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IMMOBILIZATION OF HYPERTHERMOSTABLE β AMYLASE FROM *Bacillus subtilis* DJ5 INTO GELATIN FILM BY GLUTARALDEHYDE CROSSLINKING

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ABSTRACT

β amylase isolated from *Bacillus subtilis* DJ5 were immobilized covalently in 5% and 10% gelatin matrix using glutaraldehyde as crosslinking agent. Initial screening proved enzyme immobilized in 10% gelatin with 0.8% glutaraldehyde gave higher catalytic activity (3.33 U/ml) and immobilization efficiency (88%) after 18 hour of crosslinkage at 4 °C. Moreover this matrix can be used repeatedly seven times retaining 41% enzymatic activity (1.37 U/ml) at 7th cycle. Immobilized matrix showed greater stability in presence of detergents and inhibitors compared with free enzyme system. Enzymatic activity was not compromised at any level of immobilization as both immobilized and free enzyme required same concentration of substrate (5mg/ml). But higher catalytic activity of immobilized enzyme (2.95 U/ml) than free enzyme (2.47 U/ml) indicated immobilized matrix allowed proper orientation of enzyme substrate in reaction microenvironment. Suitability of immobilized β amylase will make it more acceptable from industrial point of view.

KEYWORDS

Gelatin, glutaraldehyde, crosslinking, β amylase, immobilization

INTRODUCTION

Faithful industrial process depends on process simplicity that ultimately saves energy, labor thereby reducing overhead production cost. In that respect enzyme immobilization technology has gained tremendous popularity and has taken leading edge over free enzyme catalysis process. Several studies indicated that immobilized enzyme has shown increased catalytic activity, increased stability of enzyme[1], easy recovery of enzyme, easy separation from product minimizing or eliminating protein contamination of product, repeated or continuous use of a single batch of enzyme[2]. To a large extent this procedure prevents enzyme losses due to washout and at the same time maintains biocatalyst at high concentrations[3] and also protects enzyme from microbial contamination.

Several methods of enzyme immobilization technique used[4], covalent binding in gelatin by chemical organic cross linkers like glutaraldehyde or formaldehyde has been very effective in retaining enzymatic activity since availability and accessibility of substrate binding site of enzyme is not compromised[5]. The immobilized enzyme can contact easily with substrate and do not detach from inert support matrix maximizing all benefits of immobilization[6].

Contrary to several recent reports on alpha amylase immobilization[7-9], less attention was provided for beta amylase immobilization evidenced from fewer reports[10-13]. Exploitation of beta amylase immobilization study will make it more feasible to find greater alternatives in using this enzyme in industry.

In this present study, partially purified hyperthermostable beta amylase from *Bacillus subtilis* DJ5 has been immobilized in gelatin matrix and covalently conjugated by organic linker and effect of immobilization was tested. Optimum conditions for β amylase immobilization by this method were determined. Efficiency of enzymatic activity in both immobilized and free enzyme system was compared. Moreover stability of immobilized enzyme in different known detergents, sodium dodecyl sulfate, tween, triton X 100 and inhibitors like $HgCl_2$ and thiol group inhibitor p-chloromercuribenzoate (PCMB) were evaluated and compared with free cell system.

MATERIALS AND METHODS

(i) Microorganism:

Bacillus subtilis DJ5 (GU357825) was used in this study. This strain was isolated and subsequently mutated by physical and chemical mutagenesis in our laboratory. The strain was preserved in starch peptone medium containing (gram per liter): Peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4,7H_2O$, 0.1; NaH_2PO_4, $2H_2O$, 0.5; soluble starch (Sigma, USA), 5; agar-agar, 15; pH 6.9 and subcultured every one month.

(ii) Isolation of enzyme:

Submerged fermentation in starch peptone broth showed highest enzyme production at only six and half hours. Cell free extract obtained after centrifuging six and half hour fermented broth at 10000 rpm for 10 min was used as crude enzyme source. It was passed through a membrane (Sigma, USA) of mol cut off 100kDa and then subsequently purified partially by 20-80% chilled acetone cut. Pellet was dissolved in minimum amount of 0.1M phosphate buffer (pH 6.9) and was used as source of partially purified enzyme.

(iii) Chemicals used for immobilization:

Gelatin, glutaraldehyde, formaldehyde was obtained from Merck, Germany. Detergents (sodium dodecyl sulfate, tween, triton X 100), $HgCl_2$ and bile salt p-chloromercuribenzoate (PCMB) were obtained from HiMedia, India.

(iv) Enzyme immobilization:

Two different concentrations of gelatin powder (5 and 10% w/v) was swelled in 5 ml of 0.1 M phosphate buffer (pH 6.9) and heated at 50°C for 5 min for complete solubilization. Mixture was cooled to room temperature and enzyme (5 U/ml) was added to it and mixed thoroughly. Different concentrations (0.2-0.5% w/v) of organic cross linkers gluteraldehyde and formaldehyde were separately added in gelatin-enzyme mixture with constant stirring. It was then poured onto 90 mm petriplate and allowed to solidify to prepare thin film. It was then stored at 4°C overnight for complete crosslinking. It was then washed thoroughly with same buffer to wash any unbound enzyme and finally cut into small blocks. All subsequent experiments were carried out using the blocks.

(v) Assay of β amylolytic activity:

β amylolytic activity was measured by the method of Bernfeld (1955)[14]. Assay mixture contained 0.5 ml of 0.1M phosphate buffer (pH 6.9), 1 ml soluble starch (0.5% w/v, Sigma, USA) and 0.1 ml of enzyme. Control was prepared as same without adding substrate. The reaction mixture was incubated at 100°C for 15 min. Enzyme-substrate reaction was then stopped by addition of 1 ml 2M NaOH. Both the assay mixture and control were then allowed to boil in boiling water bath for 10 min after addition of 0.5 ml of 3, 5-dinitrosalisylic acid reagent (Merck, Germany). After cooling the assay mixture at room temperature, absorbance were measured spectrophotometrically (Elico, India) at 540 nm. Amount of maltose released (mg) was measured from standard curve of maltose. One unit (U) of β amylolytic activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute per ml from soluble starch (Sigma) under the standard assay conditions.

(vi) Process standardization:

Two different concentrations of gelatin 5% and 10% were separately mixed with 5 different concentrations of glutaraldehyde to determine optimum cross linking property that prevent enzyme leakage and also to show maximum enzymatic activity. Glutaraldehyde concentration that gave maximum enzymatic activity in different gelatin concentration was subsequently reused upto 7 cycles to determine reuse efficiency of immobilization matrix. Best matrix selected on two parameters i.e. higher production and greater reusability, was selected and tested with different concentration of substrate (1-12 mg/ml) to determine optimum substrate concentration for beta amylase activity. Other parameter i.e. optimum incubation time for maximum enzymatic activity was then determined taking maximum substrate concentration of previous test.

(vii) Effect of inhibitors and detergents:

Both bound and immobilized enzyme were mixed separately with 1% of different detergents (sodium dodecyl sulfate (SDS), tween 80, triton X 100) and were preincubated at room temperature for 30 min. Free enzyme (0.5 ml) and gelatin blocks equivalent to 0.5 ml free enzyme were mixed with 50µl Hg^{+2} (0.001M) separately and were preincubated at room temperature for 30 min. It was then assayed to determine residual activity.

(viii) Determination of immobilization efficiency:

Immobilization efficiency was determined from the difference in enzyme activity in the

solution before and after the immobilization. Efficiency was calculated as following:
Immobilization efficiency (%) = (I/ A-B) x 100 where A = added enzyme (U/g of bead); B = unbound enzyme (U/g of bead); I = immobilized enzyme (U/g of bead).

RESULTS

1. Selection of cross linker

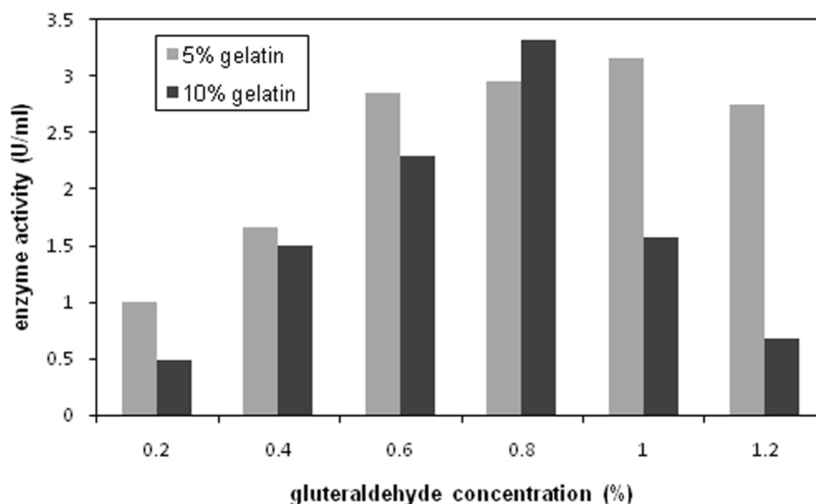
Two different organic cross linkers, glutaraldehyde and formaldehyde were separately used with different concentration of gelatin. Rapid disintegration of formaldehyde crosslinked matrix was recorded (data not shown). For further tests, only glutaraldehyde was selected as it showed higher stability and greater immobilization efficiency.

2. Optimization of parameters for immobilization

a) Effect of glutaraldehyde concentration

Variation of glutaraldehyde concentration separately with 5% and 10% gelatin showed 0.8% glutaraldehyde concentration is best for 10% gelatin immobilization (3.33 U/ml) and 1% glutaraldehyde concentration is optimum for 5% gelatin immobilization (3.16 U/ml) (Figure 1). As glutaraldehyde works as cross linking agent, lower concentration weakens gelatin matrix leading to enzyme leakage whereas increased concentration makes matrix more stronger preventing accessibility of substrate to active site of enzyme. Sharp fluctuation of enzyme activity was noted for 10% gelatin immobilization when glutaraldehyde concentrations were varied. For 5% gelatin immobilization variation of glutaraldehyde concentrations showed nearly similar pattern of enzymatic activity. From results, both 5% gelatin matrix with 1% glutaraldehyde and 10% gelatin matrix with 0.8% glutaraldehyde were chosen to determine reuse efficiency.

Figure 1
Effect of glutaraldehyde concentration on β amylase immobilization in 5% and 10% gelatin matrix.



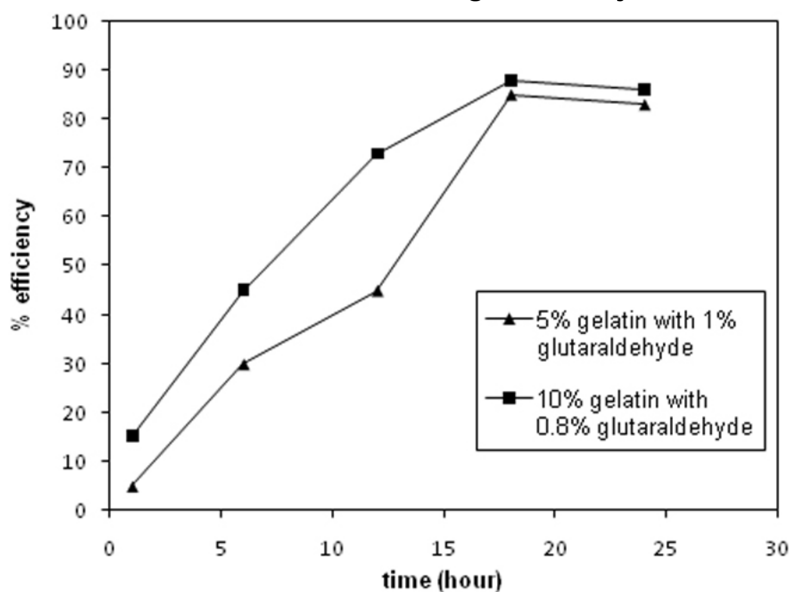
b) Effect of time on efficient immobilization

Efficient immobilization was found to be directly related with reaction time between protein and glutaraldehyde (Figure 2). Lower incubation time resulted in lesser crosslingking, eventually leading to enzyme leakage from gelatin matrix. For both 5% and 10% gelatin immobilized

matrix, similar pattern of enzyme leakage was observed but for 10% gelatin matrix, efficiency of immobilization was found little higher. In both cases, maximum efficiency was observed after 18 hour of incubation at 4 °C. Immobilization was 88% efficient for 10% gelatin matrix crosslinked with 0.8% glutaraldehyde whereas 85% efficiency was observed for 5% gelatin matrix with 1% glutaraldehyde. Prolonged incubation beyond 18 hour has shown slight inhibitory effect on enzymatic activity resulting in minor decrease in immobilization efficiency.

Figure 2

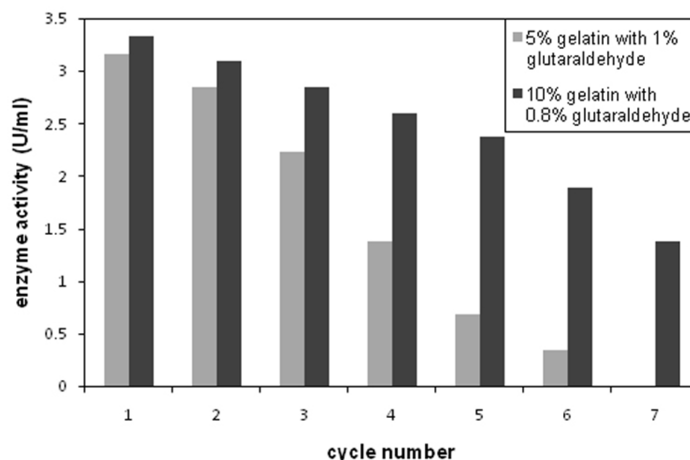
Effect of time on crosslinking efficiency at 4 °C.



c) Reuse efficiency

Two different sets of immobilization matrices were selected from previous experiment and were used repeatedly several times to determine reuse efficiency of matrices. Result indicated upto seven cycles tested, 10% gelatin immobilized enzyme showed greater reuse efficiency (Figure 3). With increase in cycle number, enzymatic activity decreases steadily indicating enzyme leakage from both matrices. The rate of disintegration of matrix was higher in 5% gelatin blocks as at 6th cycle it showed very little activity of 0.34 U/ml. At the same cycle 10% gelatin matrix still restored higher enzymatic activity (1.89 U/ml). Moreover rate of matrix disintegration was found to be very slow in case of 10% gelatin matrix. This comparative result proved that beta amylase immobilized in 10% gelatin with 0.8% glutaraldehyde is superior in both higher enzymatic activity and efficient reusability than enzyme immobilized in 5% gelatin in 1% glutaraldehyde. As enzymatic activity is not compromised due to covalent binding in 10% gelatin matrix, it was further selected for immobilization study in this work.

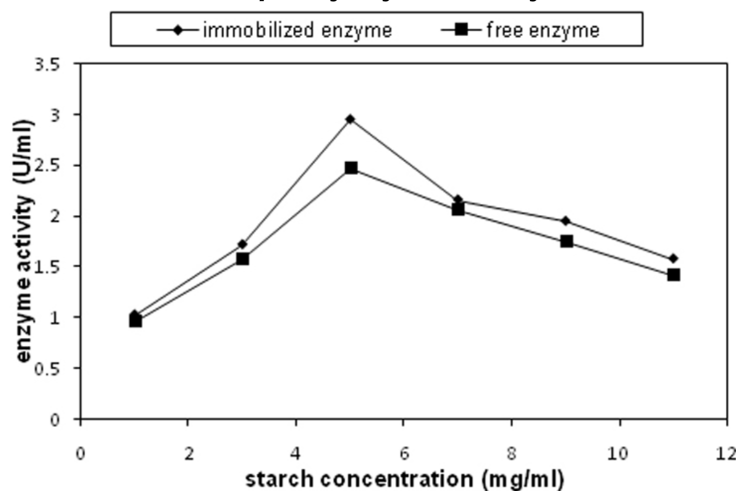
Figure 3
Reusability of immobilized β amylase from Bacillus subtilis DJ5



3. Effect of substrate concentration

Both immobilized and free enzyme was incubated for 25 min separately with different concentrations of starches to determine optimum substrate concentration for β amylase activity. Result indicated that for both free and immobilized enzyme system, 0.5% starch (5 mg/ml) was optimum for enzymatic activity (Figure 4). But immobilized enzyme has taken little advantage over free enzyme catalysis system as evidenced from higher enzymatic activity of 2.95 U/ml in respect of 2.47 U/ml for free enzyme system. With the increase of starch concentration, both systems showed decrease in enzymatic activity that indicated true substrate inhibited nature of this enzyme.

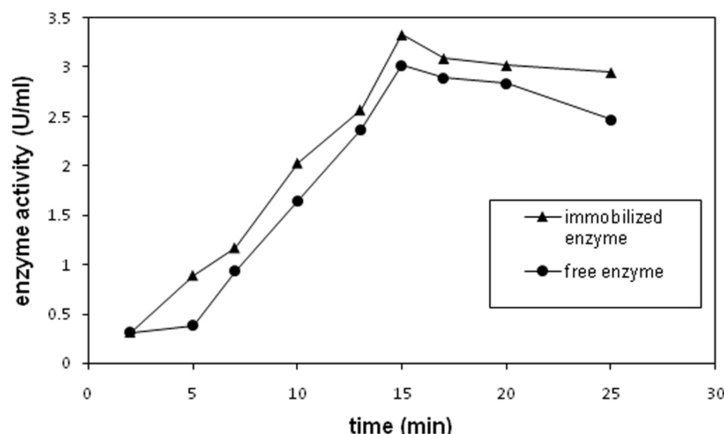
Figure 4
Effect of substrate concentration on β amylolytic activity of immobilized and free enzyme.



4. Effect of incubation time on enzymatic activity

Both immobilized and free enzyme mixed with 5mg/ml starches were incubated and tested accordingly with different time intervals (Figure 5). Maximum enzymatic activity was recorded at 15 min of incubation for both systems. At 15 min, immobilized matrix showed higher activity (3.33 U/ml) compared with free enzyme system (3.02 U/ml). Further incubation showed steady decrease in activity.

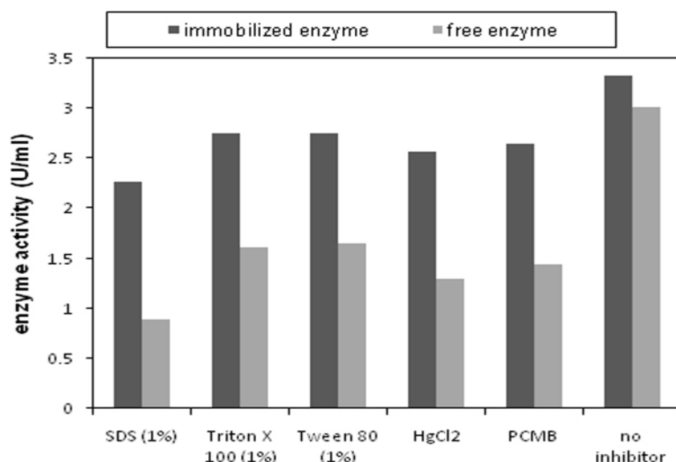
Figure 5
Determination of optimum incubation time for immobilized and free enzyme for maximum enzymatic activity.



5. Effect of detergents and inhibitor

Free enzyme is susceptible to remarkable inhibition of detergents and inhibitors (Figure 6). But interestingly gelatin immobilized enzyme resisted inhibitory effect evidenced from enzymatic activity of control. Among several detergents tested, strong cationic detergent, SDS (1%) showed highest inhibitory effect to free enzyme, reducing enzymatic activity to 29.5% (0.89U/ml). SDS probably failed to reach immobilized enzyme through crosslinking network of gelatin indicating lesser extent of inhibition i.e. 68.2% (2.27 U/ml) residual activity. 53.3% (1.61 U/ml) and 54.6% (1.65 U/ml) residual activity was observed for triton X 100 and tween 80 treated free enzyme respectively. As this enzyme is fairly stable in presence of several metal ions (data not shown), except Hg^{2+}, inhibitory effect of Hg^{2+} was only tested in this study. In presence of Hg^{2+}, free and immobilized enzyme showed 43% (1.3 U/ml) and 77.17% (2.57 U/ml) residual activity. Similar pattern of inhibition was recorded in PCMB treated enzyme.

Figure 6
Stability of immobilized and free enzyme in presence of detergents and inhibitors



DISCUSSION

Glutaraldehyde has found widespread application in enzyme immobilization technique due to commercial availability, low cost and higher reactivity with amino group of protein at around neutral pH[15] in generating thermally and chemically stable crosslinks. Though mechanism of protein crosslinking is still under investigation, glutaraldehyde probably forms a three dimensional mesh like network, holding protein within insoluble carrier (like gelatin) matrix[16]. Conditions for efficient immobilization is a trial and error method as it is completely dependent on several factors[17] like the nature of the enzyme, the concentration of both enzyme and glutaraldehyde, the pH of the solution, the temperature, and the reaction time.

Covalent binding with gelatin in this work has been performed in presence of 0.1 M phosphate buffer (pH 6.9). Choosing a buffer of neutral pH range has been justified by the findings that glutaraldehyde shows high reactivity toward proteins at around neutral pH due to presence of several reactive residues in proteins and molecular forms of glutaraldehyde in aqueous solution, leading to many different possible reaction mechanisms[18].

Low concentrations of glutaraldehyde do not form sufficient crosslinkages and higher concentration form rigid tight matrix by excluding water molecules to insolubilize the enzyme. Moreover extensive crosslinking may result in a distortion of the enzyme structure preventing accessibility and accommodation of the substrate to active site of enzyme thus affecting the retention of biological activity[19]. For 5% gelatin matrix, 1% glutaraldehyde and for 10% gelatin matrix, 0.8% glutaraldehyde has been selected as optimum concentration as higher enzymatic activity was recorded in both cases. Choice of lower or higher concentration has markedly reduced enzymatic activity in this study.

Efficient crosslinking generally requires long reaction time[20] at low temperature since reaction with glutaraldehyde with lysine residues of proteins has been found progressive with time depending on the accessibility of the ε-amino groups[21]. For both 5% and 10% gelatin immobilized matrix, crosslinking time of 18 hour was found optimum, below of which resulted in enzyme leakage from matrix.

Several reports have indicated that immobilized enzyme requires higher concentration of substrate than free enzyme for optimum activity[7,22]. Increased requirement of substrate has been explained as diffusional restriction of large substrate molecule from the bulk solution to the microenvironment of an immobilized enzyme. But in this study, same substrate concentration (5 mg/ml) has been found optimum for both immobilized and free enzyme. This indicates that matrix pores are large enough to allow easy diffusion of substrate to active site of enzyme without any leakage of enzyme molecule from the matrix. Moreover at this concentration of starch (5 mg/ml), immobilized enzyme has shown higher enzymatic activity (2.95 U/ml) than free enzyme (2.47 U/ml). This indicates a more favorable reaction condition (ordered orientation of substrate and enzyme active site) for beta amylase within matrix microenvironment. Same pattern was also observed when optimum incubation time was determined (for both immobilized and free enzyme optimum incubation time was 15 min) that further supports previous explanations. Higher concentration of substrate remarkably decreases enzyme activity for both bound and free enzyme. This indicates that this enzyme do not typically follow Michealis-Menten reaction kinetics but behaves truly as substrate inhibited enzyme[23].

Immobilized enzyme has shown greater stability in presence of detergents, heavy metals and bile salts. Such greater operational stability has been beneficial from industrial point of view as it will maximize its potential to use in textile and detergent industry. Restoration of enzymatic activity

may be due to diffusional hindrance of the large sized additive molecules to the active site of gelatin entrapped and glutaraldehyde crosslinked enzyme. Gelatin may also mask enzyme from deleterious effect of such agents.

CONCLUSION

Hyperthermostable beta amylase from *Bacillus subtilis* DJ5 immobilized in 10% gelatin crosslinked with 0.8% glutaraldehyde can be used commercially as a replacement of free enzyme system as immobilized system has shown greater operational flexibility and higher enzymatic activity.

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