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Horseradish peroxidase immobilized radiation grafted polymer matrix: A biocatalytic system for dye waste water treatment

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Abstract

Horseradish peroxidise (HRP) enzyme, an industrially and environmentally relevant biocatalyst, was covalently immobilized onto epoxy functionalized Polypropylene (PP) films, fabricated via ^{60}Co-gamma radiation induced mutual irradiation grafting of 2,3-Epoxypropyl methacrylate (EPMA) on to PP matrix. Effect of grafting parameters, such as radiation dose and monomer concentration, on the grafting yield was studied in order to optimize the radiation grafting process. Poly(EPMA)-*g*-PP films were characterized by grafting yield determination, FTIR, SEM and XPS. The catalytic activity of the immobilized enzyme HRP system was spectrophotometrically assayed using 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as a substrate. The immobilized HRP system was investigated for repeatability, storage, thermal and pH stability with respect to the free enzyme. The practical applicability of immobilized HRP in treatment of textile dye waste water treatment was examined by studying the repeated catalytic degradation of Basic Red 29 (BR29) dye. The immobilized enzyme system was found to cause ~90% degradation of BR29 over a period of 20 days and was observed to be reusable for five cycles without substantial loss in activity.

Keywords: Enzyme immobilization; Horseradish peroxidise; Radiation grafting; Catalytic degradation

1. Introduction

Enzymes are biocatalysts having excellent properties such as high activity, selectivity, specificity, etc., which facilitate their ability to perform complex chemical processes under the most benign experimental and environmental conditions. However, the use of soluble and free enzymes is limited due to issues related to their cost, reusability, stability, sensitivity to various denaturants and application in continuous reactors[1-4]. To overcome these limitations, immobilization of enzymes, in recent years, has been adopted as a strategy to promote widespread use of industrially, biologically and environmentally relevant enzymes. Immobilization not only affords enhanced stability, activity, specificity, selectivity, reduction of inhibition, but also enhances the thermal stability of the enzyme, broadens the pH range of enzyme activity and enables recovery of product with greater purity. Besides, separation and reusability of the immobilized enzymes is much more feasible compared to free enzymes, thereby, adding to the cost benefits when dealing with expensive or scarcely used enzymes[5-7].

Among the numerous enzymes that are in use, peroxidase is a class of enzyme widely distributed in different bioresources, such as microbes, plants and animals. It is an oxidoreductase enzyme, which, in the presence of hydrogen peroxide, oxidizes a wide range of phenolic compounds, such as guaiacol, catechol, pyrogallol, as well as azo dyes[8-10]. This class of enzymes, therefore, has potential industrial and environmental relevance, particularly in the field of waste water treatment by degradation of textile dyes [11] and phenolic pollutants [12], removal of peroxides from foodstuff [13], synthesis of various aromatic chemicals and diagnostic kits for enzyme immunoassay [14].

One of the novel approaches adopted to achieve irreversible immobilization of peroxidase enzymes has been the use of functionalized polymer support matrices. The

immobilization strategies employed for enzymes include physical adsorption, entrapment, crosslinking and covalent binding [15-19]. In order to achieve covalent immobilization of enzymes, polymer supports need to have some functional groups, which can be used for making covalent linkages between the enzyme and the polymer support. Radiation-induced graft polymerization, in particular, is a widely used method to produce high performance chemically functionalized polymer materials that can be utilized as support matrices for enzyme immobilization. This method offers an alternative for combining two different polymers and retaining the desirable properties of both the polymeric constituents [20-22]. Compared to conventional grafting methods, grafting achieved using ^{60}Co gamma radiation or Electron beam radiation offers some distinct advantages, such as room temperature processing, high throughput, better control over reaction parameters and the absence of any harmful chemical initiators [23]. 2,3-epoxypropyl methacrylate (EPMA) is a versatile monomer, which has been widely used to functionalize different types of trunk polymers such as films, fibers, non woven fabric and hollow fibers [24-27].

In this work, EPMA was employed to fabricate an epoxy functionalized Poly(EPMA)-*g*-PP matrix via mutual irradiation grafting process. Immobilization of enzyme HRP onto the poly(EPMA)-*g*-PP matrix was achieved through a simple one step-room temperature process to design a reusable biocatalytic system. The catalytic activity of the immobilized enzyme system was assayed spectrophotometrically using ABTS as a substrate. The practical applicability of immobilized HRP system as a reusable biocatalyst to achieve degradation of industrial dyes was demonstrated using BR29 as a model dye.

2. Materials and methods

2.1 Materials

2,3-Epoxypropyl methacrylate (EPMA) (Sigma-Aldrich, purity=97%), Horseradish peroxidase (HRP) (Sigma, type VI, salt free, lyophilized powder, 300 units/mg), and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma-Aldrich, purity >98%), Basic Red 29 (BR29) (Sigma-Aldrich, dye content=19%) and Hydrogen peroxide (6%, Merck, India) were used as received. Disodium hydrogen phosphate (Thomas and Baker chemicals, India) and potassium dihydrogen phosphate (Thomas and Baker chemicals, India) were used to make phosphate buffer solution (pH=7.4). Glycine (S.D. Fine, India) and MES (Sigma-Aldrich) were used to prepare buffer solutions of pH 3.0 and 6.0, respectively, while Tris.HCl (Sigma-Aldrich) was used to prepare buffer solutions of pH 8.0 and 9.0. Acetate buffer solution (pH=4.5) was made using glacial acetic acid (Merck, India) and sodium acetate (S.D. Fine, India) and stored at 4^0C for further use. All aqueous solutions were prepared in ultra pure water with resistivity=18MΩ.cm generated using a water purification system 'Ultraclear TWF UV' (SG Wasseraufbereitung & Regenerierstation GmbH, Germany). A micro-porous Polypropylene films (PP) (Celgard$^{(R)}$ 2400) with 25 micron thickness was used as a polymer support after washing in methanol and water. Radiation grafting experiments were carried out in a ^{60}Co gamma chamber having a dose rate of 2.0kGy/hr determined using Fricke dosimetry [28].

2.2 Radiation grafting process

Grafting of 2,3-Epoxypropyl methacrylate onto polypropylene film was carried out by mutual irradiation grafting process using ^{60}Co-gamma radiation source (Figure 1). Briefly, PP matrices of known weights were immersed for an hour in glass-stoppered tubes containing known composition of grafting solution, followed by sonication to ensure swelling/wetting of the

PP substrate in the monomer mixture to achieve uniform grafting. These tubes were subsequently exposed to ^{60}Co-gamma radiation for required radiation doses. The unwanted poly(EPMA) homopolymer formed during the mutual irradiation grafting process was removed from the grafted poly(EPMA)-*g*-PP samples, using methanol-water (1:1) mixture in a Soxhlet extraction assembly. The grafted samples were vacuum dried at 50°C and stored in desiccator for further use.

2.3. Characterization of grafted samples

2.3.1 Grafting yield estimation

Radiation grafting extent of poly(EPMA) on PP substrate was ascertained by grafting yield (G.Y.) measurement determined gravimetrically using relation (1)

G.Y (%) = [(Weight after grafting - Initial weight)/Initial weight] x 100 (1)

2.3.2 Fourier transformed infrared (FTIR) spectroscopy

Fourier transformed infrared (FTIR) spectra were recorded using an IR Affinity-1 spectrometer (Shimadzu, Japan) in diamond single reflectance ATR mode, in the range from 400 to 4000 cm^{-1}, with a resolution of 4 cm^{-1} and averaged over 50 scans.

2.3.3. Scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) images of the samples were recorded using SEMART PS-250 (PEMTRON, S. Korea) at acceleration voltages of 3.0kV using secondary electron detector. Before recording the SEM images, the sample were pasted onto a conducing surface using silver paste and coated with gold using ion sputter coater.

2.3.4. XPS analysis

The XPS analysis of samples were conducted in a UHV chamber (base pressure $<2x10^{-9}$ mbar) using a VG make, CLAM-2 model hemispherical analyzer with a non-monochromatic

twin Al / Mg X-ray source with Mg K_α line (1253.6 eV). Peak fitting of the electron spectra were performed using XPS peak fit 4.1 software. The spectrum background was subtracted using the Shirley method.

2.4. Enzyme immobilization on poly(EPMA)-*g*-PP support

Enzyme HRP was directly immobilized onto the poly(EPMA)-*g*-PP films by a single step covalent immobilization method. The amino groups present in HRP serve to form covalent linkages with the epoxy functional groups present on poly(EPMA)-*g*-PP films, as shown in the reaction scheme (Figure1). For the immobilization process, a poly(EPMA)-*g*-PP film of known weight was equilibrated with HRP solution of known concentration, prepared in a 50mM MES buffer (pH=6.0) medium at room temperature. The concentration of enzyme immobilized on the Poly(EPMA)-*g*-PP film was estimated spectrophotometrically by Bradford method, wherein the concentration of remaining enzyme in the solution after immobilization process was estimated, using BSA solution as a standard [29].

2.5. Assay of free and immobilized enzyme

The HRP enzyme activity in the free and immobilized conditions was assayed using ABTS as a substrate. HRP catalyzes the oxidation of ABTS in presence of H_2O_2, leading to the production of cation radical $ABTS^{\cdot+}$, which was followed spectrophotometrically by monitoring the OD at λ_{max}= 420 nm. The UV-visible spectra of solutions were recorded using a UV-visible spectrophotometer (Evolution 300, Thermoelectron, UK) in the wavelength region of 250-800 nm with resolution of 1nm.

2.6. Kinetic parameters K_m and V_{max}

The kinetic parameters (K_m and V_{max} values) of the free and immobilized HRP were determined in a batch system using Lineweaver- Burke Plots by measuring initial rates of the

reaction with varying concentration of ABTS (5–75 μM) in MES buffer (50mM, pH 6.0) at 25^{o}C.

2.7. Thermal and pH stability

The thermal stability analysis of the free enzyme and the HRP immobilized on poly(EPMA)-*g*-PP supports were carried out by measuring the activity of the enzyme exposed to different temperatures (10–50^{o}C) in MES buffer (50mM, pH 6.0) under optimized protocol. The effect of pH on the activity of free and immobilized enzyme systems was studied in the pH range 3.0 to 9.0 using different buffer systems at room temperature.

2.8. Storage stability and repeatability study

The activities of the free and the immobilized HRP, after storage for different time periods in MES buffer (50mM, pH 6.0) at 4^{o}C, were measured. The repeatability of immobilized HRP was studied for various reaction cycles performed over a period of 5 days in a batch operation mode.

3. Results and discussions

Polypropylene (PP) finds application in various commercial sectors due to its unique properties, such as low cost, good mechanical strength, low permeability, good chemical resistance, etc. [30]. However, for certain special applications, such as enzyme immobilization, PP needs to be functionalized with suitable chemical groups. Therefore, functionalization of the PP films was carried out by introducing epoxy group on to it via radiation grafting of EPMA. The epoxy functionalized poly(EPMA)-*g*-PP polymer film served as the support for covalent immobilization of enzymes via reaction of epoxy group of the polymer support with the amino groups of enzymes (Figure 1).

3.1. Radiation grafting of poly(EPMA) on PP film

A single step-mutual irradiation grafting process was used for epoxy functionalization of PP films. Effect of important grafting experimental parameters, such as total radiation absorbed dose and monomer concentration, on grafting yield, was investigated in order to optimize the grafting process.

3.1.1. Effect of absorbed radiation dose on grafting yield

The number of grafted chains generated in mutual grafting process is a function of the total absorbed radiation dose. The number of free radical sites generated on a trunk polymer chain increases with increase in the radiation energy absorbed by the substrate, leading to initiation of more number of grafting chains and hence higher grafting yield [31]. Expectedly, for a reaction system comprising of 10% EPMA in Methanol-water (2:3) mixture under aerated conditions, a typical plot was obtained, where the grafting yield increased abruptly in the lower dose range, followed by slow increase in the higher dose range (Figure 2). The increase was sharp initially up to a radiation dose of 1.0kGy; beyond 1.0kGy and up to 3.0kGy, the G.Y. approached a near constant value indicating attainment of saturation. This was attributed to the homopolymer formation at higher doses, which reduced the availability of monomer for grafting reaction.

3.1.2. Effect of monomer concentration on grafting yield

The concentration of monomer plays an important role in governing the grafting yield in mutual radiation grafting process. In order to investigate the effect of monomer concentration, grafting of EPMA on PP was carried out at different monomer concentrations under optimized experimental conditions (2:3 Methanol-water mixture, 0.5 kGy dose). The results are shown in figure 2 inset. At lower monomer concentration up to 5%, low grafting yield was obtained.

Significant increase in grafting yield was achieved beyond 5% monomer concentration up to 12.5%, above which saturation in the grafting yield was observed. There are two aspects related to the influence of monomer concentration on grafting yield: i) higher the monomer concentration, more the availability of the monomer for grafting reaction, leading to higher grafting yield, and ii) higher the monomer concentration, higher the chances of undesired homopolymer formation, as a major part of radiation energy gets absorbed by the aqueous monomer solution, leading to saturation of the grafting yield. Moreover, at higher monomer concentrations, increase in the viscosity of the grafting solution was observed due to homopolymer formation, which causes reduced mobility of monomer from bulk towards the backbone, and consequently, reduced the grafting yield[20]. Samples with higher grafting yield, obtained at higher monomer concentrations, were also observed to show brittleness; hence concentration of monomer was optimized at 10% (v/v) to ensure low homo-polymer formation and achieve sufficiently high grafting levels without compromising on the mechanical strength of the PP film.

3.2. Characterization of poly(EPMA)-*g*-PP samples

3.2.1 Scanning electron microscopy (SEM)

Figure 3a and 3b present the SEM images of untreated PP sample and poly(EPMA)-*g*-PP sample, respectively. The untreated PP film exhibited a highly porous and net like fibrous morphology, which provides a good support with high specific surface area for grafting, and subsequent immobilization of enzyme molecules. The surface morphology of the untreated PP film was observed to be changed after radiation grafting of poly(EPMA). The conformal layers

of grafted poly(EPMA) on the fibrous structures of the PP membrane can be clearly seen, that transformed the morphology of the membrane from more porous to less porous one.

3.2.2 X-ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectroscopy was used to examine the chemical functional groups present on the samples. Figures 4a and 4b show the XPS peak for C1s of untreated PP sample and poly(EPMA)-*g*-PP sample, respectively. The untreated PP film showed a relatively narrow and symmetric single C1 peak at 284.5eV, which corresponds to C-H/C-C of polypropylene backbone (figure 4a). This suggested that no other functional chemical groups are present on the untreated PP film. After grafting of poly(EPMA), other than C1 (284.5eV), two additional peaks C2 (286.5eV) and C3 (288.4eV) appear, which correspond to C-O epoxy/ether and C=O groups, respectively (figure 5b) [32, 33]. These additional peaks clearly confirm the grafting of poly(EPMA) on PP films. The immobilization of HRP on poly(EPMA)-*g*-PP film was evident from the additional nitrogen N1s peaks appeared at 399eV and 401.5eV (figure 4c). The major peak N1 at 399.0 eV was assigned to the free primary amino groups present in the HRP enzyme, whereas the peak N2 at 401.5eV was attributed to the N-H and N-C bonds formed subsequent to the immobilization of HRP enzyme on to poly(EPMA)-g-PP film[34,35].

3.2.3. Fourier Transformed Infrared spectroscopy (FTIR)

FTIR spectroscopy was used as a tool to confirm the grafting and the immobilization processes. Figure 5a represents the FTIR spectrum of untreated PP sample, which exhibited strong absorption peaks in the range 2950-2840 cm^{-1} corresponding to the asymmetric and symmetric CH_2 stretching modes, and peaks at 1455 cm^{-1}, 1376 cm^{-1} corresponding to the characteristic scissor deformation modes of CH_2. Figure 5b presents the FTIR spectra of poly(EPMA)-*g*-PP sample, which showed additional IR peaks at 1720cm^{-1}, 1255 cm^{-1} and 902

cm^{-1}. The peak at 1720cm^{-1} corresponded to carbonyl group, whereas the peaks at 1255 cm^{-1} and 902 cm^{-1} were assigned to the C-O vibrations of epoxy group of grafted poly(EPMA) chains[36]. Immobilization of enzyme HRP onto poly(EPMA)-*g*-PP films yielded one more additional broad peak at 1650cm^{-1} corresponding to the Amide I vibration (Figure 5c). It arises mainly from the C==O stretching vibration of the protein with minor contributions from the out-of-phase CN stretching vibration, the CN deformation and the NH in-plane bend. It was observed that the intensity of the peak at 902 cm^{-1}, corresponding to the epoxy group, decreased after immobilization of HRP enzyme.

3.3 Assay of immobilized and free HRP

The enzyme activities of the free HRP and immobilized HRP systems were assayed using ABTS as the substrate for HRP. The HRP catalysed oxidation reaction of ABTS in presence of H_2O_2 leads to the production of cation radical $ABTS^{\cdot+}$ (λ_{max}= 420 nm, ε_{420}=36000$M^{-1}.cm^{-1}$). This reaction was followed spectrophotometrically by monitoring the OD_{420nm} of the product $ABTS^{\cdot+}$ (Figure 6 inset). The results of studies on pH, temperature, storage stability and repeatability of immobilized HRP system with respect to the free enzyme system are presented in a normalized form (i.e., relative activity (%)) with the highest value of each set being assigned the value of 100% activity.

3.3.1. Influence of H_2O_2 concentration

The influence of H_2O_2 concentration on the immobilized enzyme efficacy is shown in figure 6. It was observed that activity of the enzyme increased with increase in the H_2O_2 concentration initially, and reached maximum at an H_2O_2 concentration of 1.0 mg/L, and then subsequently decreased. As explained by Wanga et al.[37], the decrease in activity at high H_2O_2

concentrations was due to oxidation of HRP itself by H_2O_2. This resulted in a decrease in the concentrations of HRP and H_2O_2 participating in the oxidation reaction of ABTS. Simultaneously, the byproduct of the oxidation reaction between HRP and H_2O_2 might also influence the microenvironment around immobilized HRP thereby inhibiting the approach of the substrate ABTS towards the enzyme and leading to a decrease in its catalytic activity [38-40]. A similar trend was found to be followed by the free enzyme. For the subsequent experiments, 1.0mg/L was taken as an optimum concentration of H_2O_2.

3.3.2. Estimation of kinetic parameters

The catalytic efficiency of an enzyme can be expressed in terms of two kinetic parameters, namely, the Michaelis-Menten constant (K_m) and the maximum reaction velocity (V_{max}). V_{max} is the maximum rate attained when all enzyme molecules are bound to the substrate, whereas, K_m represents the substrate concentration at which the reaction rate is at half the maximum rate attainable. A small K_m value indicates high affinity of the enzyme for the substrate, which implies that the rate will approach V_{max} more quickly. The value of K_m is dependent on the characteristics of both the enzyme and the substrate, as well as on the experimental conditions, such as temperature and pH.

Figure 6 inset presents the representative UV- visible spectra of ABTS /free HRP system at varying reaction time, which exhibits the oxidation of ABTS via HRP catalyzed oxidation reaction. Similar plots were obtained for varying concentrations of substrate (ABTS) with fixed concentration of HRP enzyme and H_2O_2, which were used to determine the initial rate of the reaction and subsequently, the Kinetic parameters (K_m, V_{max}) of free as well as immobilized HRP using Lineweaver- Burk linear plots, represented by equation (2).

$$\frac{1}{V_i} = \frac{K_m}{V_{\max}.[S]} + \frac{1}{V_{\max}} \qquad (2)$$

where, V_i and [S] are the initial rate and substrate concentration, respectively. K_m and V_{max} were estimated from the slope and the intercept of the $1/V_i$ *vs* 1/[S] linear plot. The K_m value of covalently immobilized HRP was found to vary significantly from that of the free enzyme. While free HRP showed a K_m value of 318.5μM, the K_m for immobilized HRP was calculated to be 753.0μM, an approximately two fold increase, which indicated a decrease in the affinity of the substrate for the immobilized HRP compared to the free enzyme. The change in the affinity may be attributed to the lower accessibility of the substrate ABTS for the active site of the immobilized enzyme [41]. The V_{max} values, however, were not found to vary significantly; free and immobilized HRP were found to have V_{max} values of 50μM/minute and 41μM/minute, respectively. This implied that immobilization of HRP onto the poly(EPMA)-*g*-PP did not significantly affect the rate at which it converted the substrate molecules to the corresponding product. HRP immobilized on to the poly(EPMA)-*g*-PP support was found to retain ~80% of the activity demonstrated by the free enzyme under identical experimental conditions. The immobilized enzyme usually has been found to show a lower activity than the same concentration of free enzyme in solution[42]. Nevertheless, unless the activity losses are very large, this disadvantage could be compensated by the reusability offered by the immobilized enzyme.

3.3.3. Storage stability and Repeatability study

Properties of enzyme system that have been generally considered to be improved via immobilization are the enzyme stability and repeatability. The free and HRP immobilized-poly(EPMA)-*g*-PP support were stored in MES buffer at pH 6.0 and 4^{o}C, and the activities of both the enzyme systems were estimated at regular intervals over a period of 15 days (Fig. 7

inset). The activity of free HRP system decreased much faster with time as compared to that of immobilized-HRP system. Activity of free HRP reduced to 65% of its initial activity in a single day, whereas the activity of immobilized HRP remained constant during the same time interval. The activity of free HRP and immobilized-HRP reduced to 41% and 93.7% of their initial activity, respectively, over a period of six days. Figure 7 presents the results of repeatability experiment of immobilized-HRP system carried out for 10 cycles over a period of 5 days stored at pH 6.0 and 4^{o}C. It can be observed that the immobilized HRP system showed merely ~10% decrease in its initial activity after 10 repeated cycles over a period of 5 days (Figure 7).

3.3.4. pH and thermal stability study

The pH and temperature of the reaction medium are the important parameters that influence the catalytic efficiency of an enzyme. Figure 8 presents the relative activity of HRP as a function of pH of the medium. It was observed that the pH of the reaction medium does not exhibit significant influence on the enzyme activity of the immobilized HRP as compared to free HRP system. The pH profiles were found to follow almost similar trend for both free and immobilized enzyme; both the systems exhibiting an optimum pH of 6.0.

Immobilization of enzyme is known to alter the optimum temperature as well as the temperature range within which the enzyme shows good activity. Hence, the effect of variation of temperature on the activity profile of free and immobilized enzyme was studied for comparison purpose. Figure 8 inset shows the temperature profile of free and immobilized HRP enzyme. The optimum temperature was found to increase substantially after immobilization of HRP, recording an increase from 25^{0}C to 40^{0}C, thereby indicating enhanced thermal stability for the HRP immobilized-poly(EPMA)-*g*-PP system. The covalent immobilization of HRP molecules on to poly(EPMA)-*g*-PP support reduces the conformational flexibility, resulting in

higher activation energy for the HRP molecule to reorganize to a proper conformation for substrate binding [43,44].

3.4 Enzymatic degradation of BR29 dye

Peroxidases have been reported to be effective degrading enzymes for different dyes, and therefore, targeted for textile dye waste water treatment application [10, 45]. In order to determine the efficacy of immobilized HRP catalytic system as a potential dye degrading agent, HRP-immobilized-poly(EPMA)-*g*-PP films of known weight were contacted with 5mL of BR29 dye (~10ppm) in presence of an optimized H_2O_2 concentration of 1.0mg/L and incubated at room temperature. The catalytic degradation of the dye was monitored spectrophotometrically by observing the decrease in OD of the absorption peak of BR29 dye at 508 nm. The OD_{508} of the reaction mixture was monitored over a definite time interval. It was observed the immobilized enzyme resulted in ~90% degradation of the dye over a period of 20 days (Figure 9a). The same immobilized HRP system was found to operate successfully over five cycles of 20 days each, with some gradual loss in activity (from ~90% to~ 65% over 5 cycles), in the degradation of BR29 dye under similar conditions (Figure 9 inset). For comparison purpose, the degradation profile of BR29 in presence of free HRP system was also studied. Free HRP system showed ~94% degradation of the BR29 dye under similar experimental conditions, which was expectedly higher than that observed in case of the immobilized HRP system (Figure 9b)[42,46]. The activity loss in case of immobilized HRP system, however, could be compensated by the reusability offered by the same.

Conclusion

Fabrication of epoxy functionalized PP films was carried out via a single step- environmental friendly- mutual irradiation grafting process and employed for the one step room temperature covalent immobilization of HRP. The immobilized HRP system could be used repeatedly for 10 cycles within five days each with only 10% reduction in the activity of the enzyme. Immobilized HRP system showed improved storage stability as compared to the free HRP system. The optimum pH for HRP system remained unaffected by immobilization process. However, the covalent immobilization of HRP certainly improved the thermal stability of the enzyme, which provides an opportunity to use the enzyme at relatively higher temperature with good catalytic efficacy. In order to test the practical applicability of HRP immobilized-poly(EPMA)-*g*-PP biocatalytic system for waste water treatment, the catalytic degradation of a model dye BR29 was successfully demonstrated. The immobilized enzyme system was found to cause ~90% degradation of BR29 over a period of 20 days and could be reused for five cycles without substantial loss in activity. Improved operational, storage and thermal stability along with reusability of HRP immobilized- poly(EPMA)-*g*-PP makes it an efficient biocatalytic system for potential environmental applications.

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Figure captions

Figure 1: Schematic diagram of radiation induced grafting process for epoxy functionalization of PP film and covalent immobilization of HRP enzyme on to poly(EPMA)-*g*-PP film.

Figure 2: Grafting yield as a function of radiation dose. ([EPMA]=10% (v/v), Solvent: methanol/water=2/3, Dose rate= 2.0 $kGy.h^{-1}$, ambience=aerated). (Inset: Grafting yield as a function of monomer concentration; Radiation dose=0.5kGy, Solvent: methanol/water=2/3, Dose rate= 2.0 $kGy.h^{-1}$).

Figure 3: FTIR spectra of (a) untreated PP, (b) Poly(EPMA)-g-PP, (c) HRP immobilized-poly(EPMA)-g-PP

Figure 4: XPS spectra of samples (a) C1s of control PP film, (b) C1s of poly(EPMA)-*g*-PP film, (c) N1s of HRP immobilized-poly(EPMA)-g-PP

Figure 5: C1s XPS spectra of samples (a) Control PP film, (b) Poly(EPMA)-*g*-PP film

Figure 6: Effect of [H_2O_2] on activity of immobilized HRP system. (Inset: UV- Visible spectra of ABTS/H_2O_2/free HRP system as a function of reaction time ([ABTS]= 50μM, [HRP]=750μg i.e. 225U, [H_2O_2]=1mg/L) (a) 0 min (b) 3 min (c) 6 min (d) 9 min (e) 12 min and (f) 15 min).

Figure 7: Activity of immobilized HRP for number of repeated cycles over a period of 5 days (Inset: Storage stabilities of the HRP enzyme system at pH 6.0 and 4^oC (a) HRP immobilized-poly(EPMA)-*g*-PP system (b) free HRP system).

Figure 8: Activity curves for HRP systems as a function of pH of the reaction medium (a) HRP immobilized-poly(EPMA)-*g*-PP system (b) free HRP system. (Inset: Temperature-activity curves for HRP system (a) HRP immobilized-poly(EPMA)-*g*-PP system (b) free HRP system).

Figure 9: Degradation profile of BR29 as a function of incubation duration with (a) HRP immobilized-poly(EPMA)-g-PP system (b) free HRP. (Inset: Degradation of BR29 in presence of HRP immobilized-poly(EPMA)-g-PP system as a function of number of cycles (20 days each)).

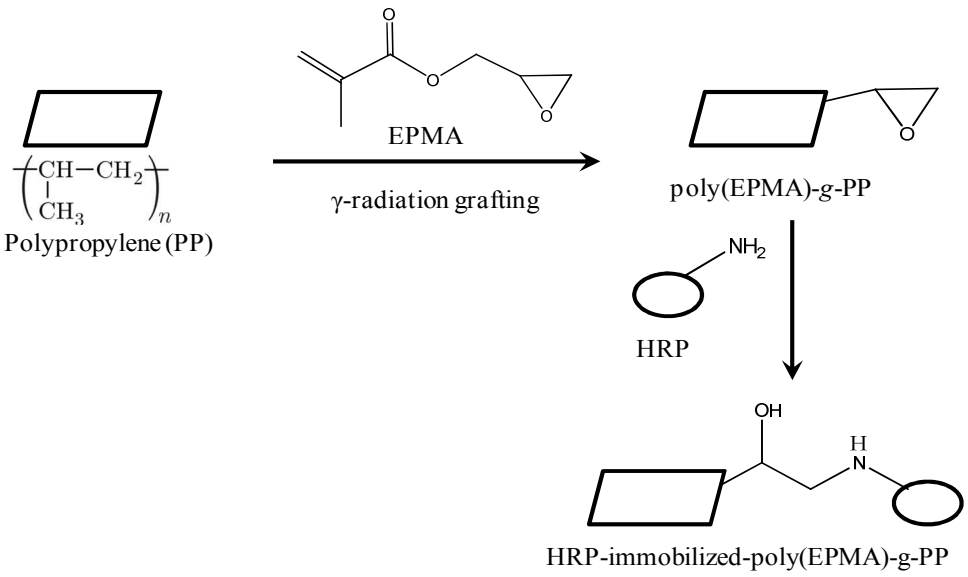


Figure 1: Schematic diagram of radiation induced epoxy functionalization of PP film and covalent immobilization of HRP enzyme on to poly(EPMA)-*g*-PP film.

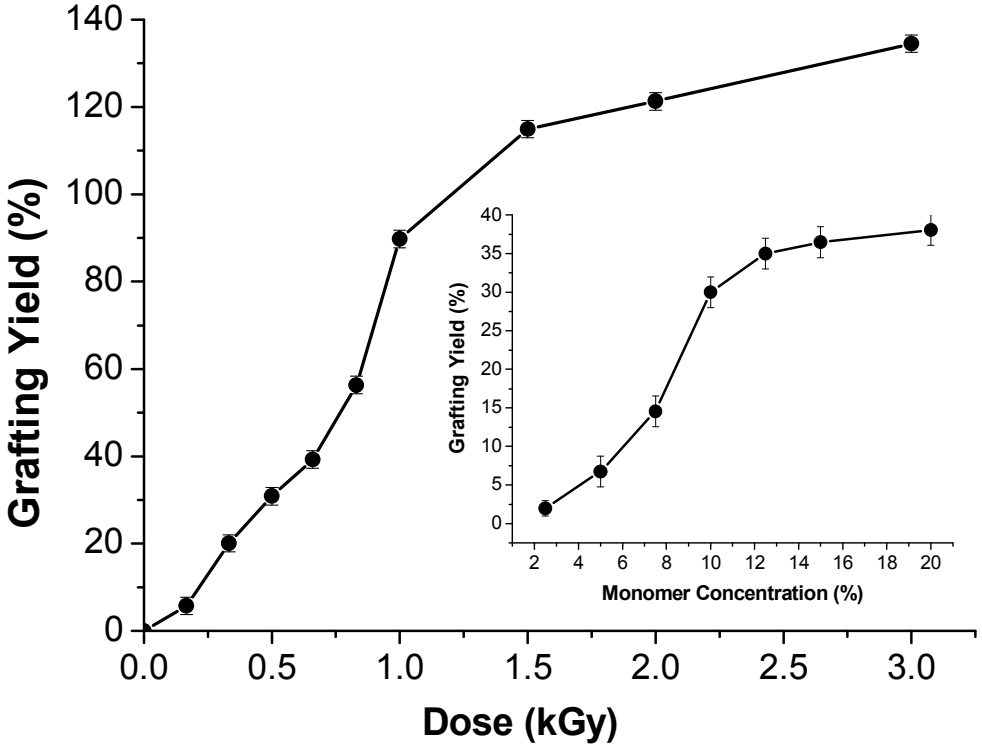


Figure 2: Grafting yield as a function of radiation dose. ([EPMA]=10% (v/v), Solvent: methanol/water=2/3, Dose rate= 2.0 kGy.h^{-1}, ambience=aerated). (Inset: Grafting yield as a function of monomer concentration. (Radiation dose=0.5kGy, Solvent: methanol/water=2/3, Dose rate= 2.0 kGy.h^{-1}).

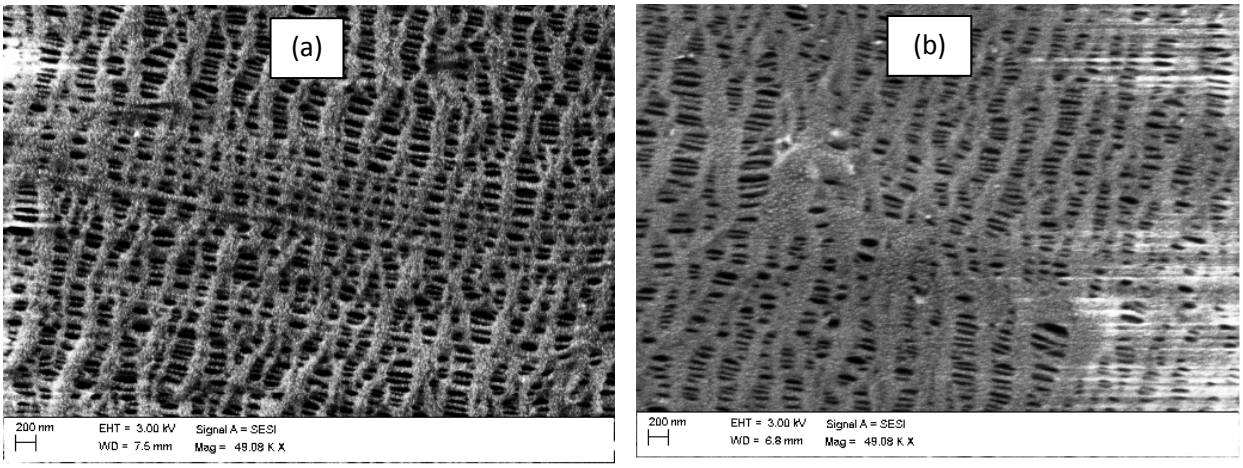


Figure 3: FESEM images of (a) control PP film and (b) Poly(EPMA)-*g*-PP film

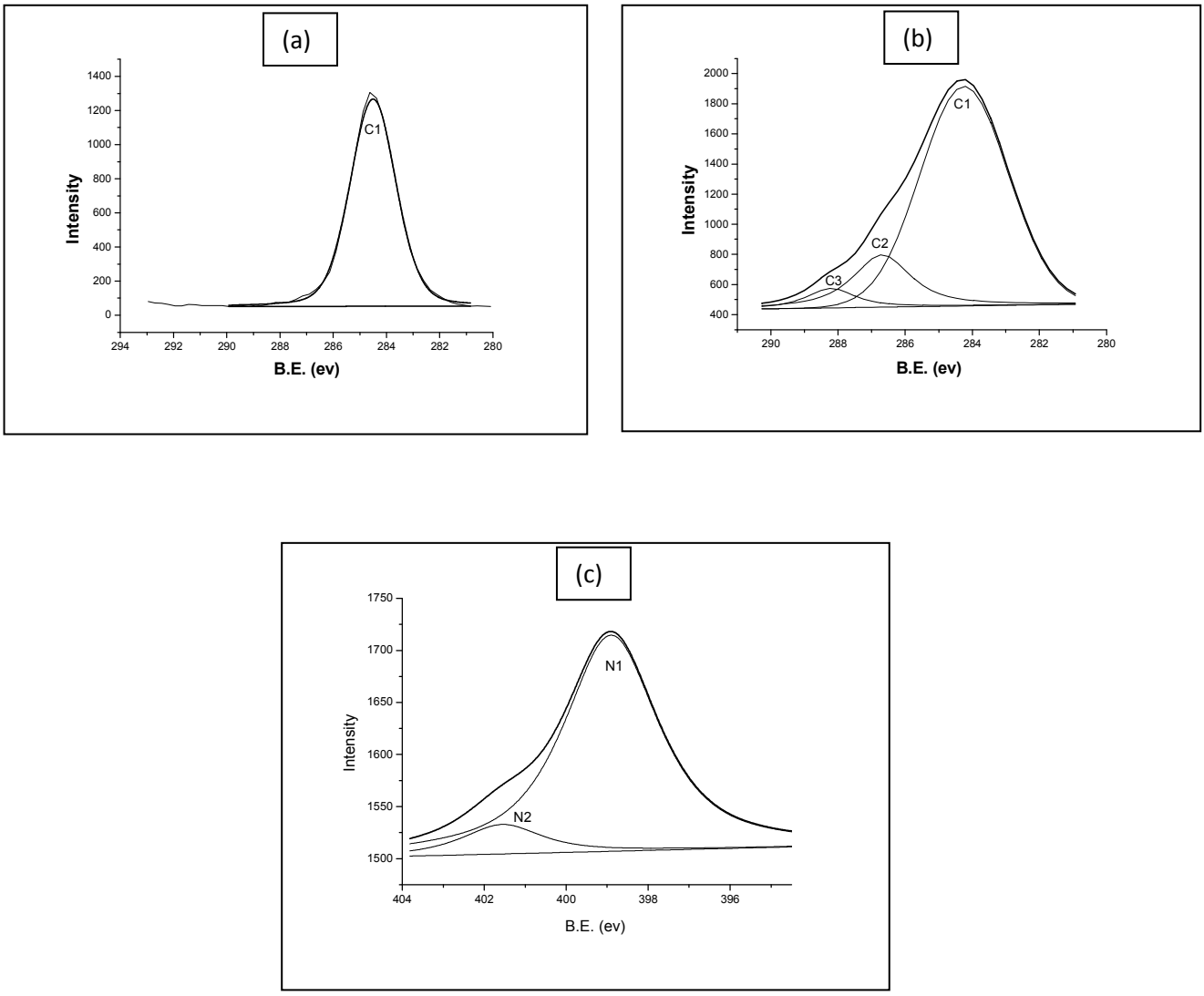


Figure 4: XPS spectra of samples (a) C1s of control PP film, (b) C1s of poly(EPMA)-*g*-PP film, (c) N1s of HRP immobilized-poly(EPMA)-g-PP

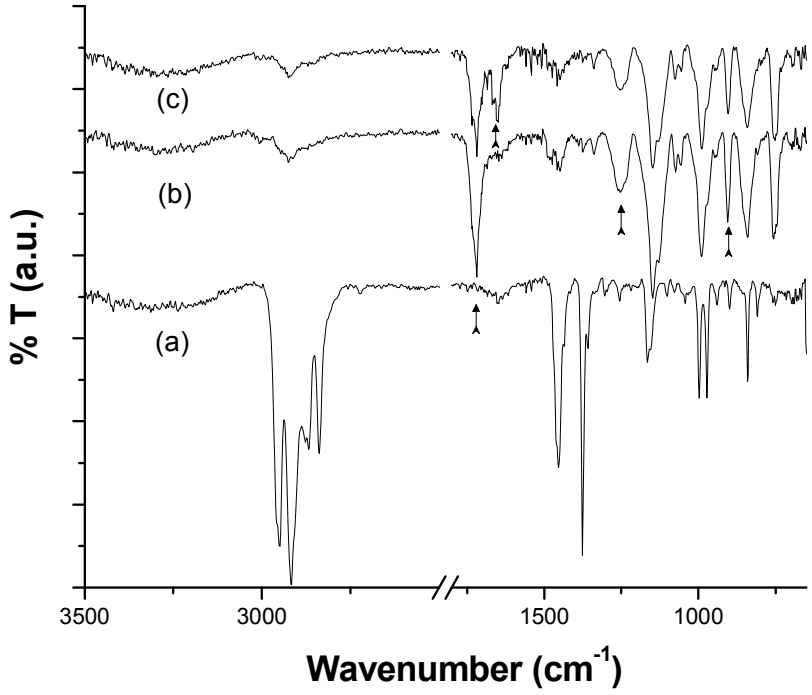


Figure 5: FTIR spectra of (a) untreated PP, (b) Poly(EPMA)-g-PP, (c) HRP immobilized-poly(EPMA)-g-PP

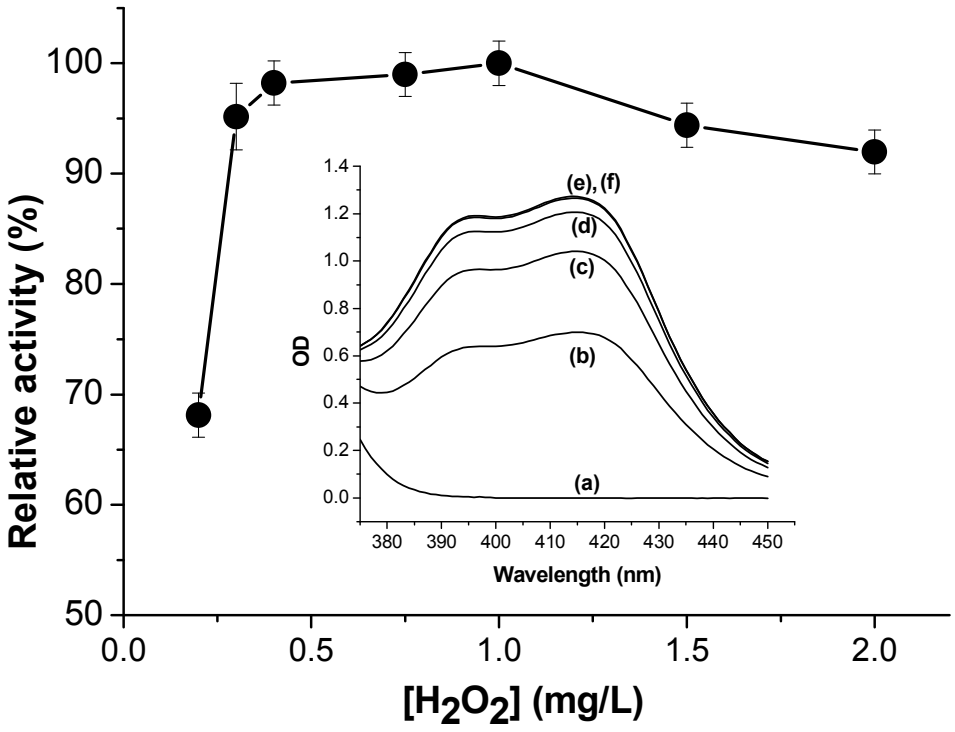


Figure 6: Effect of $[H_2O_2]$ on activity of immobilized HRP system. (Inset: UV- Visible spectra of ABTS/H_2O_2/free HRP system as a function of reaction time ([ABTS]= 50µM, [HRP]=750µg i.e. 225U, $[H_2O_2]$=1.0mg/L) (a) 0 min (b) 3 min (c) 6 min (d) 9 min (e) 12 min and (f) 15 min).

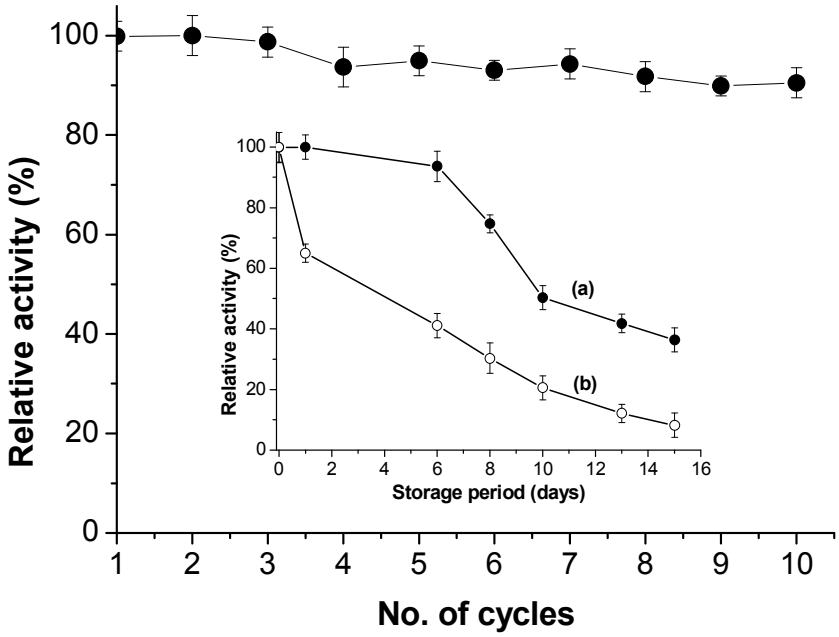
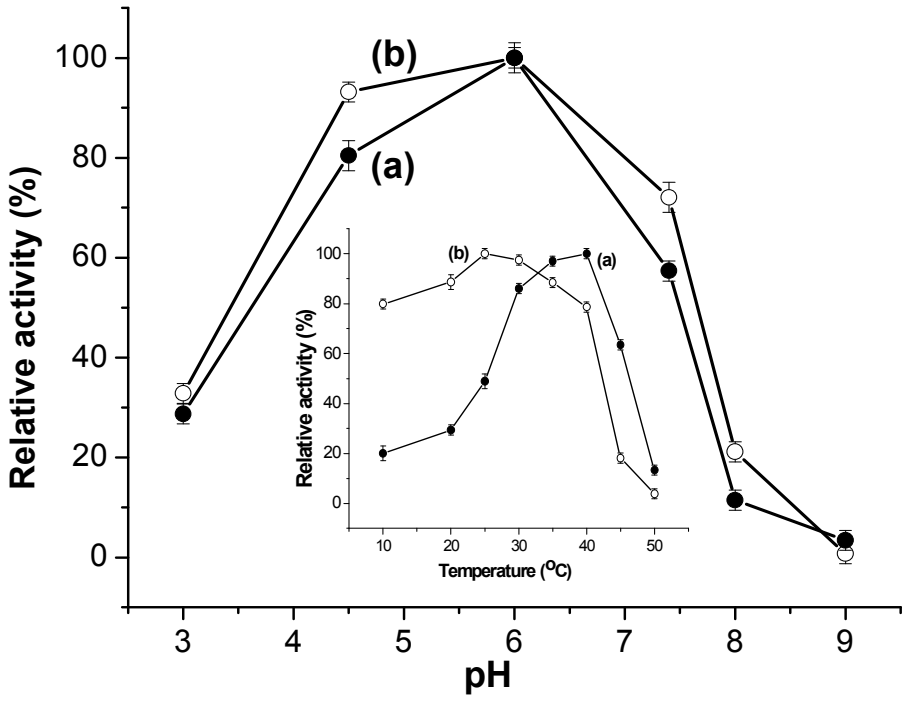


Figure 7: Repeatability analysis-relative activity of immobilized HRP for number of repeated cycles over a period of 5 days (Inset: Storage stabilities of the HRP enzyme system at pH 6.0 and 4^{o}C (a) HRP immobilized-poly(EPMA)-g-PP (b) free HRP).



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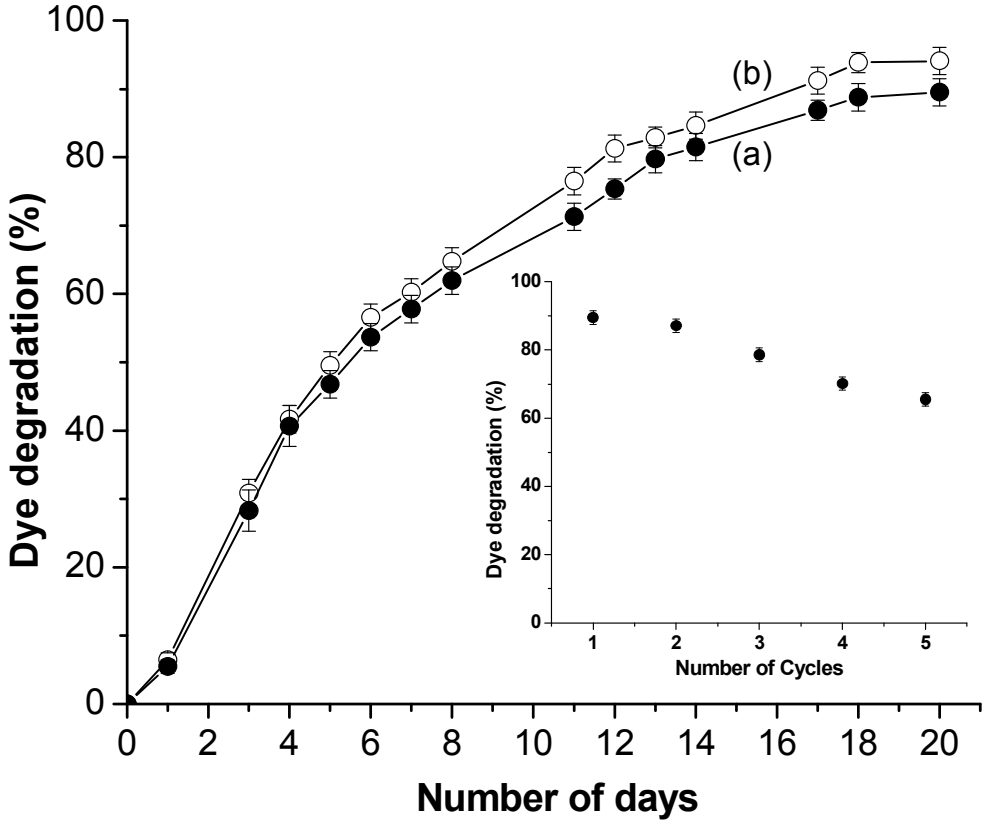

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Graphical abstract

A single step-environment friendly-aqueous based-radiation grafting process was used to fabricate epoxy functionalized polymer support for one step- covalent immobilization of enzyme HRP in ambient condition, and tested for dye waste water treatment.