Assessing the Suitability of Using Plant Latex as Immobilization Support for Horseradish Peroxidase

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Abstract— Horseradish peroxidase was immobilized onto latex from three different plants viz. *Calotropis procera*, *Euphorbia royleana* and *Alstonia scholaris* with 0.51 ± 0.01, 0.37 ± 0.01, 0.46 ± 0.01 mg/cm^2 conjugation yield and 62.07 ± 0.85, 66.1 ± 0.85, 71.24 ± 0.80 % retention of specific activity respectively. The support, before and after addition of peroxidase was characterized using scanning electron microscopy (SEM) and Fourier transmission infra-red spectroscopy (FTIR). Optimum pH, optimum temperature and changes in kinetic parameters (*E*a, *K*m and *V*max) for immobilized peroxidases were studied and found to differ from that of free peroxidase. *Alstonia scholaris* latex was most effective in stabilizing the structure of peroxidase during storage at 4°C, whereas thermal stability and reusability of peroxidase was better on *Calotropis procera* latex. Analytical use of *Calotropis procera* latex bound peroxidase for determination of phenolic content of fruit juices has also been demonstrated.

Keywords: Peroxidase, Calotropis procera, Alstonia scholaris, Euphorbia royleana, Latex, Immobilization

I. INTRODUCTION

Horseradish peroxidase catalyzes the reductive cleavage of hydrogen peroxide to oxidize both organic and inorganic compounds [1]. Peroxidases have tremendous applications in natural product and fine chemicals synthesis, medical diagnostics, detoxification, biotransformation and bioremediation of industrial effluents or decontamination of waste water containing phenols, cresols, chlorinated phenols, aromatic amines, biphenyls, bisphenols and dyes etc [2-4]. However, limited stability, difficulties in separation of catalyst after the reaction and high cost of purified enzyme limits its applications for industrial purposes. Immobilization to solid carriers is perhaps the most used strategy to overcome these problems. Immobilized enzymes can be reused and generally have improved operational stability, which reduces the cost of estimation [5-8].

In the last decades, substantial attention has been devoted to immobilization of peroxidases to modified natural organic carriers such as cashew gum polysaccharide [9], DEAE cellulose [10], calcium alginate–starch hybrid gel [11], chitosan [12,13] and calcium alginate–pectin [14]. These supports are hydrophilic and hence overcome the limitations of substrate as well as product diffusion and mass transfer frequently encountered with the use of inorganic supports. Plant latex is also an important biomaterial, which is a complex emulsion of proteins, alkaloids, starches, sugars, oils, tannins, resins and gums [15,16]. It has excellent gelling properties and coagulates on exposure to air. Moreover, it is abundantly produced in nature and is easily available. Although Koopal and Nolte [17] have reported the immobilization of glucose oxidase on commercially available latex beads for construction of glucose biosensor, application of natural plant latex as immobilization carriers for enzymes has not been explored as yet. Here, it is anticipated that biocompatible and adhesive nature of plant latex should make plant it an excellent support for enzyme immobilization.

Hence, in the present study suitability of plant latex as immobilization support was tested. Peroxidase from horseradish was chosen as the model enzyme, which was immobilized onto latex from three different plants viz. *Calotropis procera* (Family-Apocynaceae), *Alstonia scholaris* (Family-Apocynaceae) and *Euphorbia royleana* (Family-Euphorbiaceae). The kinetic parameters, thermal, storage and operational stability of the immobilized peroxidases were studied and compared with the native enzyme. The use of immobilized enzyme for determination of phenolic content of fruit juices is also demonstrated.

II. EXPERIMENTAL DETAILS

A. Fabrication of immobilization support

Plant latex was collected in eppendorf tubes during the wee hours of the morning from 3 different plants species viz. *Calotropis procera*, *Alstonia scholaris* and *Euphorbia royleana*. Latex in each of the tube was sonicated (Make: Mesonix) separately at 50% pulses for 10 min at room temperature to denature the proteases [18] and 1.0 ml of the sonicated latex was spread individually over the polyethylene strips, covering 25.0 cm^2 area. The preparations were left undisturbed for 4 h to air dry and set completely as gel.

B. Immobilization of peroxidase

One ml of peroxidase (1770 units) was spread evenly and carefully over each of the polyethylene strip supported latex membranes and dried in refrigerator overnight. Then, the adsorbed enzyme was cross linked by placing the enzyme loaded membranes in a solution of 2.5% glutaraldehyde in 0.01 M sodium phosphate buffer (pH 7.0) for 30 min at room temperature. In order to remove the unbound enzyme the immobilized enzyme preparations were washed with sodium phosphate buffer (0.01 M, pH 7.0) several times, until no protein was detected in washing. The protein content of enzyme solution and washings was determined by the method of Lowry et al. [19] using bovine serum albumin (BSA) as standard protein. The latex bound peroxidase was stored in 0.01 M sodium phosphate buffer, pH 7.0 at 4°C, when not in use.

C. Characterization of immobilized peroxidase by SEM and FT-IR analysis

Surface morphology of latex membrane with and without enzyme was studied by scanning electron microscopy (SEM LEO 440) at Amity University, Noida.

Fourier Transmission Infra Red spectroscopic analysis of latex with and without enzyme was carried out

with the help of FTIR Alpha, Bruker, Germany instrument at Department of Genetics, M. D. University, Rohtak.

D. Assay of free and immobilized peroxidases

The enzyme assay was based on Trinder's colour reaction [20]. The assay was carried out in dark and the reaction mixture consisting of 2.69 ml sodium phosphate buffer (0.05 M, pH 7.0), 0.1 ml 4-aminophenazone (50 mg/dl), 0.1ml phenol (100 mg/dl) and 0.01 ml peroxidase solution was mixed well and preincubated at 25°C for 5 min. The reaction started, after addition of 0.1 ml of 10 mM H_2O_2 solution. After incubation at 25°C for 10 min, $A_{520}$was read against control. The content of H_2O_2 utilized in the reaction was determined from the standard curve between H_2O_2 concentrations vs. A_{520}. Assay of latex bound peroxidase was done in the same way with the modification that immobilized enzyme was used in place of free enzyme, volume of buffer was raised by 0.01 ml and incubation temperature was 30°C. One unit of enzyme activity is defined as the amount of enzyme, which utilizes 1 nmole of H_2O_2/min under standard assay conditions.

E. Optimization of immobilized enzyme

All experiments related to characterization, kinetics, thermal and storage stability of immobilized peroxidases were carried out in triplicate and results were presented as means of three different experiments. Standard error was used to present the variability of data in all graphs.

F. Effect of pH

In order to know the optimum pH, activity of immobilized peroxidases was determined in the pH range of 4.0 to 8.5 using 0.01 M acetate buffer for pH 4.0 to 5.5, 0.01 M sodium phosphate buffer for pH 6.0 to 7.5 and 0.01 M borate buffer for pH 8.5 and 9.0.

G. Effect of Temperature

The effect of incubation temperature on activity of immobilized enzymes was studied by incubating the reaction mixture at different temperatures ranging from 25°C to 60°C at an interval of 5°C. Energy of activation (*E*a) was calculated from Arrhenius plot by plotting inverse of temperature (in degree Kelvin) vs. log of enzyme activity.

H. Effect of substrate concentration

Under optimum conditions of pH and temperature, the concentration of H_2O_2 was varied from 0.5 to 4.0 mM. Kinetic parameters *K*m and *V*max were also calculated by Lineweaver – Burk plot for immobilized peroxidases.

I. Thermal stability

Thermal stability of immobilized peroxidases was studied by incubating the reaction mixture at different temperatures ranging from (30°C to 65°C) at an interval of 5°C for 30 minutes and then measuring the residual activity under optimum conditions of pH, temperature and substrate concentration.

J. Operational stability

To determine operational stability, immobilized peroxidases were repeatedly assayed in batch mode till the point their activities were significantly lost. The assay was carried out under optimized conditions for each of the immobilized enzyme preparations and after each reaction run, the immobilized enzyme was washed with 0.01 M phosphate buffer (pH 7.0) to remove any residual activity.

K. Storage stability

The activity of immobilized peroxidases after storage in 0.01 M sodium phosphate buffer (pH 7.0) at 4°C was measured on alternate days for two months.

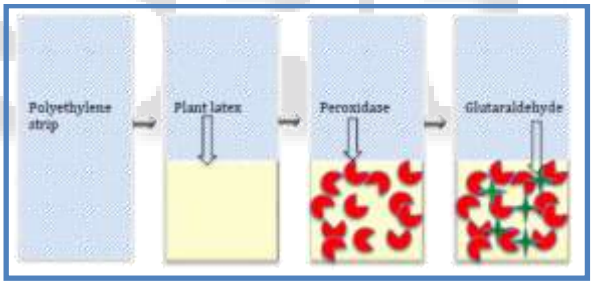
L. Use of immobilized enzyme

Total phenolic content of fruit juices was determined by immobilized peroxidase assay. Fruit juices, available commercially in tetra packs were diluted ten times before use. The reaction mixture containing 1.8 ml sodium phosphate buffer (0.1 M, pH 7.0), immobilized enzyme and 1.0 ml colour reagent (50 mg 4-aminophenazone and 100 mg phenol per 100 ml of 0.4 M sodium phosphate buffer, pH 7.0, stored in amber colored bottle at 4°C and prepared fresh every week) was incubated at 30°C for 5 min. The reaction was started by adding 0.1 ml of fruit juice and 0.1 ml of 10 mM H_2O_2 solution. After 10 min. of incubation at 30°C, the absorbance of final quinone-imine-colored product was measured at 520 nm. Total phenol contents were calculated from the standard curve of gallic acid at 750 nm.

III. RESULTS AND DISCUSSION

A. Immobilization of peroxidase onto plant latex

Scheme 1 illustrates the immobilization of horseradish peroxidase over plant latex. Horseradish peroxidase was immobilized onto plant latex from *Calotropis procera*, *Euphorbia royleana* and *Alstonia scholaris* with a conjugation yield of 0.51±0.01, 0.37±0.01 and 0.46±0.01 mg/cm^2 and with 62.07±0.85, 66.1±0.85 and 71.24±0.80 % retention of initial activity, respectively.



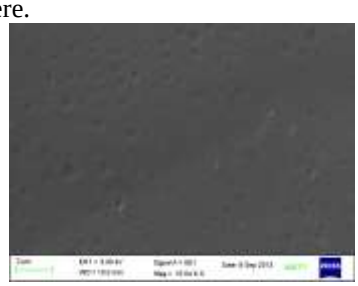
Scheme 1: Immobilization of horseradish peroxidase on plant latex.

The conjugation yield of peroxidase was highest on *Calotropis procera* latex and lowest on *Euphorbia royleana* latex, but the enzyme retained > 60% activity on both the supports. As evident from results the enzyme retained maximum activity over *Alstonia scholaris* latex, which proved that it provided the most conducive environment for enzyme immobilization. The difference in the immobilization efficiency over the three supports can only be attributed to the differences in chemical composition of latex from the three sources. However, the enzyme activity retained on all the three supports was higher than that reported on Eupergit C (15%) [21], aminopropyl glass beads (35%) [22] but lower than that reported for anodic aluminium oxide/poltethyleneimine or polyalinine composites (74%) [23] and magnetic beads (79%) [24].

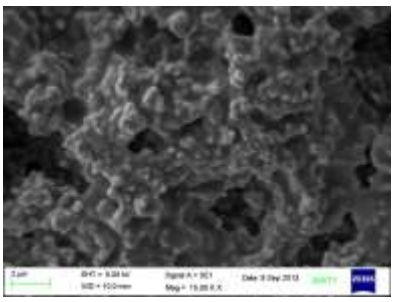
B. Characterization of latex/peroxidase conjugates

Fig. 1 depicts the morphological differences in the latex membrane after addition of peroxidase. An even latex layer is seen in Fig. 1(a) whereas the abundance of enzyme all over the latex is well illustrated by Fig. 1(b). The two

images in the Fig. 1 are easily distinguishable which confirms the immobilization of enzyme over the latex membrane. SEM images for all the three latex membranes were almost identical; hence only one for *Alstonia scholaris* is included here.



(a)



(b)

Fig. 1: Surface morphology of bare (a) and enzyme bound (b) latex surface.

C. FT-IR spectra of immobilization enzyme

The surface structures of the immobilized peroxidase were characterized by FT-IR spectra (Fig. 2). In comparison with the latex (Fig. 2, trace a), the characteristic peaks occur for the latex enzyme complex (Fig. 2, trace b) at 1641 cm^{-1} and 1249 cm^{-1} corresponding to -CONH- (amide I) and C-N stretching (amide III) respectively, suggesting the successful immobilization of enzyme on the latex. Additionally, the similarity of the spectra of latex without enzyme and the immobilized enzyme indicates that the enzyme immobilized latex retains the essential feature of its native structure on the support.

To investigate the effect of bioactivity toward immobilized enzyme, the absorption bands of enzyme amides I and II were used to provide information about the secondary structure of the polypeptide chain. The amide I peak at 1641 cm^{-1} was attributed to the peptide linkages in the protein's backbone, and the amide II at 1540 cm^{-1} was associated with the combination of N–H bending and C–N stretching. The amide I and II bands of enzyme on the latex appeared at 1641 and 1540 cm^{-1}, which was similar to the locations of the bands in free enzyme (Fig. 2, trace c).

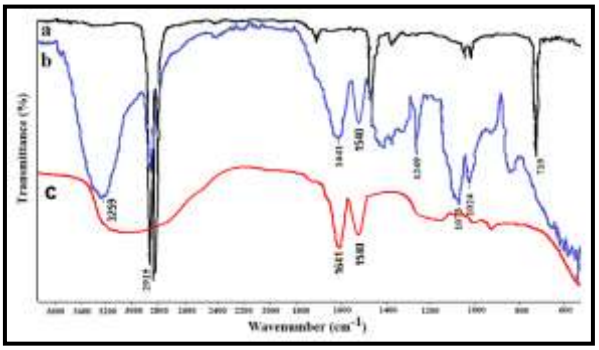


Fig. 2: FTIR spectra of latex (a), enzyme bound latex surface (b) and enzyme (c).

D. Effect of pH

The effect of pH on the activity of immobilized peroxidases is shown in Fig. 3. Optimum pH was found to be 6.0, 5.5 and 6.5 for *Calotropis procera*, *Euphorbia royleana and Alstonia scholaris* latex bound peroxidase respectively, which was loweras compared to the optimum pH of 7.0 for free enzyme [25]. The shift of pH optima of enzyme after immobilization has been a common phenomenon. It may be due to the fact that fresh latex, which is alkaline or neutral, becomes acidic rapidly on exposure to air. The formation of organic acids neutralizes the negative charge on the latex. Consequently, an enzyme attached to a charged carrier would experience a H^+ ion concentration that is higher than that of external solution. The other reason might be that the proton donating groups such as phenols and alcohols present on the support or on adjacent protein molecule might have played a role in pH shifting phenomenon [26]. Similar observations have been reported for horseradish peroxidase immobilized onto aminopropyl glass beads (pH optima 6.5) [27] and polyelectrospun fibrous membrane (pH optima 2.0 to 4.0) [28]. Out of three immobilized peroxidase preparations, *Alstonia scholaris* latex bound enzyme was found to be considerably active with< 80% retention of activity over a broad pH range from 5.5 to 7.5, whereas *Calotropis procera* and *Euphorbia royleana* bound enzyme retained only 55% and 30% activity respectively at pH 7.5.

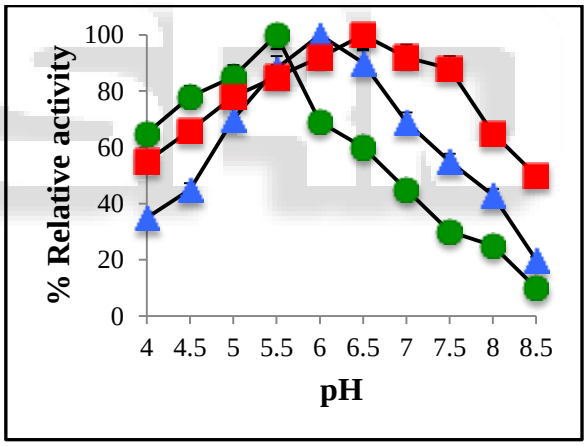


Fig. 3: Effect of pH on activity of *Calotropis procera* ( ), *Euphorbia royleana* ( ) and *Alstonis scholaris* ( ) latex bound peroxidase.

E. Effect of temperature

As evident from Fig. 4, temperature for maximum activity of peroxidase increased after immobilization onto latex from *Calotropis procera* (35°C), *Euphorbia royleana* (30°C) and *Alstonia scholaris* (30°C) as compared to free enzyme which shows optimum activity at 25°C [25]. This change signifies the protecting effect of support which might have stabilized the three dimensional structure of enzyme. An increase in the optimum temperature of peroxidase after immobilization has also been reported in literature such as on activated wool (40°C) [29], magnetic beads (35°C) [24], modified chitosan beads (45°C) [13] and nanoporous gold (40°C) [30].

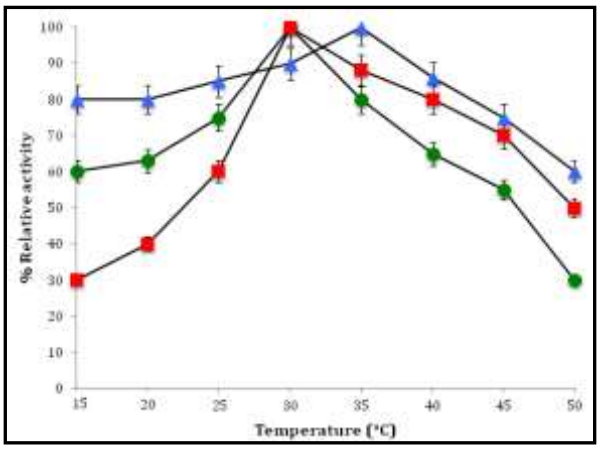


Fig. 4: Effect of temperature on activity of *Calotropis procera* ( ), *Euphorbia royleana* ( ) and *Alstonis scholaris* ( ) latex bound peroxidase.

Activation energy also determines the influence of temperature on reaction velocity. Due to restriction of conformational changes after immobilization, the energy of enzyme molecule becomes low [31]. The differential effects, which protects the enzyme against heat denaturation, leads to the lower activation energy [32], as in our studies. The activation energy (Ea) of peroxidase immobilized onto latex from *Calotropis procera*, *Euphorbia royleana and Alstonia scholaris* was calculated to be 2.22, 0.84 and 3.136 Kcal/mol respectively, which is less as compared to the free enzyme (4.58 Kcal/mol) [25]. It became clear from the results that the immobilized enzymes were less sensitive to temperature change.

F. Effect of substrate concentration

Effect of substrate concentration on the activity of immobilized enzymes peroxidases was studied in the concentration range from 0.5 to 4.0 mM. The effect of varying concentration of H_2O_2 on initial velocity of peroxidases was hyperbolic from 0.5 to 2.5 mM for *Calotropis procera* latex bound enzyme and from 0.5 to 3.0 mM for *Euphorbia royleana* and *Alstonia scholaris* latex bound enzyme, after which the reaction rate remained constant. *K*m values as calculated from Lineweaver-Burk plot were 2.4, 1.81, 2.0 and 1.71 mM for free [25], *Calotropis procera*, *Euphorbia royleana* and *Alstonia scholaris* latex bound peroxidases respectively (Fig. 5) indicating the increased affinity of immobilized enzyme for its substrate. Km of an immobilized enzyme is expected to be lower than the native enzyme, whenever the charges on support and substrate are of different polarity [33]. In addition, the kinetics of immobilized enzyme is affected by many factors such as micro environmental effects, diffusional effect, changes in enzyme confirmation, and steric hindrance [34]. *K*m values for all three immobilized peroxidase preparations in the present study are also lower than the reported *K*m values for peroxidase immobilization onto clay minerals [35], modified chitosan beads [13] and activated wool [29], indicating the suitability of plant latex as enzyme immobilization support.

*V*max value showed the amount of substrate converted to product per unit of time when the enzyme was saturated with substrate. *V*max value of free peroxidase has been reported to be 20 nmol (min mg protein)$^{-1}$ [25], while immobilized peroxidases in the present work had *V*max values of 25.0, 33.33 and 31.25 nmol (min mg protein)$^{-1}$ when bound to latex from *Calotropis procera*, *Euphorbia*

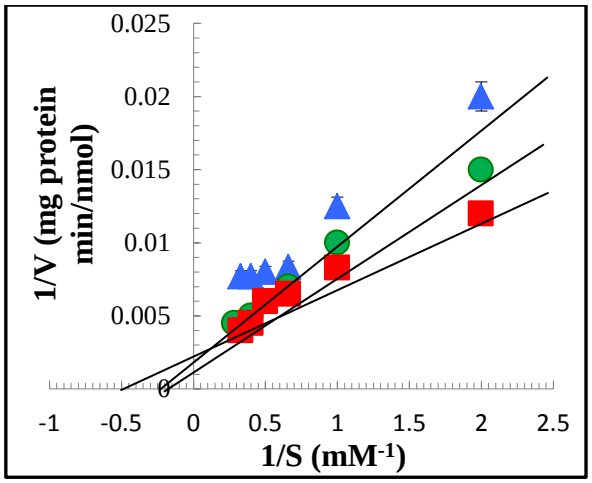


Fig. 5: Kinetic constants Km and Vmax as calculated from Lineweaver-Burk plot for the three immobilized enzymes.

royleana and *Alstonia scholaris*, respectively. Changes in *V*max could be attributed to conformational change in structure of enzyme after interaction with support and lower accessibility of the substrate to the active site of enzyme. Similar observations involving changes in *V*max values after immobilization have been reported by other researchers for immobilization of peroxidase on clay minerals [35] and arylamine glass beads [25].

G. Thermal stability of immobilized peroxidases

The stability of an enzyme usually refers to the conformational stability of the protein. The thermal stability is largely determined by the conformational stability and inactivation is caused primarily by denaturation. In order to know the thermal stability of three immobilized peroxidase preparations, they were incubated for 30 minutes at different temperatures ranging from 30°C to 65°C at an interval of 5°C. During incubation, pH and substrate concentration were kept optimum. It is clear from Fig. 5, that *Calotropis procera* latex bound peroxidase retained 100 % activity up to 45°C after which it decreased. Contrary to this, *Alstonia scholaris* and *Euphorbia royleana* latex were not so effective in stabilizing the structure of bound peroxidases as enzyme activity decreased with increased temperatures. The immobilized peroxidase retained 50 % of the initial activity at about 60°C, 45°C and 50°C for *Calotropis procera*, *Euphorbia royleana* and *Alstonia scholaris* latex respectively (Fig. 6). In addition, deformation of the latex membrane was also observed at temperatures greater than 65°C in all the three cases. The results established the superiority of *Calotropis procera* latex in stabilizing the structure of enzyme over the other two supports, which might be attributed to the presence of high amount of hydrophobic hydrocarbons in the latex of *Calotropis procera* [36]. Hydrophobic-hydrophobic interactions might have imparted structural stability to peroxidase. This is consistent with the other findings on stabilization of enzyme structure on hydrophobic supports [37].

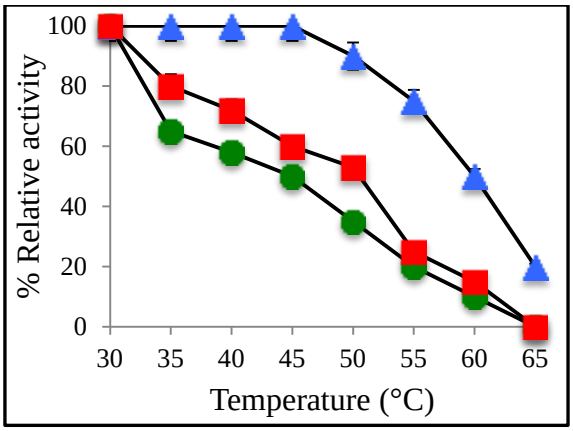


Fig. 6: Residual activity measurements of *Calotropis procera* (Euphorbia royleana ( ) and *Alstonis scholaris* ( ) latex bound peroxidase at the respective temperatures.

H. Storage stability of immobilized peroxidases

Activity of latex bound peroxidase preparations stored in phosphate buffer (0.01M, pH 7.0) at 4°C was tested on alternate days upto 60 days. As depicted in Fig. 7, activity of immobilized enzyme on all the three supports decreased with time. After one month, *Alstonia scholaris* latex bound peroxidase retained 75% of the initial activity, whereas the activity reduced to 50% in case of *Calotropis procera* and *Euphorbia royleana* latex bound enzyme. Free enzyme retained only 20% of the initial activity after one month [25], clearly suggesting the stabilizing effect of immobilization on the enzyme. However, storage stability comparison of the three latex supports established the superiority of *Alstonia scholaris* latex on which the half-life of the immobilized peroxidase was approximately 40 days.

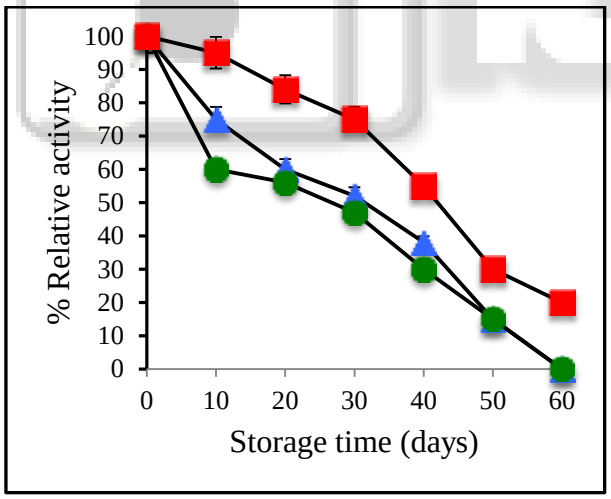


Fig. 7: Storage stability of *Calotropis procera* ( ), *Euphorbia royleana* ( ) and *Alstonis scholaris* (latex bound peroxidase.

I. Operational Stability Of Immobilized Peroxidases

A reusability study was carried out by measuring the residual activity of the immobilized peroxidases after each use. Peroxidase immobilized onto plant latex from *Calotropis procera* showed 60% activity after 50 repeated uses and the enzyme immobilized onto the latex from *Alstonia scholaris* retained 60% activity after 15 repeated uses under standard conditions, whereas in case of *Euphorbia royleana* the enzyme lost its 40% activity only after five repeated uses under standard conditions (Fig. 8).The results are either superior or comparable to the data already reported in literature for immobilization of peroxidase on activated wool (50% after 7 reuses) [29], cashew gum polysaccharide (50% activity after 9 reuses) [9], modified chitosan (65.8% after 6 reuses) [13] and chitosan-halloysite hybrid-nanotubes (50% after 5 reuses) [38].

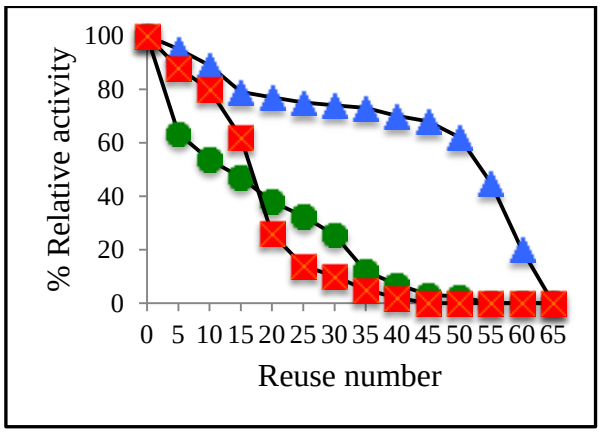


Fig. 8: Reuse capacity of immobilized peroxidases.

J. Determination of total phenolic content in fruit juices

Total phenolic content in ten different fruit juice samples was determined using *Calotropis procera* latex bound peroxidase due to its high operational stability. The phenolic content in fruit juices ranged from 0.494±0.008 to 0.798±0.01 3µmoles gallic acid per ml.

IV. Conclusion

In the present work suitability of using plant latex as immobilization support has been explored. To achieve this, horseradish peroxidase was immobilized onto latex from 3 different sources viz. Calotropis procera, Euphorbia royleana and Alstonia scholaris. Results indicated that the conjugation yield of the enzyme was highest for Calotropis procera latex (0.51 ± 0.01 mg/cm^2) but the enzyme retained maximum activity over Alstonia scholaris latex (71.24 ± 0.80). In agreement with the literature, optimum pH and temperature for activity of immobilized peroxidases was different from that of free peroxidase in all the three cases. A comparison of Km values revealed that the affinity of substrate increased in the order of Euphorbia royleana < Calotropis procera < Alstonia scholaris latex bound peroxidase. Storage stability of peroxidase on Alstonia scholaris latex was 50% more than on Calotropis procera, Euphorbia royleana latex after 50 days of storage. However, thermal stability and reusability of peroxidase was better on Calotropis procera latex. Hence, Calotropis procera latex bound peroxidase was employed for determination of phenolic content of fruit juices. It may be safely concluded that both Calotropis procera and Alstonia scholaris latex were better supports than Euphorbia royleana. The enzyme bound to Calotropis procera latex had maximum conjugation yield, thermal stability and reusability whereas Alstonia scholaris latex facilitated high retention of enzyme activity, least Km value and maximum storage stability.

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