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Hen egg white as a feeder protein for lipase immobilization

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ABSTRACT

Enzyme stabilization via immobilization is one of the preferred processes as it provides the advantages of recovery and reusability. In this study, *Thermomyces lanuginosus* lipase has been immobilized through crosslinking using 2% glutaraldehyde and hen egg white, as an approach towards CLEA preparation. The immobilization efficiency and the properties of the immobilized enzyme in terms of stability to pH, temperature, and denaturants was studied and compared with the free enzyme. Immobilization efficiency of 56% was achieved with hen egg white. The immobilized enzyme displayed a shift in optimum pH towards the acidic side with an optimum at pH 4.0 whereas the pH optimum for free enzyme was at pH 6.0. The immobilized enzyme was stable at higher temperature retaining about 83% of its maximum activity as compared to the free enzyme retaining only 41% activity at 70 °C. The denaturation of lipase in free form was rapid with a half-life of 2 h at 60 °C and 58 min at 70 °C as compared to 12 h at 60 °C and 2 h at 70 °C for the immobilized enzyme. The effect of denaturants, urea and guanidine hydrochloride on the free and immobilized enzyme was studied and the immobilized enzyme was found to be more stable towards denaturants retaining 74% activity in 8 M urea and 98% in 6 M GndHCl as compared to 42% and 33% respectively in the case of free enzyme. The apparent K_m (2.08 mM) and apparent V_{max} (0.95 μmol/min) of immobilized enzyme was lower as compared to free enzyme; K_m (8.0 mM) and V_{max} (2.857 μmol/min). The immobilized enzyme was reused several times for the hydrolysis of olive oil.

1. Introduction

Lipases (E.C.3.1.1.3) are a group of water soluble enzymes that catalyze the hydrolysis of fats and oils at an oil water interface, by the phenomenon of interfacial activation [1]. These enzymes are biotechnologically relevant and are widely used in many industries like food, fine chemicals, detergents, waste water treatment, cosmetics, paper and pulp, pharmaceuticals and leather industry [2]. However, a major limitation for the use of these enzymes in its native state during the designing of a process is its relatively low stability to temperature and pH changes, solvents, detergents [3] and product contamination of the biocatalyst [4,5]. Thus, there is a need to improve the stability of the enzyme for commercial applications.

Immobilization is one of the strategies used to improve operational stability of the biocatalyst. Better operational control, easier product recovery without catalyst contamination and better functional properties compared to the free enzyme, make immobilization the preferred method to improve enzyme stability [6]. Enzyme stabilization can be achieved through immobilization of the enzyme inside a porous support or by multipoint covalent attachment [4]. However, immobilization inside a porous support is not favorable due to diffusional limitations. Glutaraldehyde has been used extensively [7–10] as an efficient crosslinker and it promotes covalent bond between the enzyme and the matrix. However, glutaraldehyde treatment of proteins previously adsorbed on supports, establishes a multipoint covalent enzyme support attachment, leading to stable covalent immobilized enzymes which are more stable than using glutaraldehyde preactivated support [8].

Lipases have been immobilized on different materials [3,5,10–16], but most of the matrices used are polymers of carbohydrates, silica based or synthetic polymers. The use of protein-based matrices for immobilization has however, been limited [17–20]. Protein based matrices offer the advantage that they provide a wide variety of functional groups for the attachment of enzyme and the biocompatible environment helps to retain the biological activity of the immobilized enzyme [21].

Hen egg white (HEW) is a natural source of proteins with unique functional properties such as gel and foam formation. Its major attributes are its availability in a ready to use form, low cost, and nontoxic nature [21,22]. Besides, lysozyme present in HEW has been reported to provide enzyme stability [23] and bacteriolytic activity [21,22]. Use of HEW for the coimmobilization of enzymes like catalase, invertase, lysozyme and cells has been earlier established in our laboratory [21,22,24–26]. HEW is rich in lysine amino residues contributed by ovalalbumin the major protein in egg white.

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In the present study, we have utilized hen egg white (HEW) to immobilize lipase from *Thermomyces lanuginosus* using glutaraldehyde as a crosslinker. *T. lanuginosus* lipase is a single chain protein consisting of 269 amino acids with an alpha helical lid covering the active site and has a strong tendency to form bimolecular aggregates [27].

Cross linked enzyme aggregates (CLEA) are prepared by cross-linking aggregated enzyme molecules using a suitable cross-linker [28]. However, a modified process towards the preparation of CLEA has been recently demonstrated by Kim et al. [29], by absorbing and cross-linking chymotrypsin and lipase onto mesoporous silica (SBA-15). In this study, the cross-linking of lipase from *T. lanuginosus* with strong tendency to aggregate using glutaraldehyde is an approach towards CLEA preparation. HEW in addition to the beneficial features mentioned above also acts as a protein feeder, like BSA to provide additional lysine rich groups to increase the concentration of free amino groups [30]. Furthermore, the immobilization efficiency and biochemical characterization in terms of stability and reusability has been carried out.

2. Materials and methods

2.1. Materials

Lipase (triacylglycerol hydrolase (E.C.3.1.1.3) from *T. lanuginosus* with specific activity of 50 units per mg protein, was obtained from Sisco Research Laboratories (Mumbai, India) and was used without further purification. Leghorn varieties of eggs were obtained from the local market. Glutaraldehyde (25%) was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and olive oil was obtained from Loba Chemie (Mumbai, India). All other reagents and chemicals used in this study were of analytical grade.

2.2. Methods

2.2.1. Lipase immobilization

Lipase (10 mg) was mixed with egg white (20 ml) obtained from fresh eggs and was treated with 2% glutaraldehyde. The mixture was stirred to obtain a homogenous mixture and allowed to stand for 2 h at 4 °C for crosslinking. The hard gel obtained was shattered by passing through a syringe (Fig. 1A and B). The immobilized preparation was then washed to remove the excess glutaraldehyde and stored in 0.1 M phosphate buffer pH 7.0 at 4 °C [22]. For all studies, free enzyme (1 mg) was compared with HEW (2 g) produced using equivalent amount of enzyme.

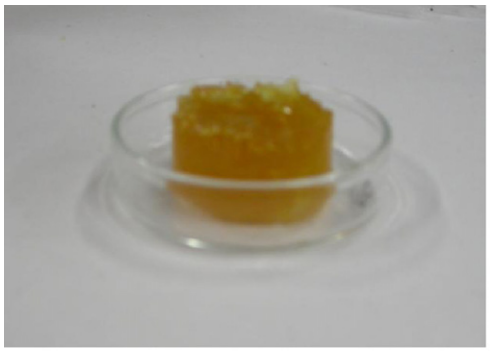
2.2.2. Activity assay

Activities of the free and immobilized lipase were assayed using olive oil as the substrate. 25 ml of olive oil and 75 ml of 2% polyvinyl alcohol solution was emulsified using a sonicator. The reaction mixture comprised of 5 ml of olive oil emulsion and 4 ml of 0.1 M phosphate buffer pH 7.0 which was preheated at 37 °C. Free or immobilized enzyme was added to the reaction mixture and the reaction was carried out for 20 min at 37 °C. At the end of the assay, the emulsion was destabilized by addition of 20 ml of 1:1 acetone:alcohol mixture and free fatty acids liberated were titrated using 0.05 N NaOH using phenolphthalein as the indicator. One unit of lipase is defined as the amount of enzyme that liberated 1 μmol of fatty acid per minute.

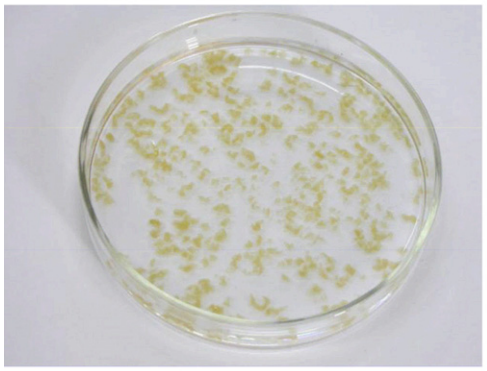
2.2.3. Immobilization efficiency

The efficiency of immobilization was determined for lipase immobilized in HEW using the following equation:

$$\text{Immobilization efficiency (\%)} = \frac{\{(\text{Activity of immobilized enzyme (U/mg)} * (\text{total weight of immobilized enzyme (mg)}\}}{\{(\text{Activity of free enzyme (U/mg)} * (\text{total weight of free enzyme (mg)}\}} \times 100$$



A. Immobilized lipase using HEW as feeder and glutaraldehyde as crosslinker.



B. Immobilized lipase after shattering through syringe.

Fig. 1. Immobilized lipase using HEW as feeder.

Enzyme units were calculated as in Section 2.2.2.

2.2.4. Effect of pH and temperature on enzyme activity

The effect of pH on the lipase activity in the free and immobilized form was determined in the pH range of 3.0–9.0 using 5 mM acetate buffer (pH 3.0–5.0), 5 mM phosphate buffer (pH 6.0–8.0), and 5 mM bicarbonate buffer (pH 9.0) at 37 °C. Hydrolytic activity for the free and immobilized enzyme was determined as mentioned in Section 2.2.2 using the different buffers.

The effect of temperature on enzyme activity was measured by determining the activity of the enzyme in free and immobilized form as mentioned in Section 2.2.2 at temperatures ranging from 20 °C to 70 °C in 5 mM phosphate buffer pH 7.0.

2.2.5. Thermostability and determination of half life

Free and immobilized enzyme preparations were incubated in substrate-free phosphate buffer (pH 7.0, 5 mM) for 30 min at temperatures ranging from 30 °C to 70 °C. After incubation, the preparation was brought to room temperature. Substrate was then added and the residual activity of the enzyme was determined as described in Section 2.2.2.

Half life determination was done by incubating the free and immobilized enzyme in 5 mM phosphate buffer for different time intervals (30 min, 60 min, 90 min, and 120 min) at 60 °C and 70 °C, followed by measuring the residual activity under standard conditions.

2.2.6. Determination of kinetic constants

The kinetic constants were determined using olive oil as substrate (in the concentrations range 3–10 mM) using free and immobilized lipase and titrating the liberated free fatty acid produced with 0.05 N NaOH as described above. The experiments were conducted at pH 7.0 and 37 °C. The apparent K_m and apparent V_{max} values for the free and immobilized lipase were calculated from Lineweaver–Burk plots.

2.2.7. Effect of denaturants

Urea and guanidine hydrochloride (GndHCl) were used as the denaturants in this study. Urea and GndHCl solutions of different concentrations were prepared by adding an appropriate volume of 8 M urea or 6 M GndHCl to phosphate buffer (5 mM, pH 7.0). The free and immobilized enzymes were incubated in different concentrations of urea and GndHCl at 37 °C for 1 h, following which the residual activity for the free and immobilized enzyme was measured in presence of the denaturant as given in Section 2.2.2.

2.2.8. Reusability of the immobilized enzyme

The reusability of the immobilized enzyme was tested for the hydrolysis of olive oil. After each reaction, the immobilized enzyme was recovered by filtration, washed with buffer and was used in the next hydrolysis reaction and compared with the first run (activity defined as 100%).

2.2.9. Storage stability

To determine the storage stability, the immobilized enzyme was stored in phosphate buffer 0.1 M pH 7.0 at 4 °C and the hydrolytic activity was measured at timed intervals.

3. Results and discussion

3.1. Immobilization efficiency

Based on the calculation as in Section 2.2.3, the immobilized lipase retained 56% of the free enzyme activity. The activity of the free enzyme was 31 U/mg powder and 8.75 U/g immobilized preparation for olive oil hydrolysis.

3.2. Effect of pH

The free lipase showed an increased activity in the pH range of 6–8 with an optimum at pH 6 whereas the immobilized lipase showed an optimum at pH 4.0 (Fig. 2). The immobilized enzyme displays an acidic shift with higher stability at pH 4.0. The microenvironment of an enzyme can influence its properties. An enzyme in solution may have a different pH optimum from that of the immobilized enzyme, depending on the surface charges on the matrix. Polycationic carriers tend to shift the pH optima of the immobilized enzyme to the acidic side [31–35]. The pH of HEW is 9.0. The presence of basic amino acids in HEW contributes to its polycationic nature. Thus making the immobilized enzyme experience an alkaline microenvironment even at acidic pH. To reduce such effect the use of appropriate buffers or buffer of higher strength may be beneficial. However, the latter is not possible since lower molarity buffer is preferable for better clarity of product assessment. Also at high molarity on account of the inherent property of *T. lanuginosus* lipase to aggregate, detection of activity is hampered. However, the use of different buffers (acetate, phosphate and bicarbonate) with good buffering near its pK_a should be beneficial. Similar results of decrease in pH optima of the immobilized enzyme have been previously reported in case of *Candida rugosa* lipase immobilized on celite [11]. Whereas, in the case of *Saccharomyces cerevisea* lipase immobilized on Mg–Al hydrotalcite the pH optima was shifted to the basic side due to the anionic nature of the support [36]. *T. lanuginosus* lipase immobilized on magnetite nanoparticles showed no change in pH optima when compared to the enzyme in free form [37]. The support thus exerts a strong influence on the pH optima of the immobilized lipase.

3.3. Effect of temperature

The optimum temperatures for free and immobilized lipase were 30 °C and 60 °C respectively, as seen in Fig. 3. The peak in such a plot results because enzymes being proteins are denatured by heat and become inactivated as the temperature is increased beyond a certain point. The apparent temperature optimum is thus the resultant of two processes, the usual increase in reaction rate with temperature and the increasing rate of thermal denaturation of enzyme above a certain temperature. Most lipases exhibit higher temperature optima in their immobilized form as compared to the free form [36–38]. Xie et al. have also observed a similar trend for immobilized *T. lanuginosus* lipase showing temperature optima at 45 °C and retaining 82% activity at 55 °C [37]. In comparison *T. lanuginosus* lipase immobilized in HEW is able to withstand even higher temperature retaining 100% activity at 60 °C.

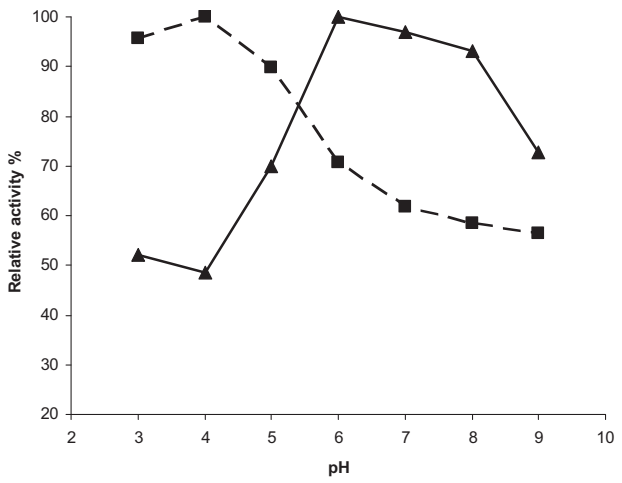


Fig. 2. Effect of pH on free (-▲-) and immobilized enzyme (–■–).

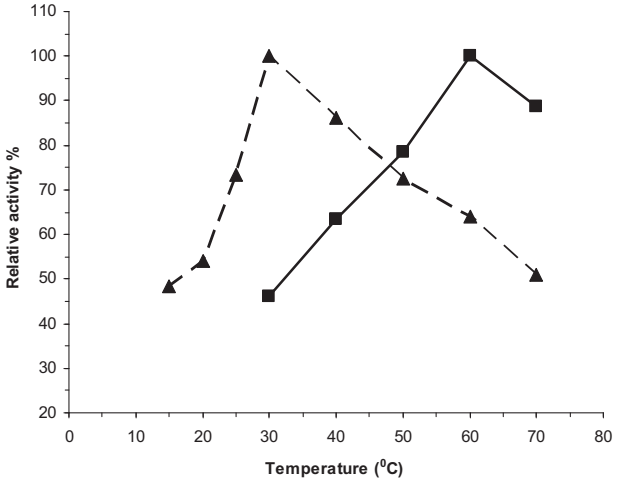


Fig. 3. Effect of temperature on free (-▲-) and immobilized enzyme (–■–).

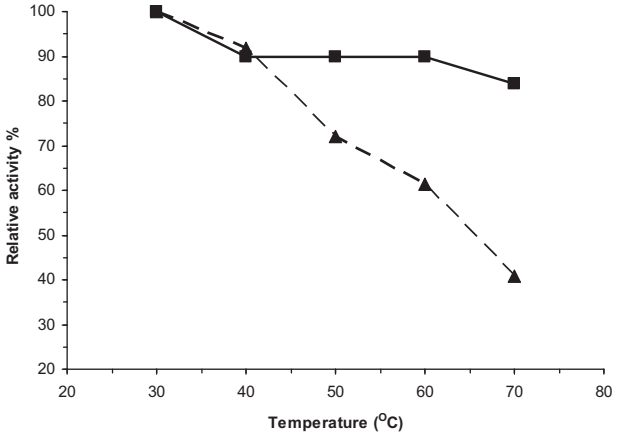


Fig. 4. Thermostability of free (-▲-) and immobilized enzyme (–■–).

3.4. Thermostability

Utilization of enzymes in process development often encounters the problem of thermal inactivation of the enzyme. At high temperature, the enzyme undergoes partial unfolding by heat-induced destruction of non-covalent interactions [39]. The relative activities of the free and immobilized lipases remained over 70% and 90% in the temperature range of 30–50 °C respectively. The immobilized lipase was found to retain its activity even at higher temperatures when compared with the free enzyme. At 70 °C, the immobilized preparation retained 83% activity, whereas the free enzyme retained only 41% activity (Fig. 4). Immobilization provided a more rigid backbone for the lipase molecules; the effect of higher temperature in breaking the interactions responsible for the proper globular, catalytic active structure became less prominent [39]. Immobilization thus, offered the microenvironment to preserve the enzyme structure from heat denaturation.

The half lives of the free enzyme at 60 °C and 70 °C was 2 h and 58 min respectively whereas that of the immobilized enzyme was 12 h and 2 h respectively. A 6-fold increase in enzyme stability at 60 °C and a 2-fold increase at 70 °C were obtained on immobilization of the enzyme.

3.5. Kinetic parameters

The Lineweaver–Burk plot for the hydrolysis of olive oil by free and immobilized lipase is depicted in Fig. 5 with a linear regression of 0.98 for the free and 0.93 for immobilized enzyme. The K_m and V_{max} of the free enzyme were 8.0 mM and 2.8 μmol/min respectively whereas the apparent K_m and apparent V_{max} of the

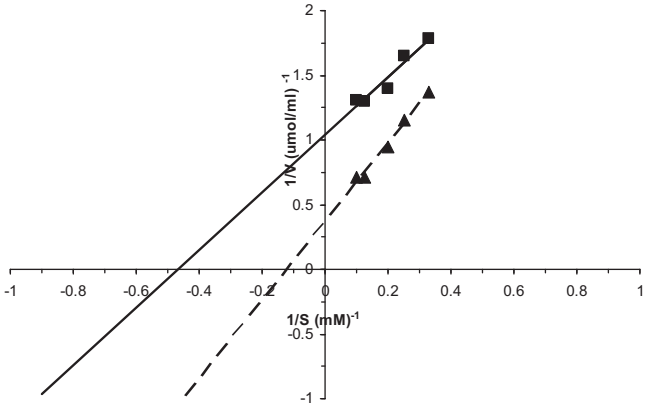


Fig. 5. Kinetic parameters of free (-▲-) and immobilized enzyme (–■–).

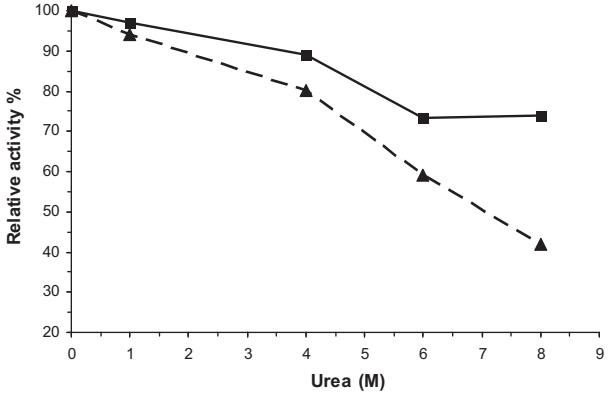


Fig. 6. Effect of urea on free (-▲-) and immobilized enzyme (–■–).

immobilized enzyme were 2.08 mM and 0.95 μmol/min respectively. Lipase immobilized in HEW showed a decrease in apparent K_m. The K_m value is known as the criterion for the affinity between enzymes and substrates. The higher affinity for substrate obtained in the case of immobilized enzyme could be possibly explained on the basis that when lipase is immobilized, the microenvironment of enzyme is hydrophobic as water availability is low, whereby the substrate which is also hydrophobic is attracted. However, in the case of free enzyme the greater extent of hydrophilicity of the medium reduces the attraction between the enzyme and substrate. This is however, not reflected in the apparent $V_{max.}$ Lipase immobilized in HEW showed a decrease in apparent V_{max}. These results suggest that the enzyme could have undergone a conformational change during the immobilization in HEW affecting the rate of the reaction. The change in orientation could lead to improper positioning of the active sites for binding the substrate. Besides the not so favorable conditions for increased reaction rate by the immobilized enzyme in this case could be because the substrate being insoluble, the immobilized enzyme may act only on the available fraction while the free enzyme could interact freely with the substrate. Similar results of a decreased apparent K_m and decreased apparent V_{max} which is not usually seen, was obtained for amylase immobilized on alkylamine glass beads by Pundir and Pundir [40] and immobilized *C. rugosa* lipase by Montero et al. [5].

3.6. Effect of denaturants

Denaturants such as urea and guanidine hydrochloride (GndHCl) are known to cause protein unfolding. To determine the effect of denaturant on enzyme inactivation, the free and immobilized enzyme were incubated and assayed in the presence of different concentrations of urea and GndHCl. The activity was expressed relative to a control sample in which urea and GndHCl were not added.

Urea and GndHCl caused a destabilization of the free and immobilized enzyme (Figs. 6 and 7). A progressive loss in the activity of the enzymes was observed with an increase in the concentration of the denaturant. However, the effect of the denaturants was more pronounced on the activity of free enzyme as compared to the immobilized enzyme. The free enzyme retained only about 42% activity in 8 M urea and 33% in 6 M GndHCl. The immobilized enzyme, was found to retain 74% and 98% activity in 8 M urea and 6 M GndHCl respectively.

Urea and GndHCl disrupt hydrophobic interactions in proteins, which are essential to maintain the secondary structure of the enzyme. Disruption of these bonds cause the peptide chains to stretch and expose essential amino acids involved in catalysis [41]. Urea and GndHCl however act differently on enzymes, and the extent of inactivation caused by each of the denaturant on the free enzyme is different. GndHCl has a higher inactivating effect

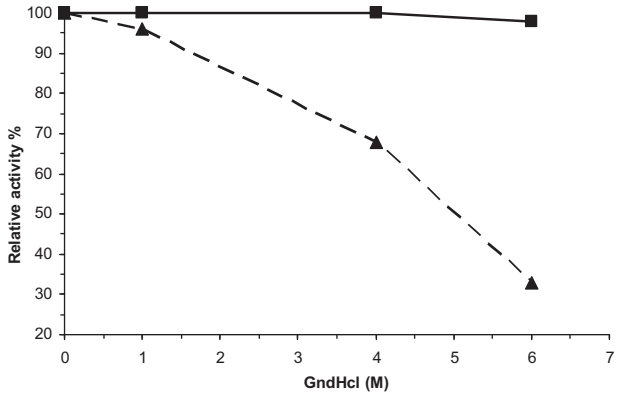


Fig. 7. Effect of GndHCl on free (-▲-) and immobilized enzyme (–■–).

as compared to urea. Our results are in agreement with the well documented fact that GndHCl is a stronger denaturant. [42]. Zhu et al. have suggested that *T. lanuginosus* lipase is stabilized by electrostatic interactions [43]. GndHCl is a salt and increasing concentration of the salt leads to an increase in the ionic strength which leads to inactivation of the enzyme by weakening the more favorable electrostatic interactions. Urea being an uncharged molecule lacks any ionic strength effects and hence affects the enzyme to a lesser extent [42,43]. The immobilized enzyme is more rigid and hence resists unfolding in the presence of denaturants. Rodrigues et al. have shown the positive effects of multipoint covalent attachment on immobilization to prevent distortion and help proper refolding of the enzyme in presence of denaturants [44].

3.7. Reusability

The reusability of immobilized lipase is important to determine the economic viability of a system. The reusability of the immobilized lipase was tested and it was found to retain up to 45% activity after 7 reuses. The decrease in the hydrolysis after seven reuses could be due to leakage of enzyme from the matrix or due to the formation of a thin film layer of emulsion over the immobilized matrix. The film prevents the access of substrate to the enzyme [45]. To confirm this possibility, the matrix was washed with acetone after each reuse and then rinsed in buffer and used in the next round for olive oil hydrolysis. The use of acetone, removed the emulsion coating the matrix preventing substrate access to the enzyme. The immobilized enzyme retained up to 70% activity after 12 reuses. Thus the immobilized enzyme could be reused without much loss in activity.

3.8. Storage stability

The immobilized lipase exhibited good storage stability, retaining up to 75% activity after 30 days. The high storage stability of the immobilized enzyme makes it favorable for application in industries.

4. Conclusion

In the present study, HEW was used as a proteinic feeder for the immobilization of lipase. The immobilized preparation can be easily prepared, reused and stored. The use of HEW for immobilization was found to stabilize the enzyme to changes in system parameters such as pH, temperature and denaturants such as urea and GndHCl. The immobilized enzyme exhibited thermal stability with a higher half life and operational stability. This technique of immobilization opens up a new possibility towards CLEA preparation and can find application in transesterification of oils.

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