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Preparation and characterization of cross linked enzyme aggregates (CLEAs) of *Bacillus amyloliquefaciens* alpha amylase

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Abstract

Stabilization of enzymes is one of the major challenges in biocatalytic processes. Alpha amylase from *Bacillus amyloliquefaciens* was immobilized as cross-linked enzyme aggregates (CLEAs). Alpha amylase was aggregated using ammonium sulfate. The resultant aggregates on cross-linking with glutaraldehyde produced insoluble catalytically active cross-linked enzyme aggregates. The effects of precipitation and cross-linking were studied and immobilized alpha amylase was characterized. Seventy percent ammonium sulfate saturation, 2% (v/v) glutaraldehyde, were used; 6 h cross-linking reaction at room temperature was performed and 100% activity recovery was achieved in CLEAs with enhanced thermal and acidic condition stabilities. The cross-linked enzyme aggregates exhibited pH optima of 6.0 and higher temperature optima of 60°C. Although after immobilization maximum velocity of enzyme reaction did not change, substrate affinity of the enzyme increased. Alpha amylase CLEAs retained 65% activity after 4 reuses with 30 min of each reaction time. The Scanning electron microscopy analysis showed that morphology of CLEAs substantially changed after 4 reuses.

Keywords: Enzyme immobilization, cross linked enzyme aggregates, carrier free, alpha amylase, ammonium sulfate, glutaraldehyde.

Introduction

α-amylase (EC 3.2.1.1) is of great importance in present day biotechnology with applications ranging from food, baking, brewing, fermentation, detergent applications, textile desizing, paper industries, etc. (Alva et al. 2007; Pandya et al. 2005). The industrial application of enzymes is often hampered by a lack of availability, high price and limited stability under operational conditions. These drawbacks can be overcome by immobilization of the enzyme thereby rendering it more stable and easy to recover and recycle [Sheldon et al. 2005]. Recently several efforts have been taken to immobilize α-amylase by binding it to solid carriers [Turunc et al. 2009; Reshmi et al. 2007; Egwim and Oloyede 2011; Dincbas and Demirkan 2010]. However, immobilization of enzymes with solid carriers results in diluted enzyme activities, decreasing space–time yields and lowering catalyst productivity. Recently solid carrier-free enzyme immobilization methods are gaining importance due to clear advantages: highly concentrated enzyme activity in the catalyst, high stability and the low production cost due to the elimination of an additional solid carrier [Fernandes et al. 2005].Among these methods, cross-linked enzymes aggregate (CLEA) is a simpler one in which soluble enzyme is aggregated by agents such as inorganic salts or organic solvents without undergoing denaturation and subsequently cross linking of formed aggregates using bifunctional agent such as suitable dialdehyde, usually glutaraldehyde [Cao et al. 2000]. Immobilization via cross-linking of enzyme molecules with bifunctional cross-linking agents does not suffer from disadvantages such as diluted enzyme activities and lower catalyst productivity. Since the molecular weight of the cross-linking agent is negligible compared with that of the enzyme, the resulting biocatalyst essentially comprises 100 wt% protein [Schoevaart et al. 2004]. As CLEAs are heterogeneous catalysts, they can be easily recovered from reaction mixture by centrifugation [Park et al. 2010].This method does not require highly purified enzyme [Mateo et al. 2004]. So far, CLEAs of various enzymes such as acetyl xylan esterase, lipases, laccases, invertase, subtilisin, horseradish peroxidise, etc. have been prepared [Montoro-García et al. 2010; Kartal et al. 2011; Sheldon et al. 2010; Talekar et al. 2010; Sangeetha and Abraham 2008; Šulek et al. 2011].However, no study has been reported on CLEAs of *Bacillus amyloliquefaciens* alpha amylase in the literature yet. Thus in the present work, alpha amylase CLEAs were prepared using alpha amylase produced by *B. amyloliquefaciens* considering the industrial importance of the enzyme. Conditions of CLEA preparation were optimized. The prepared CLEAs were characterized in terms of optimum temperature and pH, kinetic parameters and compared with those of soluble alpha amylase.

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Materials and Methods

Chemicals

Starch and glutaraldehyde (25%) were obtained from Sigma (St. Louis, USA). Ammonium sulfate and DNSA (3, 5-Dinitrosalycyclic acid) were purchased from Merck (Darmstadt, Germany). Other reagents used were of analytical grade and obtained either from Sigma or Merck.

Alpha amylase production

The *Bacillus amyloliquefaciens* NCIM 2829 was grown in 250 ml shake flask containing 100 ml medium : Starch, 1(g/l); Peptone, 6(g/l); $MgSO_47H_2O$, 0.5(g/l); KCl, 0.5 (g/l); pH 6.5.After fermentation of 30 h biomass was removed by centrifugation and remaining supernatant was used as crude soluble alpha amylase.

Preparation of CLEA

Saturated ammonium sulfate solution was added up to the final concentration of 70% saturation in 2 ml crude enzyme solution to precipitate the enzyme. After 30 min, glutaraldehyde was added slowly to the final concentration of 2 %(v/v) to cross link enzyme precipitate. The mixture was kept at 20°C for 6h cross linking reaction with continuous stirring at 150 rpm. Subsequently, the mixture was centrifuged at 13,000g for 15 min at 4°C. The recovered pellet was washed three times with 0.1M sodium phosphate buffer, pH 7 to remove unreacted glutaraldehyde and suspended in buffer. Activity recovery in CLEAs was calculated as given in following equation.

Activity recovery (%) = (Total activity of CLEA in units/Total crude enzyme activity used for CLEA Preparation in units) × 100.

Assay of alpha amylase

Alpha amylase activity was determined using starch (1%, w/v) as a substrate [Dekker 1977]. The amount of reducing sugar (estimated as maltose) released was measured using 3, 5-dinitrosalicylic acid [Miller 1959]. One unit (U) of enzyme activity is defined as the amount of enzyme required to release 1µmol of reducing sugar per minute at 37°C.

Optimization of CLEA Preparation

Different ammonium sulfate saturations (10-70%), glutaraldehyde concentrations (0.5-2% v/v) and time of cross linking reaction (2-6 h) were used during CLEA preparation from crude alpha amylase solution. The optimum values of these parameters were determined to achieve 100% activity recovery of alpha amylase in CLEA form.

Effect of pH and temperature on alpha amylase activity

The pH optimum of soluble alpha amylase and CLEA was studied in the pH range of 3-9 using 0.1M buffers (pH 3-5, sodium citrate buffer; pH 6-8, sodium phosphate buffer; pH 9, NaOH/glycine buffer). The effect of temperature on enzyme activity of soluble alpha amylase and CLEA were determined in the temperature range of 30-90°C.

Kinetic Analysis

Kinetic parameters of soluble alpha amylase and CLEA were estimated by measuring initial reaction rates using different starch concentrations in the range of 0.2 - 3 mg/ml at predetermined optimum pH and temperature of each form. K_m, V_{max} values of soluble alpha amylase and CLEA were calculated from nonlinear regression fitting of the initial reaction rates corresponding to different starch concentrations with Graph Pad Prism software.

Reusability of alpha amylase CLEA

To investigate the effect of immobilization on reusability of alpha amylase CLEA, the CLEA after each cycle of 30 min reaction time at 60°C was washed with buffer and then suspended again in a fresh reaction mixture to measure enzyme activity. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%.

Results and Discussion

Optimization of CLEA preparation

During CLEA preparation alpha amylase from crude solution was precipitated using saturated solution of ammonium sulfate. As the saturation of ammonium sulfate was increased, activity recovery in CLEA also increased and 100% activity recovery was obtained at 70 % ammonium sulfate saturation (Fig.1). Precipitation decreases the surface area that is in contact with solvent and hence increases the stability of proteins. Furthermore, precipitation conditions may result in more active conformation of proteins [Lo´pez-Serrano et al. 2002]. It was reported that CLEAs of tyrosinase were coarse grained and less structured at low ammonium sulfate concentration, whereas at high ammonium sulfate concentration, they were fine grained and more structured with many cavities, which permits better mass transfer [Aytar and Bakir, 2008].The orderly arranged proteins are cross linked by the addition of glutaraldehyde as a cross linking agent and their conformation is stabilized [Cao et al. 2000]. Therefore, CLEAs of alpha amylase retained all of the free enzyme activity at higher ammonium sulfate concentration because of above mentioned factors.

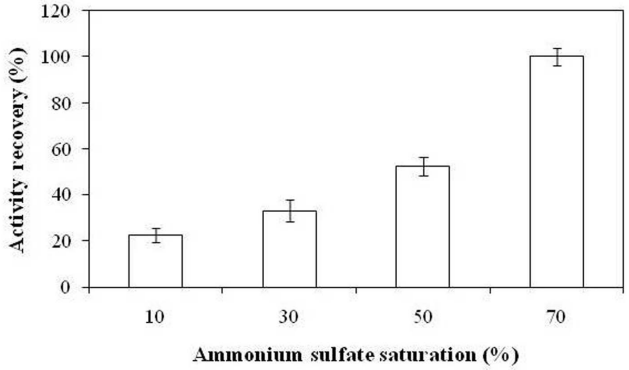

Figure 1. Effect of ammonium sulfate concentration on activity recovery in CLEA.

The concentration of the cross linking agent also had a significant effect on the activity of CLEAs [Aytar and Bakir 2008]. If too little cross-linker is used the enzyme molecule may still be too flexible while too much cross-linker can result in a loss of the minimum flexibility needed for the activity of enzyme [Sheldon et al. 2010].So the concentration of cross linking agent must be optimized to recover 100% activity in CLEA. Concentration of glutaraldehyde was optimized using 70 % ammonium sulfate saturation during precipitation. As shown in Figure 2 Activity recovery in CLEAs was increased with increase in glutaraldehyde concentration. At 2% (v/v) glutaraldehyde, all of the free enzyme activity was recovered in CLEAs.

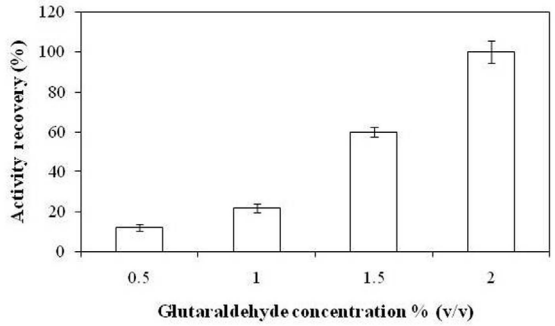

Figure 2. Effect of glutaraldehyde concentration on activity recovery in CLEA.

Since cross linking is reaction, time required to recover 100% activity in CLEA during cross linking is important. The initial cross-linking experiments were performed for 12 h. The time required to recover 100% activity in CLEA can involve a compromise between efficient cross-linking and enzyme stability during the procedure [Sheldon et al. 2010]. Therefore, the cross linking was performed with optimized precipitant and cross linker concentrations as shown in above experiments. Figure 3 Shows the effect of cross linking time on activity recovery in CLEA.As the time of cross linking reaction was increased activity recovery was increased. After 6 h cross linking reaction, 100% activity recovery was obtained.

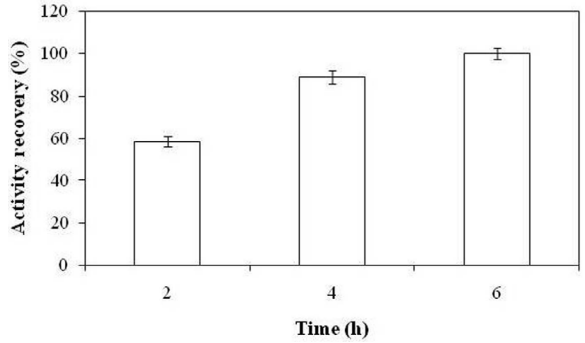

Figure 3. Effect of cross linking time on activity recovery in CLEA.

Effect of pH and temperature on alpha amylase activity

The pH is one of the major parameters capable of shifting enzyme activities in reaction mixture. Immobilization usually results in shift of optimum pH due to conformational changes in enzymes. The effect of pH on activity of both soluble alpha amylase and CLEA is given in Figure 4. Optimum pH values were 7 and 6 for soluble alpha amylase and CLEA respectively. This shift in optimum pH could be resulted from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site, which was caused by the newly formed interactions between basic residues of enzyme and glutaraldehyde during cross linking [Aytar and Bakir 2008].

The activity of enzyme is also strongly dependent on temperature. The soluble alpha amylase activity increased with temperature and maximum activity was observed at 45°C (Fig.5). The optimum temperature of soluble alpha amylase was shifted to 60°C after CLEA formation. As was evident from the data, CLEA possessed temperature resistance than the soluble enzyme. This shift in optimum temperature can be due to covalent bond formation between proteins caused by glutaraldehyde during CLEA preparation which might decrease the conformational flexibility of

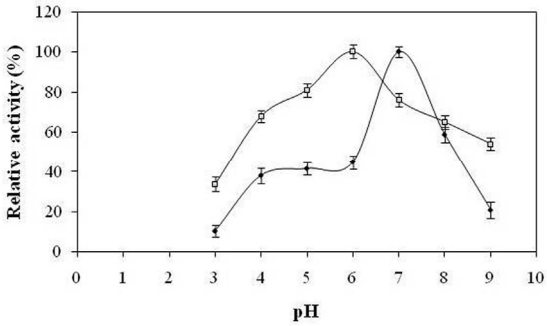

Figure 4. Effect of pH on activity of soluble alpha amylase (♦) and alpha amylase CLEA (□).

enzyme and protects it from distortion or damage by heat exchange [Aytar and Bakir 2008; Sangeetha and Abraham 2008].

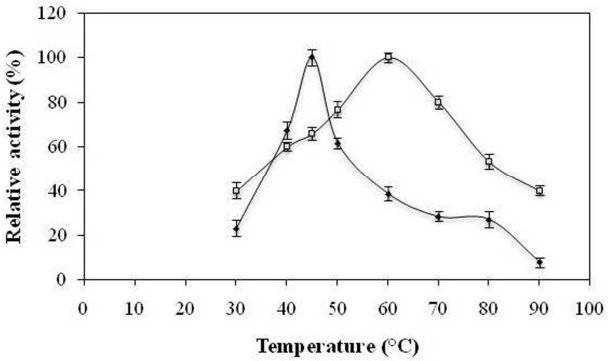

Figure 5. Effect of temperature on activity of soluble alpha amylase (♦) and alpha amylase CLEA (□).

Table 1: Kinetic parameters of soluble alpha amylase and CLEA.

Enzyme	V_{max} (µmole/min)	K_m (mg/ml)
Soluble alpha amylase	0.174 ± 0.011	2.748 ± 0.027
CLEA	0.179 ± 0.023	0.3245 ± 0.013

Kinetic analysis

Kinetic parameters of soluble and CLEAs of alpha amylase were determined by measuring initial reaction rates for each form with varying amounts of starch. For both soluble and CLEA forms Michaelis-Menten kinetic behavior was observed. As shown in Table 1, V_{max} values of both forms are equal, which indicated the rate of starch hydrolysis was not changed after CLEA preparation.

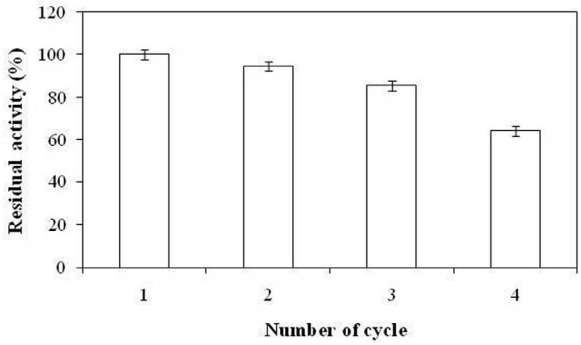

Figure 6. Reusability of CLEA.

The K_m value shows the affinity of enzyme for its substrate. Lower the K_m value more is the affinity of enzyme for its substrate. In case of CLEA, lower value of K_m was observed. It suggests that

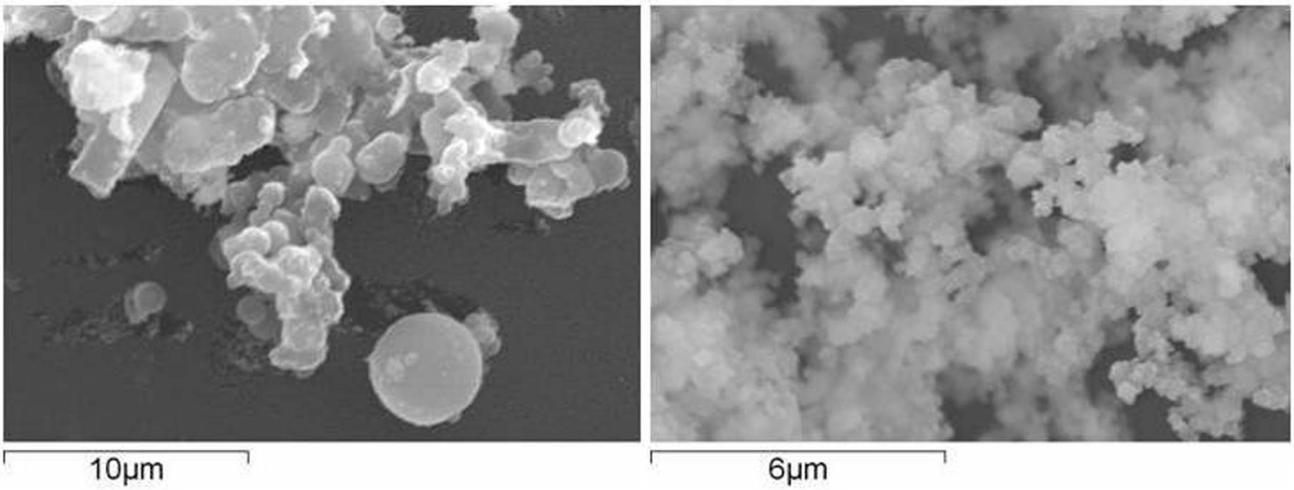

Figure 7. Scanning electron microscope images of CLEA (A) Alpha amylase CLEA and (B) Alpha amylase CLEA after four cycles of use. (Magnification 10000 X)

conformational changes due to immobilization help the enzyme to suitably orient its active site towards the substrate [Aytar and Bakir 2008; Sangeetha and Abraham 2008].

Reusability of alpha amylase CLEA

The main advantage of cross linked enzyme aggregates is that it leads to a reusable enzyme preparation. Reusability of enzyme is a key factor for its cost-effective industrial use. CLEAs can be reused either by filtration or centrifugation. The reusability of CLEAs was studied up to 4 cycles. After each cycle, CLEA was separated from the reaction mixture by centrifugation, washed and used again in the next cycle. As shown in Figure 6, the activity of CLEA decreased to approximately 65% of its original activity over four cycles. Comparison of scanning electron microscopic (SEM) pictures of the CLEA before and after four times use in batch reaction shows that the decrease in activity is accompanied by a substantial change in morphology (Fig.7).

Conclusion

CLEAs of alpha amylase from *B. amyloliquefaciens* were prepared with complete soluble enzyme activity recovery. It was demonstrated that the factors such as concentrations of ammonium sulfate, glutaraldehyde and cross-linking time had significant effects on activity recovery in CLEA. Activity recovery was increased with increasing these factors. Temperature and pH optima of alpha amylase in CLEA form were shifted; this could increase the applicability of the enzyme. Moreover, after immobilization, substrate affinity of alpha amylase was significantly increased. The results of reusability experiment demonstrated that the CLEAs retained 65 % of activity after four cycles of use. Hence, we conclude that alpha amylase CLEAs clearly have practical utility in the hydrolysis of starch.

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