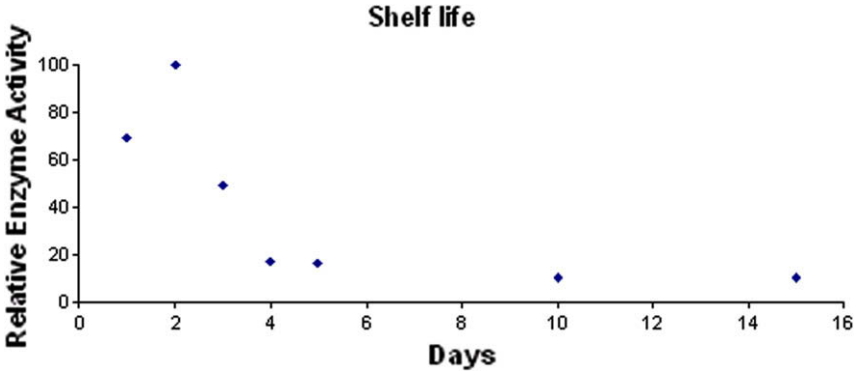


Fig. 6. Shelf life of enzyme bio-sensor.

1.4×10^{-3} S/cm at RT with RH ~50%. Chitosan/AA matrix reveals high temperature resistance as determined by TGA. Polymer electrolyte matrix protects entrapped cholesterol oxidase, hence the enzyme shows higher stability over broader temperature and pH ranges.

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