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Research Article

PREPARATION AND CHARACTERIZATION OF CROSS-LINKED ENZYME AGGREGATES (CLEAs) OF *AEROMONAS CAVIAE* AU04 PROTEASE

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ABSTRACT

Protease from *Aeromonas caviae* AU04 was immobilized as cross-linked enzymes aggregates (CLEAs) with salt precipitation and glutaraldehyde as cross-linker. $(NH_4)_2SO_4$ was the most suitable precipitant with 90% of the activity recovery. Immobilization was carried out for 8 h with agitation at 30°C and pH 8.0.Maximum cross-linking was obtained at 70 mM glutaraldehyde and 6 h of cross-linking time at 30°C. It was able to retained activity after 24 h in the presence of non-polar solvents at 30°C. The CLEAs was reused for 10 cycles retaining about 42% of the activity. It showed enhanced storage stability at 4°C when compared to the native form. SEM image of the freshly prepared CLEAs showed a coarse-grained appearance and more structured. It was able to degrade the native chicken feather after 35 h of incubation. These results suggest a potential CLEAs application for various industries.

Keywords: Protease, *Aeromonas caviae*, Cross-Linked Enzyme Aggregates (CLEAs), Glutaraldehyde, operational stability

1. INTRODUCTION

Demand for hydrolytic enzymes has increased in recent years, notably for proteases due to its diverse applications in different industrial domains, such as the leather, detergent and food industries. Among the different industrial enzymes, proteases account for about 60% of the total commercially available enzyme [1]. Protease is found in all organisms and the preferred source being the microbes because of their fast growth rate in the confined space and can be genetically manipulated to generate new enzymes with altered properties that are beneficial for numerous applications [2]. Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus* [3, 4] due to their high pH and temperature stability and have gained more interest in the field of bioengineering and biotechnological applications. However, the drawbacks of the free protease, such as poor operational properties such as stability, recyclability, and difficulties in separating from products, limit its application in industry. To overcome the weaknesses, immobilization techniques are widely employed.

Cross-linked enzyme aggregates (CLEAs) are the recently developed immobilization strategy to enhance the operational stability and re-usability of the enzymes. Moreover, this method of immobilization without the use of support is gaining importance because they have the benefits of high volumetric productivity and low production cost. This methodology combines both purification and immobilization in a single step where the enzyme are precipitated from an aqueous solution by adding a salt or a water-miscible organic solvent or polymer followed by cross-linking of the physical aggregates of enzyme molecules with a bifunctional agent [5, 6]. Enzyme immobilization via cross-linking with bifunctional cross-linkers reagent does not suffer from limitations such as diluted enzyme activities and lower catalyst productivity. The resulting biocatalyst essentially comprises 100 wt% protein as the molecular weight of the cross-linker agent is negligible when compared to that of the enzyme molecule [7]. CLEAs are easily recoverable by centrifugation because they are heterogeneous catalyst. Several reports are there on the immobilization of alpha-amylase [8], lipase [9] and α-L-rhamnosidase [10].

In the present work, we have studied the preparation of the CLEAs protease and the biochemical characterization of the CLEAs. Further, the CLEAs was used for the degradation of the chicken feather and for the bloodstain removal in cotton cloth.

2. MATERIAL AND METHODS

2.1. Precipitation of Enzyme for Immobilization

The protease enzyme was precipitated by different organic solvents such as acetone, ethanol and *tert*-butyl alcohol and ammonium sulfate. The enzyme was precipitated with ammonium sulphate between 0-60% saturation. Chilled organic solvents (acetone, ethanol and *tert*-butyl alcohol, 5ml each) was added drop-wise separately to samples of the crude enzymatic extract (10 ml) with shaking and kept for 6 h at 4°C for complete precipitation of enzymes and then centrifuged for 15 min at 12,000 rpm. The supernatant was discarded and precipitate was re-dissolved in 50 mM Tris-HCl buffer, pH 8.0.

2.2. Preparation of CLEAs

Cross linked enzyme aggregate was prepared by the drop-wise addition of varying amount of glutaraldehyde (10-100 mM) in 3 ml of enzyme precipitate and the mixture was agitated at 150 rpm for different time (1-8 h) at pH 8.0 and 30°C. The mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed until there was no trace of enzyme activity in the supernatant, the aggregate was stored in 50 mM Tris-HCl buffer (pH 8.0) at 4°C. The percentage activity recovery of protease was determined by Equation

Activity recovery (%) =

$$\frac{\text{Total activity of enzymes in CLEAs (U)}}{\text{Total initial activity of free enzymes (U)}} \times 100$$

The protease activity was assayed using the microassay described by Cabral et al. [11] using azocasein as the substrate with slight modifications.

2.3. Characterization of the immobilized enzyme

2.3.1. Structural Characterization by Scanning Electron Microscopy

The shape and surface morphology of CLEAs was examined using scanning electron microscopy (SEM). The SEM image of CLEAs was obtained on JEOL JSM-6360(Germany) instrument at 10 kV. Samples were dried under vacuum and then placed on a carbon tape over a microscope slide to coat with gold particles using a sputter coater.

2.3.2. pH and thermal Stability of CLEAs

The pH stability the CLEAs was determined by incubating the immobilized enzyme in 50 mM buffer of desired pH (Sodium acetate (pH 4.0-5.0), Sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 7.0-8.0) and Glycine-NaOH (pH 9.0-12.0)) for 3 h at 37°C. The CLEAs was assayed to determine the protease activity with azocasein as substrate.

Thermal stability of the CLEAs was compared with that of the free enzyme. The CLEAs was incubated without substrate at 40, 50, 60, 70, and 80°C for 5 h. After incubation, CLEAs was assayed to determine the protease activity with azocasein as substrate in 50 mM Tris-HCl buffer. The residual activity of the CLEAs was determined by taking the activity at zero hour as 100%.

2.3.3. Determination of kinetic parameters

The kinetic parameters were calculated from the initial rate activities of the free enzymes and CLEAs using various concentrations (2 to 20 mg/ml) of azocasein under the specified assay conditions at predetermined optimum pH and temperature. K_m and V_{max} values were calculated from non-linear regression fitting of the initial reaction [12].

2.3.4. Effect of Organic Solvent on Activity of CLEAs

The stability of CLEAs in organic solvent was carried out by incubating 5 mg CLEAs in various organic solvents for 24 h at 30°C in the presence of 30% organic solvent with constant agitation at 150 rpm. Dimethyl sulfoxide (DMSO), dichloromethane (DCM), toluene, n-hexane, acetonitrile, 1-propanol, ethanol, methanol, n-heptane, n-octane, n-nonane and n-decane were used and the solvents were chosen according to the hydrophobicity and log *P* values. After the incubation period, the assay of CLEAs was done by the standard assay procedure. The activity was compared with the original activity and the activity retention was calculated. The activity of the immobilized enzyme in an aqueous buffer was taken as the control (100%).

2.3.5. Determination of Reusability and Storage of CLEAs

The CLEAs was separated from the reaction mixture by filtration after the activity assay and followed by washing with water and simultaneously with 50 mM Tris-HCl buffer pH 8.0 after each usage. Then it was suspended again in a fresh reaction mixture to measure the enzyme activity. The procedure was repeated until the enzyme lost 50% of its original activity.

The storage stability was determined by incubating the CLEAs in Tris-HCl buffer (50 mM, pH 8.0 at 4°C for 3 months). Aliquots were periodically withdrawn for assaying its activity. The residual activity was measured by the standard assay procedure.

2.3.6. Application on the Chicken Feather Degradation

The keratinolytic activity of the CLEAs was studied using chicken feathers from a slaughter house. The feathers were washed with ethanol and autoclaved. Disintegration was studied by incubating the feathers with the CLEAs supplemented with 0.1% v/v β-mercaptoethanol (β-ME) at 50°C for 48 h on a rotary shaker at 120 rpm [13]. The level of degradation was observed periodically during the treatment.

3. RESULTS AND DISCUSSION

3.1. Effect of Different Precipitants on the Recovery of the Enzyme

The influence of varying precipitants towards the recovery of the enzyme is shown in Fig. 1. Activity recovery of the protease CLEAs was increased with increased in ammonium sulphate concentration in the enzyme solution during the precipitation. 40-60% saturation showed the highest protease precipitation (90%), while above 60% saturation protease activity decreased. When 1-propanol was used as a precipitant, protease activity of the aggregates decreased. The assay was performed in duplicates and the data is represented with standard error.

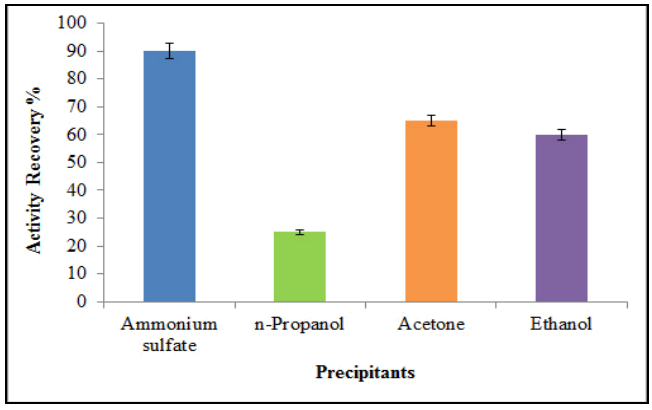

Fig. 1: Effect of different precipitant on the activity recovery of CLEAs protease

The preparation of CLEAs depends on various factors such as precipitant, glutaraldehyde concentration, cross-linking time etc. The preparation of CLEAs is carried out by first precipitating the enzyme by the addition of salts, organic solvents or non-ionic/ionic polymers to aqueous solutions of proteins, followed by cross-linking with a bifunctional reagent [14].

In the present study, 40-60% ammonium sulphate saturation showed the highest protease precipitation (90%), while above 60% saturation protease activity decreased. Similar results have also been observed in the precipitation of esterase from *Bacillus subtilis* 0554 (BSE) [15].

Most of the water miscible organic solvents screened do not precipitate the protease enzyme as large amount of enzyme was left in the solution indicating that ammonium sulphate was the most preferable precipitating agent. The denaturation of enzymes was observed during the precipitation by organic solvent, which might be due to hydrophobic interaction between the solvent and the nonpolar groups of the enzyme [16].

3.2. Effect of Glutaraldehyde Concentration on CLEAs Preparation

It was observed that the activity recovery increased with an increase of glutaraldehyde concentration. Below 60 mM, the enzyme recovery was lower due to insufficient crosslinking, with little insoluble aggregates resulting in unstable CLEAs and release of free enzyme to the reaction. As shown in figure 2, the enzyme retained its complete activity (99.1±3%) in 70 mM glutaraldehyde. As the glutaraldehyde concentration increased above 70 mM, the protease activity recovery decreased to 30%.

The most commonly used cross-linker is glutaraldehyde as it is economical and easily obtainable in large quantities, low toxicity in the relevant reactions, commercial availability and ease of manipulation in addition to its high reactivity compared with other reported cross linkers. It also reacts rapidly with amine groups at neutral pH and is more efficient than other aldehydes in generating highly stable intra and intermolecular cross links [17].

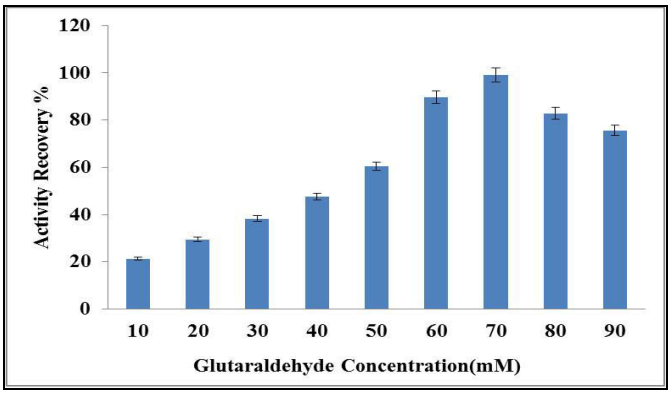

Fig. 2: Effect of varying glutaraldehyde concentration on the activity of CLEAs

With increasing concentration of glutaraldehyde, the protease activity decreases which may be because of excessive cross-linking, resulting in a loss of the flexibility of enzyme, limits the mass transfer leading to the reduced enzymatic activity [18].

3.3. Effect of Cross-linking Time for the CLEAs Preparation

The optimal cross-linking time was determined to obtain a firm and stable CLEAs, after subjecting the enzyme to cross-linking with 70 mM of glutaraldehyde for various time intervals between 1 and 8 h at 37°C as shown in Figure 3. When the time of cross-linking increased, it resulted in significant increase in the enzyme activity with a maximum recovery of 98% at 6 h and no enzyme activity was detected in the supernatant. Further crosslinking, the enzymatic activity was reduced to 79% in 7 h and up to 60% in 8 h.

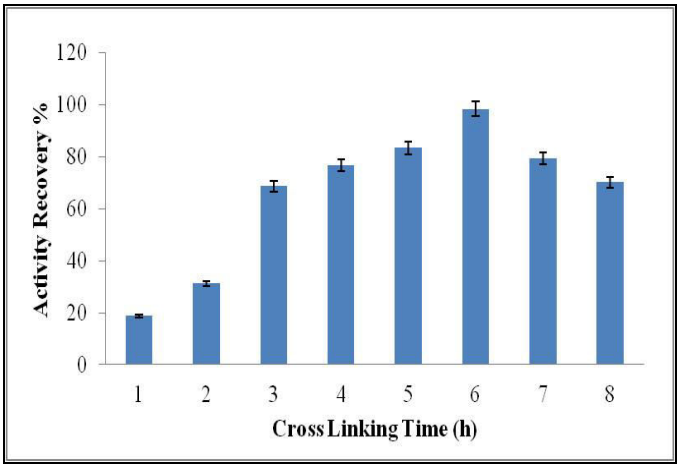

Fig. 3: Effect of cross-linking time on the relative activity of CLEAs

Cross-linking time for the maximum recovery of the enzymes in CLEAs is an important factor in the preparation of stable CLEAs.

A shorter cross-linking time results in inadequate cross-linking, leading to poor activity recovery and less stable CLEAs. However, if the cross-linking time is prolonged it restricts the enzyme flexibility and hinders the enzyme activity due to intensive cross-linking, therefore decreases the activity recovery of protease CLEAs [19]. The enzyme involved in this study was able to retain the maximum amount of protein with short period of incubation with the cross-linker.

3.4. Characterization

3.4.1. Structural Characterization by Scanning Electron Microscopy

The scanning electron microscope images of CLEAs prepared with the use of 60% ammonium sulfate saturation and 70mM glutaraldehyde shown in Figure 4. SEM of the CLEAs before first cycle shows that it has a coarse grained appearance, more structured than type 2 but do not have the ball-like appearance of type 1 either [20].

Based on morphology, CLEAs are classified as type 1 and type 2 aggregates. In type 1 aggregates, CLEAs appear as fine-grained structures with many cavities whereas in type 2 aggregates, they are course-grained structures with fewer cavities. Therefore the particle size and morphology of CLEA must be controlled [8]. In this study, the CLEAs have a coarse-grained appearance, more structured than type 2 but do not have the ball-like appearance of type 1 either. Similar structure of CLEAs in the presence of bovine serum albumin has also been reported for lipase [19].

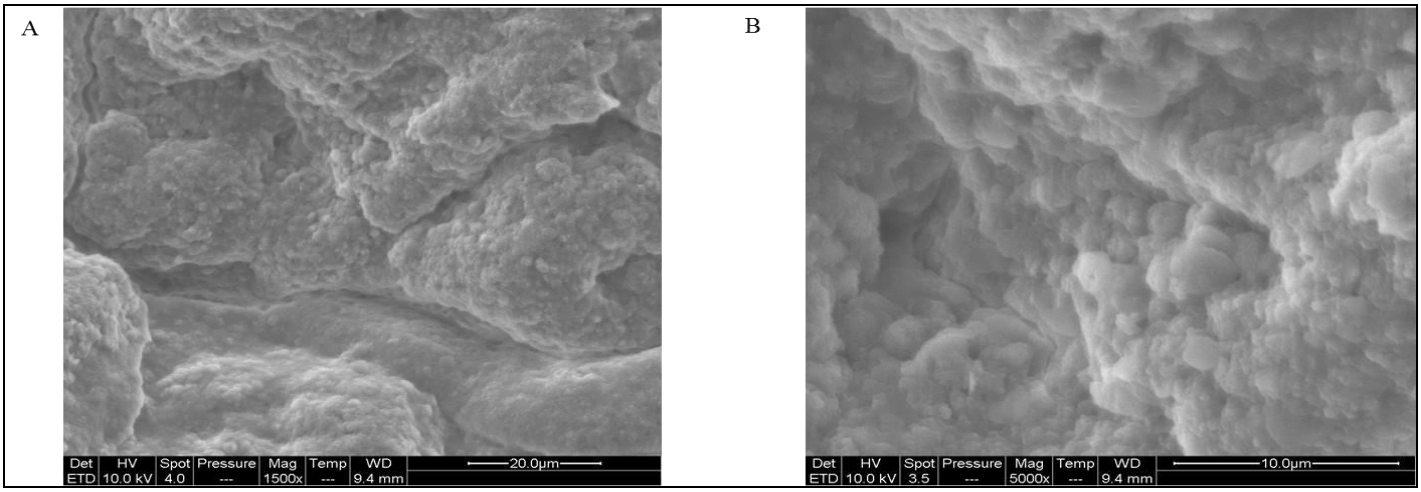

Fig. 4: Scanning electron microscope images of CLEAs prepared with 60% ammonium sulphate saturation and 70mM glutaraldehyde at 1500x and 5000x magnification

3.4.2. Effect of pH on the Activity of Protease CLEAs

The effect of pH on the activity of CLEAs showed that the optimum pH of the CLEAs was pH 9.0 after cross-linking as shown in Figure 5. The pH stability test showed that the CLEAs were highly stable over a broad pH range, showing 100% of their original activities between pH 6.0-10.0 for 5 h when compared with the free enzymes [21].

The CLEAs was active in the pH range of 5.0–10.0, with the optimum pH at 9.0 after cross-linking. It retained more than 80% activity at pH 6.0 while at pH 7.0 and 8.0 more than 90% of the relative activity respectively was retained.

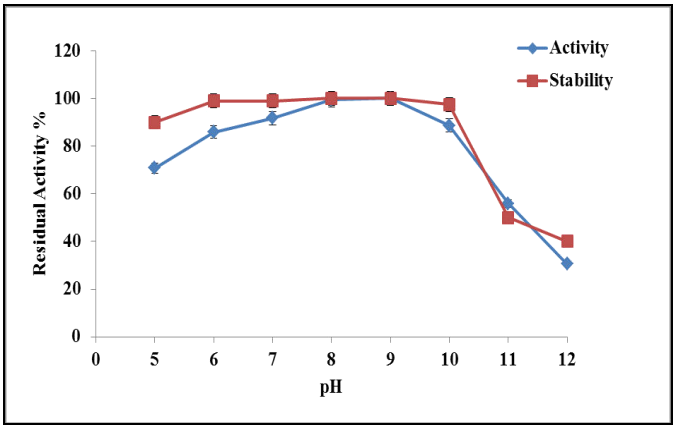

Fig. 5: Effect of pH on the activity of CLEAs

At pH 10.0, it retained about 88 % of the activity while at pH 11.0; there was 50% loss of the activity. The reaction of cross-linker with the enzyme would have linked all the available amino groups on the surface of the enzyme, and hence the acidic groups on the enzyme surface lead to negative charge to the enzyme, ultimately shifting the optimum pH to the higher values. The result corroborated with CLEAs of recombinant poly-3-hydroxybutyrate depolymerase from *Streptomyces exfoliatus* [22]. The CLEAs were highly stable over a broad pH range between pH 6.0-10.0 retaining 100% of the residual activity after 5 h of incubation with the respective buffer. It retained more than 45% of the residual activity at pH 11.0 after 5 h of incubation. Similar results were also seen in the cross-linked tyrosinase aggregates in aqueous and non-aqueous media, which at extreme pH the CLEAs maintained higher stability [23].

3.4.3. Effect of Temperature on the Activity of CLEAs

The optimum temperature of CLEAs was 60˚C (Figure 6 (a)) retaining almost 100% of the relative activity when compared with free enzyme [21]. Further increase in the temperature led to reduction in the relative activity to 60% at 70˚C and up to 55% at 80˚C.

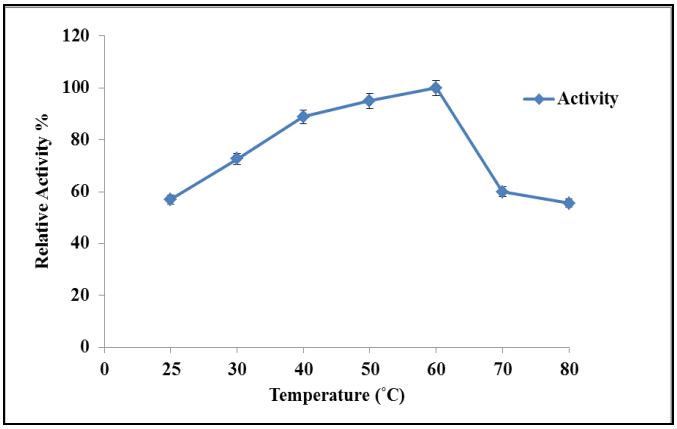

Fig. 6 (a): Effect of temperature on the CLEAs protease

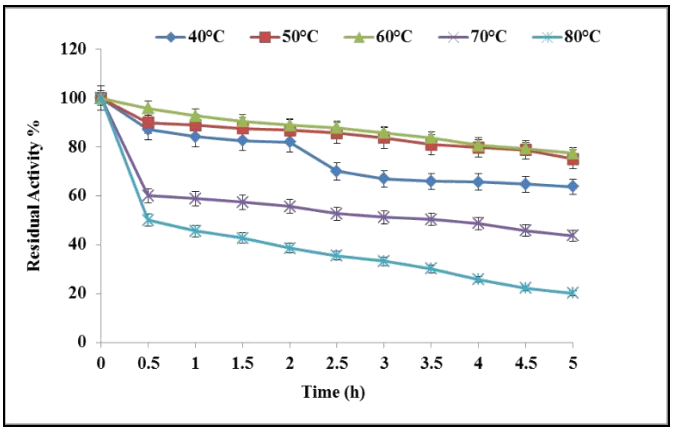

Fig. 6 (b): Temperature stability for the CLEAs protease

The CLEAs were thermostable up to 70˚C and above this temperature; the enzyme activity started decreasing as the incubation time increases as shown in Figure 6(b). The CLEAs was more than 90% stable at 50˚C and 60˚C even after 2 h incubation with slight reduction in the stability with further incubation till 5 h. At 80˚C, the CLEAs retained about 50% of the residual activity after 1 h of incubation, but loss of enzyme activity occurred when the time of incubation were increased at the same temperature. Results showed that thermal stability of CLEAs was higher than the native enzyme [21].

For any industrial application, the thermal stability of immobilized preparation is of great importance. The optimum temperature for the CLEAs was 60˚C retaining almost 100% of the relative activity when compared with free enzyme [21] when examined at different

temperatures 40°C-80°C at different time intervals. Similar result was observed in cross-linked Transglutaminase (TGase) [24] which showed the optimum temperature for reaction shifted to higher temperatures.

The CLEAs in this study was found to be thermostable, retaining more than 90% stable at 50°C and 60°C after 2 h incubation with a slight reduction in the stability with further incubation till 5 h. After 5 h of incubation, CLEAs retained about 45% residual activity at 70°C while at 80°C it 50% residual activity was retained which decreases with further increase in incubation time. Similar studies were reported on improved thermostability, at 65°C, after 30 min of incubation, cross-linked tyrosinases [25]. The increased thermal stability may be due to additional ionic and hydrophobic contacts between the enzyme molecules and also the ordered arrangement of the molecules by inter and intramolecular cross-links within and between the aggregates, and gives rigidity to the three-dimensional arrangement of the molecules in the CLEAs [26].

3.4.4. Determination of kinetic parameters

Kinetic analysis showed that both the free enzymes and the CLEA form followed the Michaelis-Menton kinetic behavior. CLEA was found to have equal Vmax and Km values which show that the rate of azocasein hydrolysis is same as that of the free enzymes as shown in table 1. CLEAs and free enzymes have a Vmax value of 127.2 and 122.4 U/mg while Km values of 0.74 and 0.70 mg/ml respectively. Similar result was also observed in the case of CLEAs of *Bacillus amyloliquefaciens* where the Km and Vmax values of both free enzymes and CLEAs are similar [27].

Table 1: Kinetic parameters of free enzymes and CLEAs

Enzyme	Km(mg/ml)	Vmax(U/mg)
Free Enzyme	0.74	122.4
CLEAs	0.70	127.2

3.4.5. Effect of Organic Solvent on CLEAs

The stability of CLEAs in different organic solvents was studied and is shown in Table 2. The stability of the CLEAs in the presence of organic solvents was significantly improved as compared with the free enzymes [21]. CLEAs retained higher activity in non-polar solvents like n-hexane, n-heptane, n-octane, n-nonane and n-decane of 87%, 83%, 86%, 90% and 90% respectively after 24 h of incubation at 30°C. The immobilized enzymes showed low activity in polar solvents like DMSO, methanol, ethanol and 1-propanol. There are various advantages of using enzymes in organic solvents or aqueous solutions containing organic solvents compared with in water such as elimination of microbial contamination in the reaction mixtures or increase solubility of the nonpolar substrates.

Table 2: Effect of different organic solvent on the CLEAs protease

Organic solvent (30%)	Relative Activity (%) Incubation for 24 hours
Control	-
DMSO	18.8 ± 0.5
Methanol	16.5 ± 0.2
Ethanol	12.3 ±0.3
n-propanol	9.5 ± 0.2
Acetronitrile	15.5 ±0.3
Toluene	72.0 ± 0.2
Dichloromethane	80.7 ± 0.3
n-hexane	88.6 ±0.1
n-heptane	82.7 ± 0.5
n-octane	85.7 ± 0.3
n-nonane	90.2 ± 0.5
n-decane	90.2 ± 0.2

The presence of organic solvents may distort enzyme molecules or they may become competitive inhibitors with enzymes, leading to changes in the reaction kinetics and substrate specificity [28]. The stability of CLEAs in different organic solvents was studied and the presence of organic solvents has significantly improved when compared with free enzymes. CLEAs retained higher activity in non-polar solvents after 24 h of incubation at 30°C while in polar solvents it showed lower activity.

The decrease in enzyme activity in these polar solvents is due to the stripping of water from the surface of the enzyme by competing through hydrogen bonds between the atoms in the protein. Cross-linking increased the rigidity of the enzyme molecules and therefore reduced the unfolding of the three-dimensional structure of the protein.

3.4.6. Reusability and storage of the CLEAs

The reusability of the CLEAs was tested upto 10 cycles as shown in figure 7. The activity of the CLEAs decreased as the number of cycle increased. The results show that about 42% of the activity was retained after the 10 cycles

of use. It retained its activity upto 90% till the 6th cycle, which reduced to 75% in the 7th cycle. As the CLEAs is reused further the enzyme's activity was reduced leading to substantial change in morphology as shown in Figure 8.

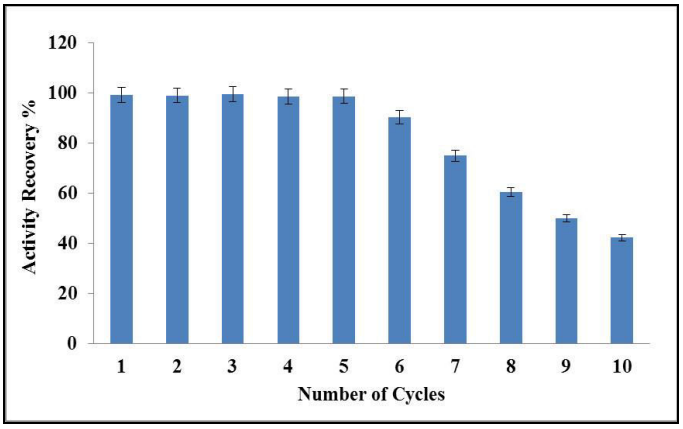

Fig. 7: Reusability of the protease CLEAs

The storage stability of the CLEAs protease and the free enzyme was determined at 4°C for 90 days in buffer as shown in Figure 9. The activity of the CLEAs just after preparation was taken as the initial 100% activity. More than 50% of the original activity disappeared completely for the free enzyme after 10 days when stored in the buffer solution. The CLEAs proteases were fully active while complete loss of activity was observed in case of free enzymes after 60 days of incubation [20]. CLEAs protease retained 60% of its residual activity even after 90 days of storage.

CLEAs has offer a unique properties for recycling and reuse which is another important reason to immobilize costly enzymes which facilitates their recovery from the reaction medium, resulting in the simplification of downstream processing [29]. The CLEAs in this study was reused for 10 cycles retaining 90% activity till 6th cycle.

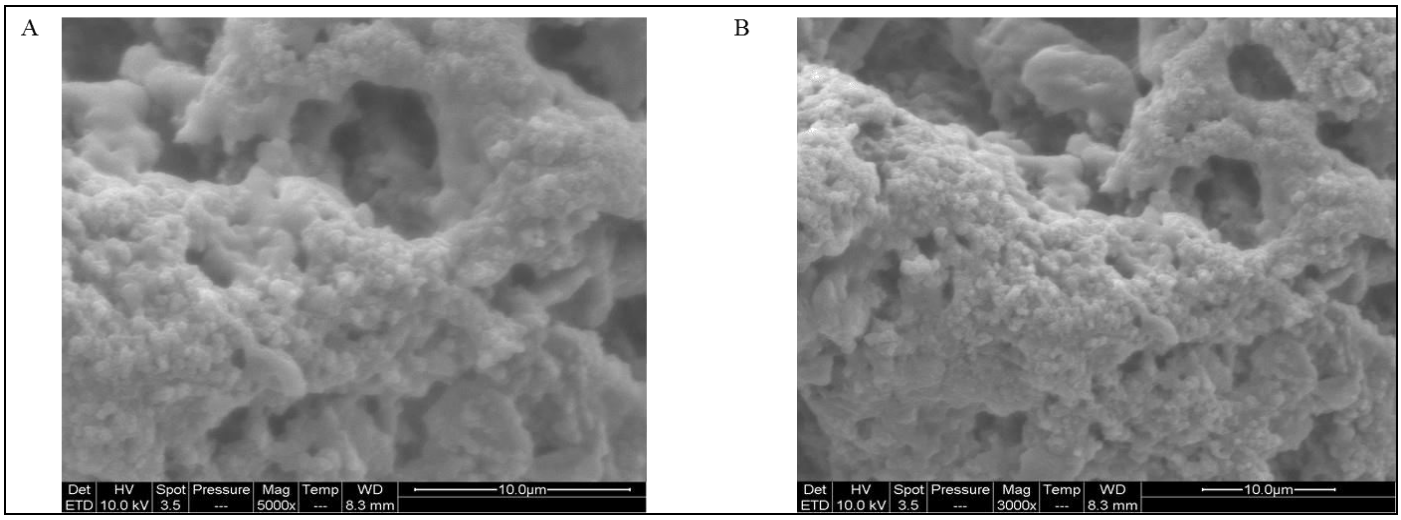

Fig. 8: Scanning electron microscope images of used CLEAs at 3000x and 5000x magnification

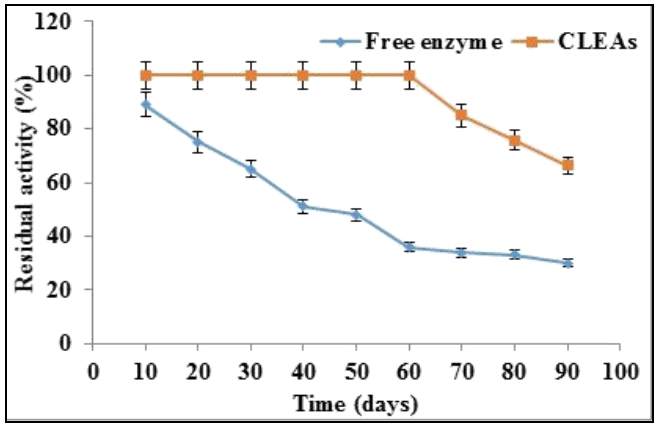

Fig. 9: Storage stability of the free and CLEAs enzyme for 90 days

As the immobilized enzymes is reused further the enzyme's activity was reduced with 15% and 50% loss of activity in 7th and 10th cycle respectively. The loss in activity might be due to inactivation of CLEAs upon reuse rather than leaching out of enzyme activity causing conformational changes which distort the active site [30]. Storage stability study showed that the CLEAs were able to retained 100% of the initial activity even after 60 days of incubation when compared with the free enzymes which loses 50% of the activity after 10 days of the incubation. This clearly indicates that the immobilization of enzymes via glutaraldehyde coupling on the supports resulted in a significant storage stability compared to the free counterpart. CLEAs of laccases

from *T. villosa* and *T. versicolor* showed similar result [31].

3.4.7. Chicken feather degradation by CLEAs protease

The CLEAs was studied to determine its ability to degrade the native chicken feather. The feathers were incubated at 50°C and was observed periodically for 48 h. Complete degradation of the feather was observed only after 35 h as shown in Figure 10 while free enzymes shows complete degradation at 24 h [21]. The feather degradation study using the CLEAs showed that the complete degradation of the feather was observed at 35 h when compared with free enzymes where the complete degradation was at 24 h. This may be due to less availability of the substrate to the immobilized enzyme. The control feather incubated without the CLEAs showed no degradation even at 48 hours of incubation.

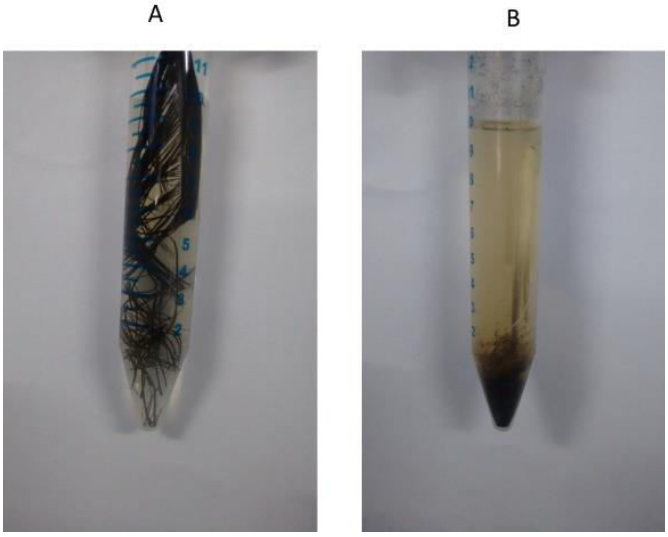

Fig. 10: Keratinolytic Activity of the CLEAs protease A) Feather incubated in buffer without enzyme B) Feather degraded by CLEAs after 35 h

4. CONCLUSION

In this paper, the preparation conditions of cross-linked enzyme aggregates (CLEAs) of protease were optimized and studied. SEM analysis shows that CLEAs has a coarse-grained appearance. They exhibited better stability and dispersibility in alkaline pH, higher temperature, and organic solvents. Such improved operational performance of the immobilized protease gives various industries a very promising biocatalyst.

5. ACKNOWLEDGEMENT

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