7.1. Overview

Enzymes as biocatalysts are exclusively preferred in various biotechnological applications including clinical diagnostics, biotransformation reactions and the enzymatic end-point assay of biochemicals in bioanalytical chemistry because of its high substrate specificity and catalytic activity (Tomassetti et al., 2013; Bhatia, 2018; Devlin, 1959). Chox has well established applications in the field of biosensors and bio-analytical chemistry. Chox plays a key role in the specific estimation of cholesterol in human serum by enzymatic method. Chox based assay method for the detection of cholesterol is highly specific, selective and have no chemical substitute for the reaction it catalyzes. Despite of their high specificity, selectivity and no use of corrosive reagents, extreme pH or temperature ranges; the enzyme-catalyzed reactions suffer awful shortcomings of high cost and low stability.

Horseradish peroxidase (HRP, E.C.1.11.1.7) has been widely used and investigated for analytical purposes (Murugaiyan et al., 2014). Hydrogen peroxide (H_2O_2) is liberated as a by-product in the oxidation reaction of cholesterol which is catalyzed by Chox. HRP is coupled with this H_2O_2 in the presence of a chromogen (reducing agent) to produce a colored adduct for the estimation of Chox. In the clinical pathology, this enzymatic assay catalyzed by Chox and HRP is used for the indirect estimation of serum cholesterol. The use of soluble HRP in the estimation of Chox by the enzymatic endpoint method makes this assay method expensive due to its poor reusability and low stability. Enzyme immobilization technology offers a straightforward and most effective solution to reduce the cost of this biochemical assay by increasing the reusability and storage stability of soluble HRP.

The use of specific reaction conditions and their non-reusability in the reaction mixture adds cost to the enzymatic assays they catalyze. Moreover, their possible inactivation caused by various physical and chemical agents viz. pH, temperature, organic solvents, detergents, and salts pose a question on operational stability and storage stability of these enzymes. Immobilization of soluble enzymes to a suitable support matrix is the most influential and efficient means to optimize the specific properties of enzymes (catalytic activity, stability, and specificity), minimize the enzyme inhibition effects, and aids in the recovery and reuse of the biocatalyst and is therefore highly significant from the economic point of view (Elhakeem et al., 2014; Mohamad et al., 2015).

Covalent binding technique essentially prevents enzyme leaching from the carrier surface thereby increasing its reusability and longevity in storage conditions (Sheldon and Woodley, 2017. The use of magnetic nanoparticles (MNPs) for enzyme immobilization imparts easy separation property to the biocatalyst from the reaction mixture. The conjugation of MNPs with a biopolymer such as chitosan protects the nanoparticles from erosion and provides promising functional groups for enzyme attachment. Chitosan has unique biochemical properties including biocompatibility, bioactivity, biodegradability andnon-toxicity making it an excellent biopolymer for both coating the MNPs as well as enzyme immobilization (Zhang et al., 2009)**.** The reactive functional groups (free hydroxyl and amino groups) of chitosan support both adsorption and covalent linkage. Graphene oxide (GO) is an amphiphilic macromolecule with a hydrophobic plane and hydrophilic edges (Dreyer et al., 2010). GO possess multiple functional groups (epoxy, hydroxyl, carboxyl and carbonyl groups) on its surface, readily available to interact with the enzymes thus making it an ideal support for enzyme immobilization (Gao, 2015). GO also enhances

the integrity of the synthesized biocatalyst by providing high mechanical strength. Covalent immobilization of the enzyme on the support surface by using glutaraldehyde as a crosslinking agent increases the enzyme attachment thereby increasing the immobilization efficiency.

In the present work, we prepared GO coated Fe_2O_3/chitosan beads and activated the beads by crosslinking with glutaraldehyde treatment. The objective is to use these beads as a carrier support for HRP immobilization by covalent binding technique, and the application of these GO coated magnetic chitosan beads for increasing the reusability of HRP in Chox assay. Some optimal operational conditions for immobilized HRP were established and the stability of immobilized HRP under various conditions was studied.

7.2. Experimental

7.2.1. Chemicals Used

Cholesterol was purchased from Sigma Aldrich Pvt. Ltd. Chitosan (deacetylation degrees higher than 85%), Horseradish Peroxidase and single layer Graphene oxide nanopowder were purchased from Sisco Research Laboratories, Mumbai, India. All the chemicals used were of analytical grade and were used without purification.

7.2.2. Preparation of Magnetic Nanoparticles

Magnetic iron oxide nanoparticle was prepared by the thermal co-precipitation method described by Moghaddam and Aliahmad with necessary modifications (Moghaddam and Aliahmad, 2013). A mixture of the aqueous solutions of Fe_2Cl_3 (100 ml, 1 M) and $FeSO_4$ (100 ml, 0.5 M) was used for the synthesis of maghemite nanoparticles. The above mixture was heated up to 90˚C along with the addition of NH_4OH (25% v/v of ammonia) drop-wise with vigorous stirring until the alkaline condition (pH 10.0) was

reached. The color of the mixture slowly turns to black while reaching the alkaline pH. The mixture was continued to stir for at least 30 min and centrifuged at 5,000 rpm for 5 min. The black precipitate obtained was washed with deionized water till neutral pH of the supernatant, dried in a hot air oven at 60˚C and crushed using a pestle and mortar. The black powder so obtained was Fe_3O_4 nanoparticles (magnetite). For the synthesis of Fe_2O_3 (maghemite) nanoparticles, the black powder was kept at 800˚C in a hot air oven for 14-16 h till it turned to reddish brown color. The reddish-brown powder was cooled to room temperature and crushed again on pestle and mortar.

7.2.3. Preparation of Magnetic Chitosan Beads

Chitosan was dissolved in acetic acid (25 ml, 2% v/v) with the help of a magnetic stirrer overnight to assure no lump formation and until a clear transparent solution is obtained. The discharge of trapped air bubbles was done by sonicating the chitosan solution for 20 min. Fe_2O_3 nanoparticles prepared in section 2.2 were treated with ethanol to remove any moisture in it and air dried. Fe_2O_3 (2 g) was added slowly to the chitosan solution; vigorously stirred for 1 h till the nanoparticles were homogeneously dispersed in the chitosan solution to form a blend. The chitosan-Fe_2O_3 blend was used for preparing chitosan beads having magnetic properties with the help of a 5 ml syringe in NaOH (100 ml, 1 M) under continuous stirring. The beads were kept in NaOH solution for 4 h for hardening and then washed with deionized water until neutral pH of the washings was obtained.

7.2.4. Coating Graphene Oxide on Magnetic Chitosan Beads

After extensive washing with deionized water, the magnetic chitosan (MC) beads (100 mg/ml) were transferred to an exfoliated and homogeneous solution of GO (stock

solution 0.4 mg/ml) and kept under stirring overnight. The MC beads were separated from the GO solution using an external magnet. The GO solution can be reused for the second cycle of GO coating on MC beads. The MC beads were extensively washed for removal of excess GO from the beads surface. The GO coated MC beads were sonicated for 1 h in a bath sonicator. The GO coated MC beads (GOMC beads) were washed with deionized water and freeze-dried for 6-8 h.

7.2.5. Immobilization of HRP on GO coated Magnetic Chitosan Beads

The activation and functionalization of GOMC beads were carried out by glutaraldehyde (GA) (2.5% v/v) treatment at 25˚C for 8 h. The aldehyde functionalized GOMC beads were separated using an external magnet and washed at least five times to remove any unreacted GA residues. The obtained GA activated GOMC beads were stored in potassium phosphate buffer (50mM, pH 7.0).

HRP was covalently immobilized on the surface of GOMC beads by the GA activation method (Wang et al., 2012). The GA-activated GOMC beads were immersed in a cocktail solution of HRP (10mg/100 beads) and phosphate buffer (50mM, pH 7.0) followed by overnight incubation at 30˚C, 200 rpm in an orbital shaker (Orbitek, Scigenics Biotech Pvt. Ltd., Chennai, India)for immobilization. After completing the immobilization procedure, the peroxidase immobilized graphene oxide coated magnetic chitosan beads (PGOMC) were separated using an external magnet. The PGOMC beads were washed four to five times with phosphate buffer (50mM, pH 7.0) to remove the unbound peroxidase till no protein was detected in the washings. The PGOMC beads were preserved in phosphate buffer (50mM, pH 7.0) in a refrigerator at 4˚C for further studies and freeze-dried (6-8 h) before structural characterization.

The protein concentration was determined spectro-photometrically according to the method described by Bradford (Bradford, 1976). Bovine serum albumin was used as a standard protein for the construction of the calibration curve. The amount of immobilized protein was evaluated by taking the difference between the initial protein concentrations of the prepared enzyme solution subjected for immobilization to that of the residual protein left in the same enzyme solution after immobilization.

$$\text{Immobilization Efficiency (\%)} = \left(\frac{\text{Activity of Immobilized Enzyme}}{\text{Initial Activity of the Soluble Enzyme}}\right) \times 100 \quad \text{….. (7.1)}$$

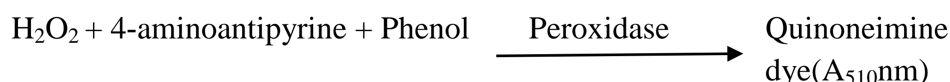
7.2.6. Characterization of the HRP loaded Magnetic Chitosan Beads

Surface morphology of the synthesized beads was imaged with a Zeiss EVO 18 scanning electron microscope (Zeiss, Germany) using 20 kV accelerating voltage. X-ray diffraction (XRD) spectrum of the synthesized material was obtained for the crystallization phase analysis (Rigaku diffractometer). The measurement was done in the Bragg's angle (2θ) range from 10°-80°, at scanning rate of $5^{\circ}\,\text{min}^{-1}$, Cu Kα radiation (λ = 0.15418 nm) using Rigaku Miniflex 600 D Tex ultra. Magnetization curve of the synthesized GOMC bead support material was obtained using a vibration sample magnetometer (VSM, Riken Denshi Co. Ltd., Japan) at room temperature. The analysis of functional groups attached to carrier support and the enzyme was done using Fourier transform infrared spectroscopy (FTIR). The samples were scanned in the range of 4000–400 cm^{-1} using FTIR spectrophotometer (Shimadzu, Japan).

7.2.7. Activity assay of Free and Immobilized HRP

The estimation of peroxidase activity was done by coupling of H_2O_2 with 4-aminoantipyrine and phenol in the presence of peroxidase to form Quinoneimine; a pink colored chromogen with absorption maxima at 510 nm. The amount of Quinoneimine

produced is directly proportional to the amount of peroxidase present (Sarika et al., 2015). 1ml of reaction mixture consisted of 0.05 M potassium phosphate buffer pH 7.0, 0.0025 M 4-aminoantipyrine with 0.17 M phenol and soluble or immobilized HRP; pre-incubated for 3 min at 30˚C. The reaction was initiated by adding 0.0017M hydrogen peroxide to the pre-incubated reaction mixture and the incubation continued for 10 min at 30˚C. The reaction was terminated by heating the samples in a boiling water bath for 2 min followed by subsequently transferring the samples in an ice-bath for color development. The PGOMC beads were magnetically separated before the termination of the reaction. Blank was prepared by taking the reaction mixture containing all the components excluding HRP (free or immobilized). Absorbance was recorded at 510 nm by discontinuous spectrophotometric method (UV 1800 Spectrophotometer, Shimadzu, Japan). One unit of activity was defined as the amount of HRP required to hydrolyze one micromole of hydrogen peroxide per minute at 30˚C and pH 7.0.

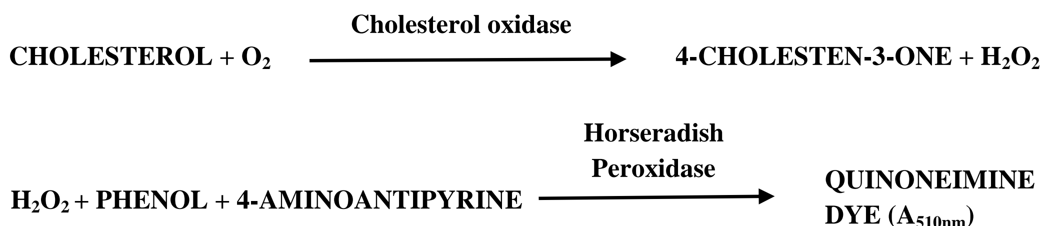


7.2.8. Application of PGOMC Beads for Cholesterol oxidase assay

The prepared PGOMC beads were examined for their efficacy to estimate Chox in the reaction mixture containing cholesterol as a substrate by the modified method of Allain et al (Allain et al., 1974). The reaction conditions for the Chox assay were optimized in our previous study (Sahu et al., 2019). Cholesterol (50 μL; 6 g/L) dissolved in Triton X-100 5% (v/v) and dimethyl formamide was added to 1 ml of reaction mixture containing 1mM 4-aminoantipyrine, 5 mM phenol, and sodium phosphate buffer (20 mM, pH 7.0). The as-prepared PGOMC beads (mentioned in section 2.5) loaded with 10 U HRP (4 beads) were

added to the above mentioned reaction mixture and pre-incubated for 5 min at 30˚C. 100 μL of crude Chox extracted from supernatant of the fermentation broth of *S. olivaceus* MTCC 6820 was added to the pre-incubated reaction mixture to start the reaction and the incubation continued for 10 min at 30˚C. The PGOMC beads were separated by an external magnet before the termination of the reaction. The reaction was terminated by placing the samples in a boiling water bath for 2 min and then immediately placed in an ice bath for 2 min for color development. Absorbance was recorded at 500nm (UV 1800 Spectrophotometer, Shimadzu, Japan). Blank was prepared by adding an inactivated enzyme to the reaction mixture. One unit of cholesterol oxidase activity was defined as the amount of enzyme that converts 1μmol of cholesterol into H_2O_2 per minute at 30˚C.

The use of immobilized HRP in the enzymatic assay method of Chox can be explained by the following biochemical reaction. The oxidation of cholesterol is catalyzed by the Chox enzyme to produce H_2O_2; the degradation of H_2O_2 is further catalyzed by HRP to produce Quinoneimine dye having absorption maxima at 510 nm.



7.2.9. Properties of Immobilized HRP

7.2.9.1. Effect of pH

The study of optimal pH for both the free and immobilized HRP was done by measuring the enzyme activity over the pH range of 4 to 9 using the following buffers

(0.05 M): sodium acetate buffer (pH 4.0 to 5.5), potassium phosphate buffer (pH 6.0 to 7.5), and Tris-HCl buffer (pH 8.0 to 9.0). Blank was prepared using potassium phosphate buffer pH 7.0. Free and immobilized HRP along with respective pH buffers were incubated for 10 min at 30˚C and the enzyme activities were measured as mentioned in section 2.6.

7.2.9.2. Effect of Temperature

The study of optimal reaction temperature was carried out by measuring the enzyme activities in the temperature range of 25 to 85˚C. Both the free and immobilized HRP was incubated for 10 min at different temperatures along with the reaction mixture using potassium phosphate buffer (0.05M, pH 7.0) and enzyme activities were measured as mentioned in section 2.6.

7.2.9.3. Thermo-stability

The thermal stability studies were carried out at three different temperatures viz. 35˚C, 55˚C, and 75˚C. Both the free and immobilized enzymes were incubated using phosphate buffer (0.05 M, pH 7.0) at the respective temperatures for 2 h. 100 µl samples from each temperature variable were tested for peroxidase activity using phosphate buffer pH 7.0 (refer section 2.6).

7.2.9.4. Effect of metal ions

The effect of metal ions on both free and immobilized HRP was studied in the presence of chloride and sulphate salts of different metal ions viz. NaCl, KCl, $MgCl_2$, $CaCl_2$, $HgCl_2$, $PbCl_2$, $ZnCl_2$, $CdCl_2$, $MnCl_2$, $NiCl_2$, $CuSO_4$, $CsCl_2$. The enzyme solution immobilized was incubated with 2mM solution of different metal ions for 15 min and then the enzyme activity assay was done for both the free as well as immobilized HRP. The enzyme activity in the absence of metal ions was taken as 100%.

7.2.9.5. Stability in various detergents and organic solvents

Free and immobilized HRP (2.5 U) were incubated overnight with 50% (v/v) suspension of organic solvents viz., isopropanol, ethanol, methanol, and acetonitrile and non-ionic detergents Triton X-100, tween-20, and Tween-80 at 30˚C. Samples from each test tube were tested for enzyme activity as mentioned in section 2.7. The enzyme activity assayed in the absence of these compounds was considered as control (100%) for the calculation of residual activity.

7.2.9.6. Reusability and Storage Stability of the Immobilized HRP

The activity assay for Chox using PGOMC beads was performed for 10 consecutive cycles according to the method mentioned in section 2.7. After the completion of each cycle, the same PGOMCS beads were separated magnetically, washed with phosphate buffer (50 mM, pH 7.0) and reused in the new reaction cycle without any treatment. The activity of freshly prepared PGOMC beads was taken as 100%. The storage stability of immobilized HRP was monitored by storing the PGOMC beads in a refrigerator for 90 days at 4˚C. The activity of POMC beads was checked at an interval of every 10 days.

7.3. Results and Discussion

7.3.1. Immobilization

In the present study, we prepared GO coated magnetic chitosan beads for HRP immobilization and characterized the immobilized enzyme. The magnetic chitosan beads were prepared, coated with GO and activated with glutaraldehyde. The immobilization of HRP was successful with nearly 80 ± 1.35 % immobilization efficiency. The maximum amount of bound protein was 0.08 mg/bead. The immobilized enzyme was characterized for optimum functional range and stability. Glutaraldehyde activation of chitosan beads

provides multipoint attachment sites for the enzyme. Literature suggests that the conjugate of Fe_2O_3-GO mimics peroxidase-like activity, which may be the reason to enhance the HRP activity (Song et al., 2016). This support matrix may have a positive synergistic effect over HRP immobilization. Moreover, coating GO on magnetic chitosan beads with sonication allowed the smaller size GO to well-disperse onto the chitosan beads surface, providing uniformity inactive sites throughout the beads. With the high immobilization efficiency of ~80%, the GO coated magnetic chitosan beads proved to be excellent carrier support for HRP immobilization.

7.3.2. Characterization of Immobilized HRP with Carrier Support

7.3.2.1. SEM

The SEM image of the HRP immobilized GO coated magnetic chitosan bead is displayed in **Fig 7.1 (a, b and c)**. Spherical beads with an average size of 1.5 mm were formed **(Fig 7.1 a)**. The SEM image gives an idea about the surface morphology of the PGOMC bead, which indicates the non-porous structure of the bead surface **(Fig 7.1 b)**. The compactness in the surface morphology may be attributed to the GO coated over the surface of the bead increasing the mechanical integrity relatively **(Fig 7.1 c)**.

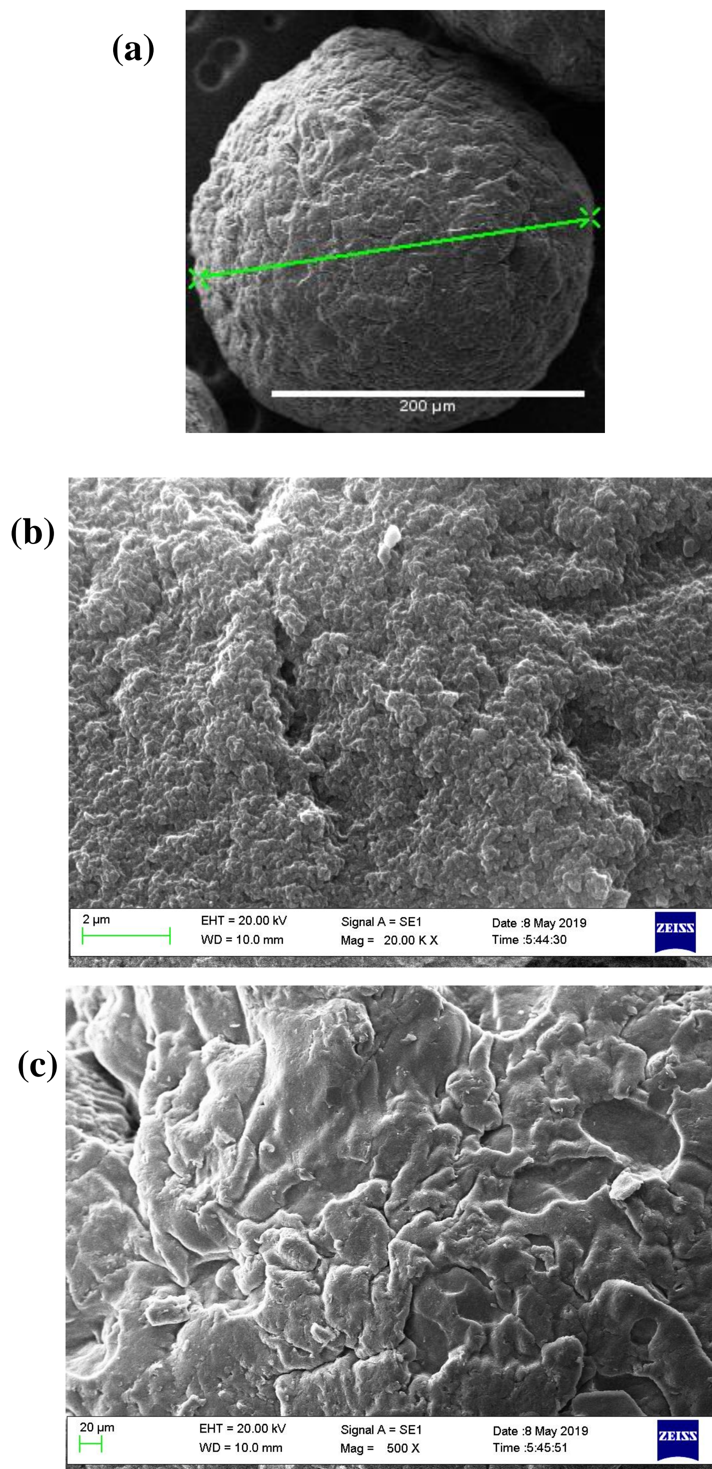


Figure 7.1 SEM image of (a) HRP immobilized GO coated magnetic chitosan bead

(b) Surface morphology of the GOMC bead (20.00 KX magnification)

(c) Surface morphology of the GOMC bead (500 X magnification)

7.3.2.2. FT-IR Analysis

The beads were analyzed by FTIR spectroscopy to figure out the interactions between Fe_2O_3, Chitosan, Fe_2O_3-Chitosan, Fe_2O_3-Chitosan-GO, and Fe_2O_3-Chitosan-GO-Peroxidase, presented in **Fig 7.2.** The peak at 552 cm^{-1} indicates the presence of iron oxide nanoparticles (NPs) while the sharp peak at 634 cm^{-1} corresponds to the characteristic Fe-O vibrational mode of iron oxide NPs (**Fig 7.2A)** (Khan et al., 2012). The FTIR spectra of chitosan show the trough at 1382 cm^{-1} and 1658 cm^{-1} which are due to the N-H bending vibrations and –C=O stretching of the amide-I group respectively **Fig 7.2B** (Wang et al., 2017; Qian et al., 2017). The band at 1067 cm^{-1} corresponds to the C-O stretching vibration due to the presence of saccharide structure in chitosan (Singh and Kumari, 2014). However, it has been reported that the bands appearing in the region of 960 cm^{-1} to 1180 cm^{-1} correspond to the saccharide peaks of chitosan and was consistently maintained (Fig 7.2 B, C, and D). The new band present at 1454 cm^{-1} in the FTIR spectrum of the Fe_2O_3-Chitosan shows the C-O stretching of the primary alcoholic group (**Fig7.2C**). The spectrum of Fe_2O_3-Chitosan depicts the presence of all the characteristics bands of chitosan. This indicates that Fe_2O_3 NPs were completely covered with chitosan which confirmed the formation of Fe_2O_3-chitosan nanocomposite (Alagappanand Sairam,2014). The band at 1067 cm^{-1} and 1454 cm^{-1} was constantly maintained in **Fig 7.2D** which represents the C-O-C groups present in GO. The new bands observed at 1658 cm^{-1} and 1542 cm^{-1} are attributed to the –CONH– (amide I) and amide II vibrations **Fig 7.2E** representing the characteristic transmittance of HRP (Chang and Tang, 2014). These results ascertain the immobilization of HRP on the surface of GOMC beads.

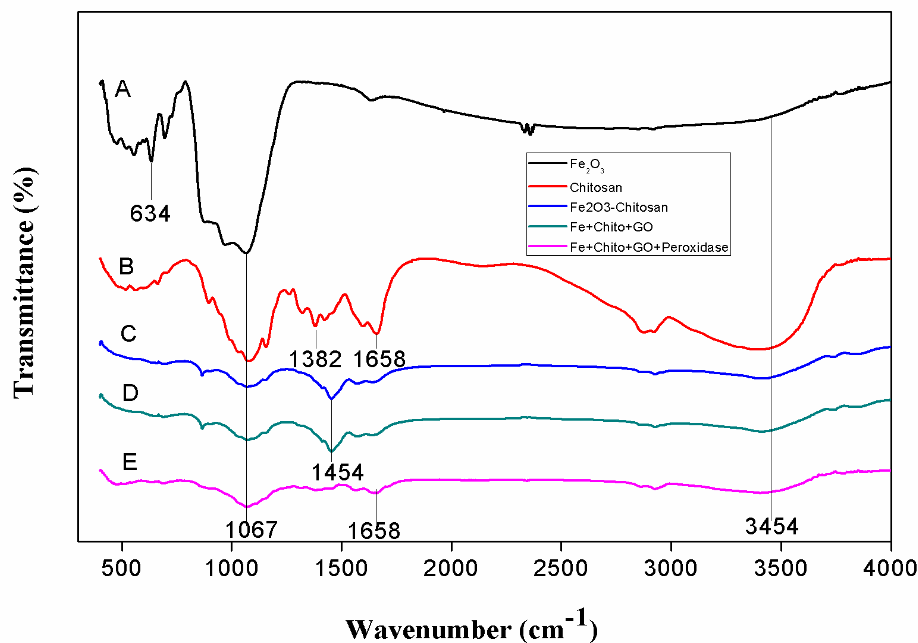


Figure 7.2: **FT-IR Spectra of (a) Fe_2O_3 (b)Chitosan (c) Fe_2O_3-Chitosan (d) Fe_2O_3-Chitosan-Graphene Oxide and (e) Fe_2O_3-Chitosan-Graphene Oxide-HRP**

7.3.2.3. X-ray Diffraction

The XRD patterns of Fe_2O_3, Fe_2O_3-chitosan, and immobilized HRP are shown in **Fig 7.3.** For Fe_2O_3 nanoparticles, the peaks at $2\theta = 30.1°, 35.4°, 43.1°, 53.2°, 56.9°$, and $62.5°$ correspond to (220), (311), (400), (422), (511), and (440) planes of Fe_2O_3, respectively. After coating with chitosan and immobilization of HRP, the same characteristic peaks exhibited, which revealed that the magnetic nanoparticles uphold the crystalline structure of Fe_2O_3.

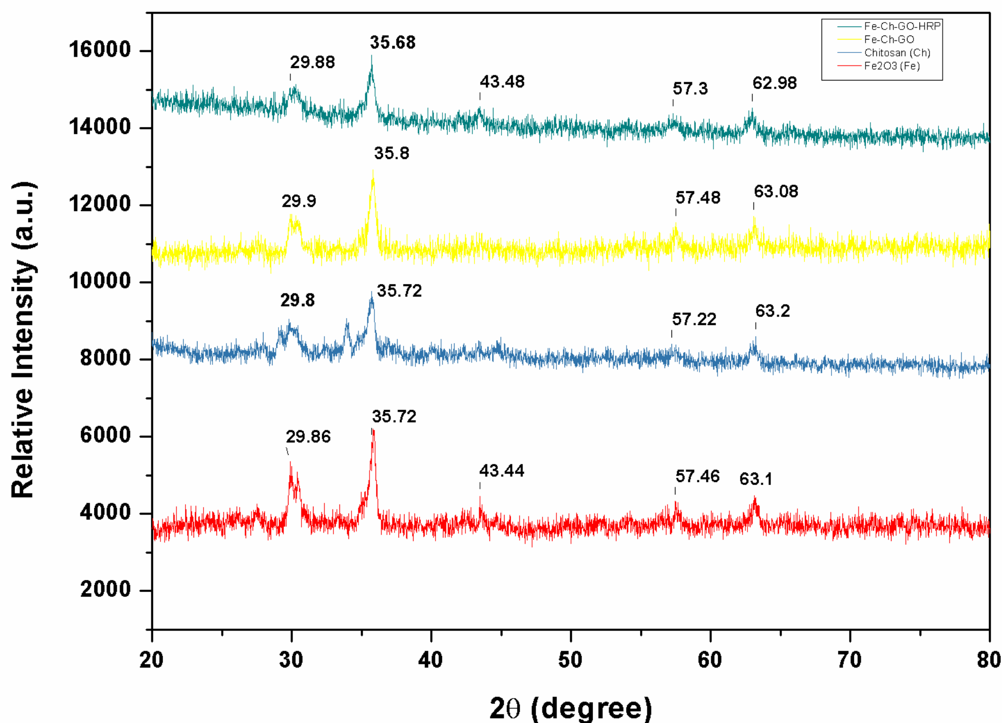


Figure 7.3: **X-ray diffraction of (a) Fe_2O_3 (red line) (b) Chitosan (blue line) (c) Fe_2O_3-Chitosan-Graphene Oxide (Yellow line) and (d) Fe_2O_3-Chitosan-Graphene Oxide-Peroxidase (sea green).**

7.3.2.4. Vibrating Sample Magnetometer (VSM) measurements

A vibrating sample magnetometer (VSM) was used to measure the magnetic property of the Fe_2O_3 nanoparticles. The magnetization curve of Fe_2O_3 nanoparticles at room temperature is displayed in **Fig 7.4**. The value of saturation magnetization (Ms) for Fe_2O_3 was found to be 19 Am^2/kg**.** The synthesized Fe_2O_3 sample showed super-paramagnetic behavior with 0.015 T coercivity and 0.0018 Am^2/kg remanence values. **Fig 7.4** displays a fine hysteresis loop which depends upon the size of the nanoparticles. With the decrease in particle size, there is a decrease in magnetic domains per particle. The magnetic nanoparticles exist in a single domain below a critical diameter; in this condition the

behavior of particles is super-paramagnetic. The super-paramagnetic nanoparticles experience fluctuations due to thermal energy which tend to randomize the moments of nanoparticles in the absence of an applied magnetic field; resulting in negligible coercivity and remanence (Moghaddam and Aliahmad, 2013; Sellmyer and Skomski, 2006). This indicates the fine crystallite size of γ-Fe_2O_3 nanoparticles in the range of 20 - 50 nm (Dutz and Hergt, 2012.

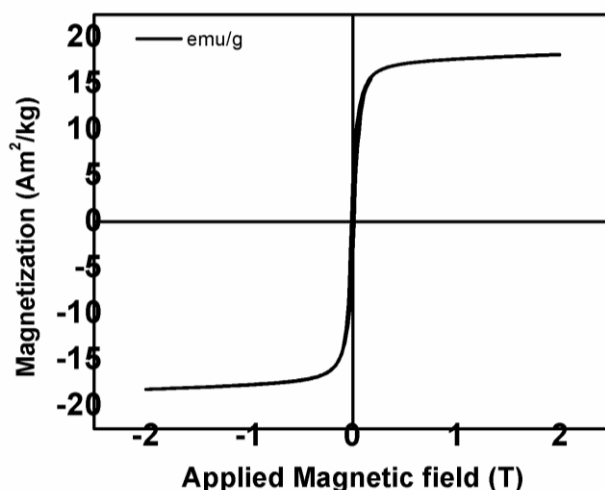


Figure 7.4: Magnetization curve of the Fe_2O_3

7.3.3. Properties of Immobilized HRP

Enzymes often exhibit altered kinetic behavior after immobilization which may be attributed to the changes in the microenvironment of the immobilized enzyme provided by the support matrix.

7.3.3.1.Effect of pH on free and immobilized HRP

The effect of pH on the activity of free and immobilized HRP was studied by incubating with different buffers in the pH range 4 – 9 at 30˚C and the results are presented in **Fig. 7.5a**. The pH profile of the immobilized HRP showed that it was most active from pH 6-8. However, the optimum pH for the activity of free and immobilized HRP were 6.5

and 7.0 respectively. Similar results were found, where the pH (6.5) for free HRP was shifted to (7.0) for immobilized HRP (Yu et al., 2019). A possible reason for this small pH shift may be attributed to the conformational changes in the three-dimensional structure of the enzyme protein due to the surface properties of the carrier support after immobilization. The outer surface of the PGOMC bead is rich in free amino groups due to the presence of chitosan, which absorbs H^+ ions from the reaction mixture resulting in lower pH value in the vicinity of the immobilized enzyme. In order to account for the conformational change brought about by the immobilization, the pH is raised to a certain degree facilitating the enzyme to function properly (Chang and Tang, 2014). This shift in the pH optima of the immobilized HRP shows its adaptability towards the environmental alkalinity, which is favorable for its application in the cholesterol oxidase assay (Sahu et al., 2019).

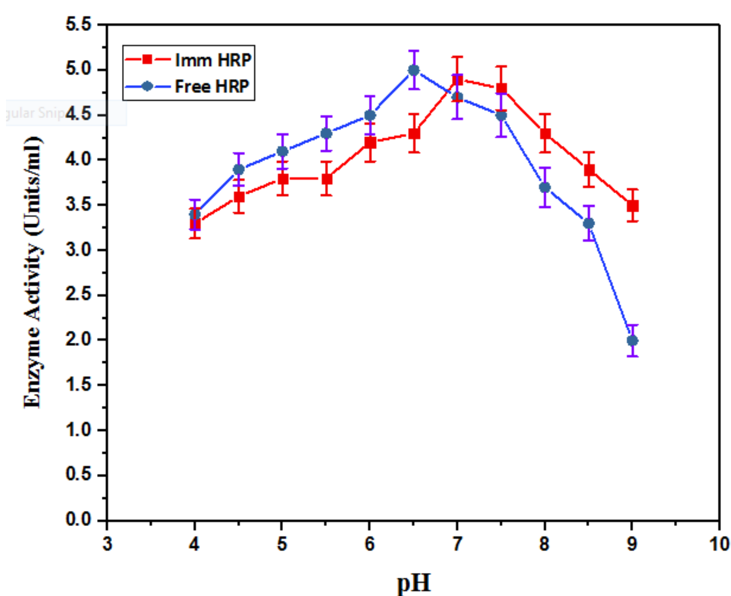


Fig 7.5 (a) Effect of pH on activity of free and Immobilized HRP

7.3.3.2. Effect of temperature on free and immobilized HRP

The optimum temperature of the free and immobilized HRP was determined by measuring the enzyme activity at a variable temperature ranging from 25˚C to 85˚C at pH 7.0 (presented in **Fig 7.5 b**). The immobilized enzyme was active over a wide range of temperature viz. 35˚C - 65˚C. An elevation in the optimum reaction temperature of the free HRP (50˚C) as compared to the immobilized HRP (55˚C) was observed, which is following the earlier reported literature (Mohamed, 2013). This increased heat resistance might be possible because of the support matrix providing a higher degree of cross-linking sites for the attachment of enzyme protein in their active orientation, which shields the HRP from inappropriate structural changes at high temperature. It is well established that if subjected to thermal exposure, enzymes in the soluble form are more prone to the unfolding of protein native three-dimensional conformation thus affecting the protein structure-function relationship which is minimized in case of immobilized enzymes (Querol et al., 1996).

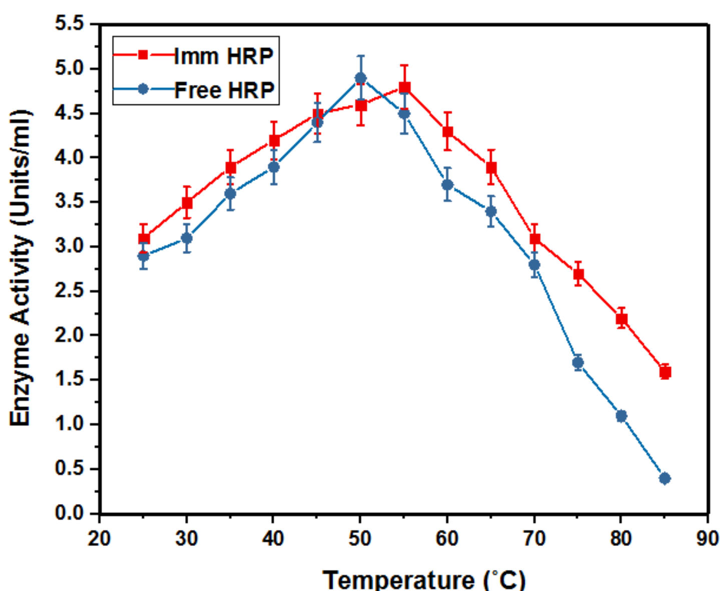


Fig 7.5 (b) Effect of temperature on activity of free and Immobilized HRP

7.3.3.3. Thermo-stability

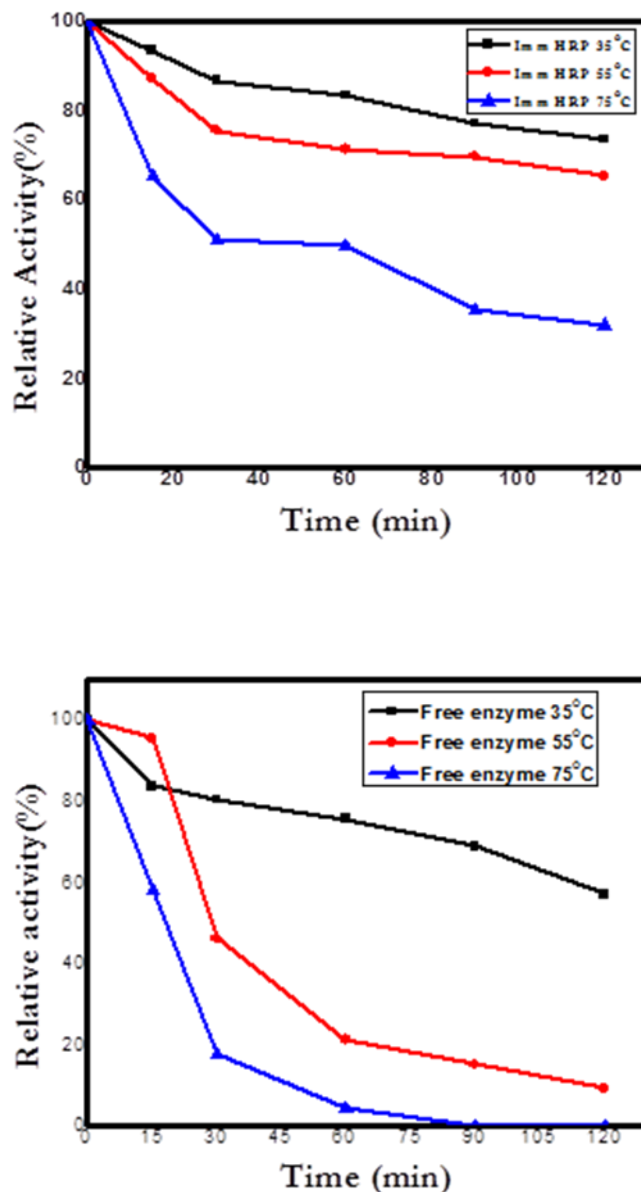


Figure 7.6 Thermostability of (a) Immobilized HRP (b) Free HRP

The thermal stability of free and immobilized HRP was studied at three different temperatures 35˚C, 55˚C and 75˚C for 2 h. The immobilized HRP maintained 80% activity for 90 min at 35˚C, retained more than 70% activity for 90 min at 55˚C, and 50% activity up to 60 min at 75˚C; **Fig 7.6 (a)**. On the other hand, free HRP retained more than 75%

relative activity for 60 min at 35˚C, around 45% and 20% of the relative activity up to 30 min at 55˚C and 75˚C respectively; **Fig 7.6 (b)**. The activity of the enzyme at pH 7.0, 30˚C assayed for 10 min was considered as 100% for the calculation of relative activity. An improvement in the thermal stability of the PGOMC beads was observed which was attributed to the covalent attachment of enzyme groups with the free amino ends of chitosan and oxides of GO. The working temperature window of the immobilized HRP was widened with respect to time which shows that the GOMC was a good support matrix for electrode preparation using immobilized HRP increasing its stability and applicability in electrochemical biosensors.

7.3.3.4. Effect of detergents, organic solvents, and metal ions

Chox assay essentially requires the use of various non-ionic detergents and organic solvents for the dissolution of cholesterol (water-insoluble) in the reaction mixture. Therefore, the stability studies of the enzyme in the presence of organic solvents and detergents are very important. The activity of free and immobilized HRP was determined in the presence of ethanol, methanol, isopropanol, acetonitrile, Triton X-100, tween – 20, and tween - 80. The results are illustrated in **Table 7.1 and 7.2**. The free and the immobilized HRP showed a very similar behavioral pattern for the organic solvents and detergents, however, the immobilized HRP exhibited more stability in the presence of these chemicals. The relative activity of the immobilized HRP was increased in the presence of methanol, ethanol, and isopropanol whereas it was reduced in the presence of acetonitrile. Triton X - 100 and tween – 20 enhanced the relative activity of both the free and immobilized HRP. The significant stability of the immobilized HRP in ethanol, Triton X – 100 and tween – 20 with relative activity ≥ 150% proved that the PGOMC beads were

highly suited for the estimation of cholesterol oxidase, as they are most commonly used solvents in the cholesterol oxidase assay.

The effect of different metals on free HRP and HRP immobilized on GOMC beads were studied and summarized in Table 2. Mg^{2+}, Ca^{2+}, Zn^{2+}, and Mn^{2+} showed strong activation effects on immobilized HRP. Zn^{2+} strongly inhibited the free HRP while Cs^{2+} inhibition was strong for both free and immobilized HRP. Pb^{2+} inhibition effect was more on free HRP while Hg^{2+} and Cu^{2+} completely inhibited both the free and immobilized HRP. The results indicated that the HRP immobilized on GOMC beads had more resistance towards metal ions than the free HRP.

Table 7.1 Effect of organic solvents on the stability of immobilized and free HRP

Chemicals	**Relative activity (%)**	
	Immobilized HRP	**Soluble HRP**
Control	100.23 ± 0.25	100.36 ± 0.32
Isopropanol	107.82 ± 0.15	35.61 ± 0.70
Ethanol	156.45 ± 1.33	56.57 ± 0.95
Methanol	101.13 ± 0.80	33.19 ± 1.0
Acetonitrile	71.59 ± 0.69	23.57 ± 1.07
Triton X - 100	167.20 ± 1.59	69.43 ± 1.36
Tween - 20	142.6 ± 1.45	54.04 ± 1.78
Tween - 80	101.93 ± 1.69	33.46 ± 1.23

All the experiments of enzyme activity measurement were independently performed in triplicate and the results were presented in terms of mean and standard deviation.

Table 7.2 Effect of Metal Ions on the Stability of Immobilized and Free HRP

Metal ions	**Relative activity (%)**	
	Soluble HRP	**Immobilized HRP**
Control	100.13 ± 0.32	100.16 ± 0.76
NaCl	116.37 ± 0.60	118.5 ± 1.32
KCl	110.52 ± 0.58	116.0 ± 1.34
$MgCl_2$	98.71 ± 1.07	176.79 ± 0.94
$CaCl_2$	95.57 ± 4.27	174.03 ± 0.90
$HgCl_2$	-	-
$PbCl_2$	60.54 ± 1.15	74.00 ± 1
$ZnCl_2$	23.71 ± 0.81	145.55 ± 1.35
$CdCl_2$	178.39 ± 0.97	187.18 ± 1.25
$MnCl_2$	84.51 ± 0.67	198.28 ± 1.27
$NiCl_2$	132.12 ± 1.03	137.13 ± 1.09
$CuCl_2$	-	-
$CsCl_2$	37.4 ± 0.55	30.82 ± 0.76

All the experiments of enzyme activity measurement were independently performed in triplicate and the results were presented in terms of mean and standard deviation.

7.3.4. Reusability of Immobilized HRP

One of the major advantages of the immobilized enzyme is the reusability, which can effectively reduce the cost of the applications. The operational stability of the HRP immobilized on GOMC beads was evaluated by performing repeated cycles of cholesterol oxidase assay. **Fig 7.7** displays the reusability assay of immobilized HRP for estimation of cholesterol oxidase. The relative activity of the immobilized HRP reduced with the increasing number of reuses. The immobilized HRP retained ~90% of the residual activity after 12 times repeated reuse in the cholesterol oxidase assay. The enzyme activity obtained in the first cycle was considered as 100% activity. The number of reuse represents a more satisfying performance when compared to recent researches **(Table 7.3)**. These results may be attributed to the covalent attachment of enzyme to the support after glutaraldehyde activation. Also, the GO coated on the magnetic chitosan beads enhanced mechanical integrity of the beads, which made them non-brittle and allowed easy separation from the reaction mixture (Lau et al., 2014). GO sheet has also been reported as an excellent matrix for enzyme immobilization, which prevented HRP from easy leach out from the surface of the GOMC beads (Zhang et al., 2010). The magnetic separation and the enhanced mechanical strength of the chitosan beads proved very handy and practical in the reusability assay.

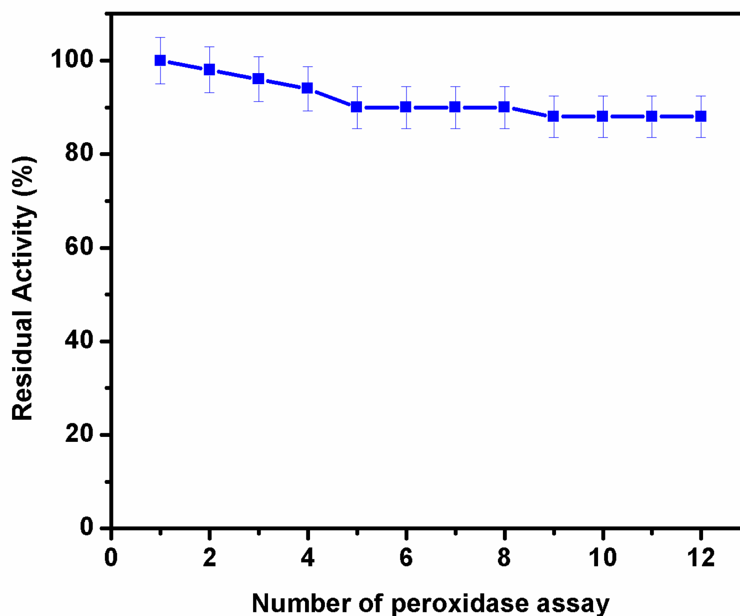


Fig 7.7 Effect of repeated use of immobilized enzyme on activity of Peroxidase

Table 7.3 Comparison of repeated use of HRP immobilized on different carriers after ten times reuse

Carrier material	Relative Activity (%)	Reference
Graphene oxide/Fe_3O_4	41	Chang and Tang, 2014
Amine-rich nanoporous BC	74.6	Yu et al., 2019
Chitosan-g-PEA	58	Monier et al., 2010
Fe_2O_3-Chitosan-Graphene oxide	90	Present study

7.3.5. Storage stability

The study of storage stability is a very essential factor to be taken into account for reducing the cost of the immobilized enzyme by the assessment of immobilization efficiency(Liu et al., 2006). Free and immobilized HRP were stored in a refrigerator at 4ºC

in potassium phosphate buffer (pH = 7) and the enzyme activity was examined at every 10 days interval. The enzyme activity at day zero was considered as 100% and the relative activity was determined. It is evident in **Fig 7.8,** after 30 days of storage**;** the relative activity of immobilized HRP was 93.72% whereas that of the free HRP was ~72%. After 60 days of storage, the relative activity of the immobilized HRP was 60.97% better than the free HRP (~50%). The free HRP loses about 72% activity whereas the immobilized HRP retains more than 70% of its relative activity and shows good stability upon storage for 90 days. **Table 7.4** displays a comparative analysis of the storage stability of HRP immobilized on different carriers, which demonstrate excellent storage stability of HRP immobilized on GOMC beads. These results indicate that the storage stability of the HRP immobilized on GOMC beads are greatly enhanced after immobilization, which is due to the covalent attachment of the enzyme with carrier support.

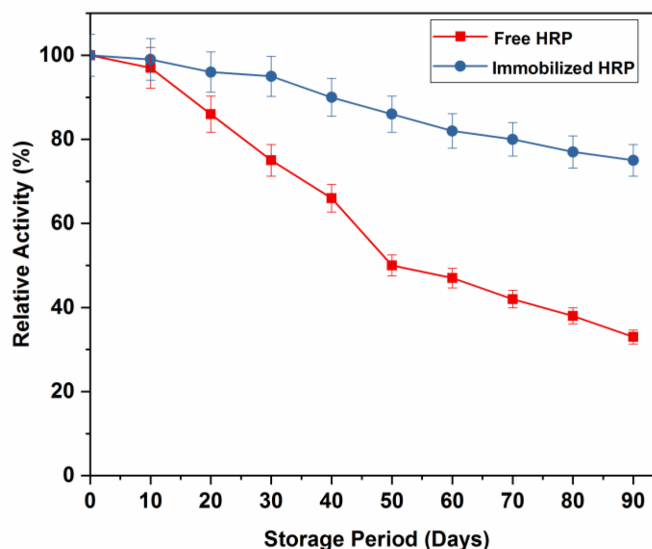


Figure 7.8 Storage stability of free and immobilized HRP

Table 7.4 Storage stability of immobilized HRP on different carriers

Carrier	**No. of days of storage**	**Relative activity (%)**	**Reference**
PET-g-AAm fiber	60	61	Temoçin and Yiğitoğlu, 2009
$RGO{\cdot}NH_2$	35	55	Vineh et al., 2018
Fe_3O_4@PAA-6-arm-PEG-NH_2	60	71.0	Xiea et al., 2019
Fe_2O_3-Chitosan-Graphene oxide	30; 60	93; 85	Present study

7.4. Conclusion

The preparation of GOMC beads and its characterization was studied. Covalent immobilization of HRP onto the surface of GOMC beads was accomplished with glutaraldehyde activation and the application of PGOMC beads for cost-effective Chox assay was investigated. The immobilization of HRP was successful with ~80% immobilization efficiency and the maximum amount of bound protein was 0.08 mg per bead. The immobilized HRP retained ~90% residual activity after being used for up to 12 cycles of Chox assay, which gave a more satisfying performance as compared to the previous report by other scientists, thus providing an economic advantage for the fermentative production and purification of Chox in this research work. By increasing the reusability of HRP we are the pioneer to use low-cost technology for HRP mediated estimation of Chox.