Immobilization and optimization of bacterial α- amylase and its comparative study with pure α- amylase

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Abstract: The objective of the present study was to isolate amylase producer from various sample and to investigate immobilization of amylase in calcium alginate beads and its comparative study with pure commercially available α-amylase. Various immobilization conditions like different curing time, sodium alginate concentration, calcium chloride concentration were studied and optimized. Optimization of pH and temperature carried out for free and immobilized enzyme. Effect of bead size, thermal stability, reusability, storage stability of immobilized form was investigated. Effectiveness of free and immobilized enzyme in stain removal was also checked. Investigation yield optimum results like 120 minutes curing time, 3% sodium alginate, 1M calcium chloride for AP7 and 120 minutes curing time, 4% sodium alginate, 1M calcium chloride parameters gave optimum yield for pure α-amylase. Optimum pH for free and immobilized AP7 was 7 and for pure α-amylase they were 6.6 & 7 respectively for free and immobilized enzymes. Optimum temperature for free and immobilized AP7 was 70˚C& 60˚C respectively, for free and immobilized pure α-amylase is 70˚C & 60˚C respectively. From the different bead size 1.5mm size gave higher rate of enzyme activity. Both enzymes are thermally stable for 60 minutes at 60˚C, reusable after 3cycle, stable in storage condition showing activity after 4 cycles. Both enzyme kinetic analyses showed increased value while immobilized when compared to free form. Both free and immobilized enzyme shows efficient stain removal when mixed with detergent when compare to enzyme or detergent alone.

Keywords: α-amylase, immobilization, entrapment, calcium alginate beads, comparative study

INTRODUCTION

The α-amylase (EC 3.2.1.1) enzyme which hydrolyzes starch to malto oligosaccharides is the of great importance in the present day biotechnology with application ranging from food, baking, brewing, fermentation, detergent applications, textiles desizing, paper industries, etc.[1,2]. Starch degrading amylase enzymes which breakdown the starch into sugars by acting on α-1,4-glycosidic bonds[3]. Amylases are obtained from various origins like plant, animal, bacteria and fungal. Microorganisms are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production [4, 5]. Microbial amylases are available commercially, and they have almost completely replaced chemical hydrolysis of starch in the starch processing industry [6]. Amylolytic enzymes account US$ 225 million of the US$ 2 billion annual worldwide sales of industrial enzymes. A-amylase seems to be the most versatile enzymes in the industrial enzyme sector and account for approximately 25% of the enzyme market [7, 8]. The use of enzymes in a free form is very uneconomical because the enzymes generally cannot be recovered at the end of the reaction. These drawback can be overcome by immobilization of the enzyme thereby rendering it more stable and easy to recover and recycle [9,10]. Immobilized enzymes pave the way to industrial development of continuous enzyme reactors. This procedure prevents enzyme losses due to washout a end at the same time maintains enzymes at high concentrations in order to reduce the cost of the enzyme [11].

Generally, enzymes are immobilized by physical adsorption, ionic binding, covalent binding, cross-linking and entrapment methods [12]. Entrapment method consists of enclosing the enzyme in an aqueous solution inside a semi permeable membrane capsule. In this immobilization technique, the particle structure allows contact between the substrate and enzyme to be achieved, and it is possible to immobilize several enzymes at the same time [13].Calcium alginate has been explored for enzyme and cell immobilization by entrapment and it is widely used most commonly in the form of spherical beads because bead preparation is a safe, easy, and rapid process [14]. Immobilized enzymes are preferred over their native counterparts because of their potential for repetitive use. Immobilization of enzyme allows for easy separation of the enzyme from the products and for

recycling of the enzyme. Immobilized enzymes are used in the food technology, biotechnology, biomedicine and analytical chemistry [15, 16].

MATERIALS AND METHODS

A.Materials Starch broth medium, nutrient agar medium supplemented with starch, tendler's fermentation medium, starch, sodium alginate, calcium chloride were purchased from Finar Limited (Mumbai). Pure α-amylase (Diastase) and DNSA (3, 5-Dinitrosalycylic acid) were purchased from Himedia (Mumbai). All the reagents and chemicals used were of analytical grade.

B.Sample collection

5 different samples like soil sample, potato surface, rice mill soil etc. were collected. Samples were transferred to sterile plastic bag and processed on the same day of collection.

C. Isolation of amylase producing microorganisms

One gram of the sample was mixed to 9 ml of sterile distilled water. After that 1ml mixture was transferred to sterile starch broth for enrichment and incubated at room temperature for 24 hours. After enrichment serial dilution was done up to 10^{-6} and spread plated into nutrient agar plate supplemented with 1% starch. Then the plates were kept for incubation at room temperature for overnight. After incubation plates were observed for the growth and for the confirmation plates flooded with Gram's iodine to produce deep colored starch- iodine complex.

D.Screening of higher amylase producer

Screening of higher amylase producer was done by putting spot of culture on starch agar surface. Then plates were incubated at room temperature for 24-48 hours. After different time interval plates were observed for the growth. By comparing zone diameter of isolates; highest zone giving isolate was selected for further study.

E.Extraction of crude amylase

After 48 hours, fermented broth was centrifuged at 10,000rpm for 15 minutes to obtain supernatant. Supernatant was used as crude enzyme for enzyme activity.

F.Preparation of enzyme stock solution

Freeze-dried α-amylase (Diastase) was added to 1M sodium phosphate buffer (pH 7.0) to the concentration of 1mg/ml. Prepared fresh every time.

G. Enzyme Assay

Amount of reducing sugar released was measured by following DNSA (DNSA (3, 5-Dinitrosalycylic acid) method. [17]. 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.

H. Immobilization of Enzyme

1 ml of enzyme solution is added in 9 ml of melted and cooled sodium alginate solution. The total volume of matrix and enzyme mixture being 10 ml. Solution was mixed well. The mixture was taken into syringe, and beads were formed by dropping the solution drop wise into 50 ml of calcium chloride solution at least from 1 feet height. Beads were allowed for hardening in calcium chloride solution for 120min in refrigerator. Formed beads were recovered by filtration and dried. The filtered calcium chloride solution was collected for enzyme activity determination.[18, 19]. % Immobilization yield was calculated by following equation:

Immobilization yield (%) = (Activity of immobilized enzyme/A-B) × 100

Where A is the activity of free enzyme added, and B is the activity of remaining enzyme in washed water and filtered calcium chloride solution. Both A and B were evaluated from the amount of reducing sugars produced enzymatically in the corresponding solutions.

I. Effect of time

After 24-48 hours of incubation time period crude amylase was extracted from the fermentation medium. Immobilization yield (%) was determined at 24 and 48hours.

J. Optimization of immobilization parameters

Various curing time (3o, 60, 90, 120, 150, 180minutes), sodium alginate concentration(1%, 2%, 3%, 4%, 5%, 6% w/v), calcium chloride concentration (0.5M, 1M, 2M, 3M, 4M, 5M) were studied to achieve higher % immobilization yieldand were optimized.

K. Optimizing pH and Temperature for both free and immobilized enzyme

For the optimization of pH, enzyme activity was determined in the different pH such as 5.8, 6.2, 6.6, 7.0, 7.4, 7.8 using 1M sodium phosphate buffer. Then enzyme activity was checked by enzyme assay and enzyme activity(µg/ml) were calculated. To optimize the temperature for free and immobilized enzyme, enzyme activity were performed in the temperature range of 30-90˚C and enzyme activity were checked by enzyme assay and enzyme activity (µg/ml) were calculated.

L. Effect of Bead size

For obtain different bead size, drop sodium alginate solution and enzyme mixture through three gauge of needles (18, 22, 26 gauge) into calcium chloride solution. The resulting beads were of 2.8, 2.0, 1.5 mm diameter respectively.

M. Thermal Stability of free and immobilized enzyme

Free enzyme and beads are exposed to 60˚C for different time interval time period (0, 15, 30, 45, 60minute). Then enzyme assay was performed using exposed free enzyme and beads and residual activity was checked for each exposure. Enzyme activity at 0 min consider as 100%.

N. Reusability of immobilized enzyme

Enzyme activity of alginate beads is checked. Then beads are recollected and washed with distilled water, and dried. After drying beads were again transferred to next fresh batch. These steps are repeated several times. Relative enzymatic activity is calculated for each cycle by considering results of first cycle as 100%.

O. Storage stability of immobilized enzyme

Alginate beads of enzyme formed under optimized conditions, and enzyme activity is checked. After 1st cycle beads are recollected and washed properly air dried then beads are stored at 4˚C in deionized water. After 48 hours beads are again suspended in fresh reaction system for enzyme activity. These steps are repeated several times. The residual activity was calculated by tiling the enzyme activity of the cycle as 100%.

P. Kinetic Analysis

For free and immobilized enzyme kinetic parameters were estimated using different starch concentration in the range of 0.1-1.0mg/ml. K_m, V_{max} values of free and immobilized enzyme were calculated using Michaelis - Menten curve and Line weaver burk plot.

Q. Application of free and immobilized enzyme

Clean white cotton cloth were stain equally with food gravy and were completely dried. A cloth piece with dried stain was left untreated and considered as a "control". Other cloth pieces were treated separately at room temperature. The following sets were prepared and studied:

1) Control
2) Distilled water + stained cloth
3) Free enzyme + stained cloth
4) Detergent + stained cloth
5) Immobilized enzyme + stained cloth
6) Free enzyme + stained cloth + detergent
7) Immobilized enzyme + stained cloth + detergent

RESULT AND DISCUSSION

Table 1: Results of starch hydrolysis after 48 hours of incubation

Isolates	Zone diameter (mm)
AP1	26mm
AP2	9mm
AP3	20mm
AP4	22mm
AP5	19mm
AP6	20 mm
AP7	27mm
AP8	26mm

A. Enzyme Assay

Enzyme assay was performed by DNSA method for selected 3 isolates (AP1, AP7and AP8). Among them AP7 gave higher enzyme activity. Therefore, AP7 isolate was selected for further study.

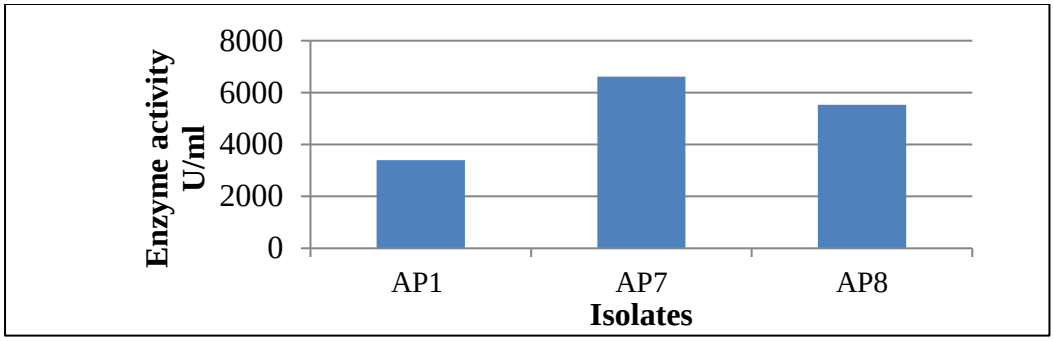

Figure 1: Enzyme activity given by 3 isolates

B. Results of Effect of Time

From the below figure, it was observed that the higher activity was obtained after 48hours of fermentation, than 24 hours.

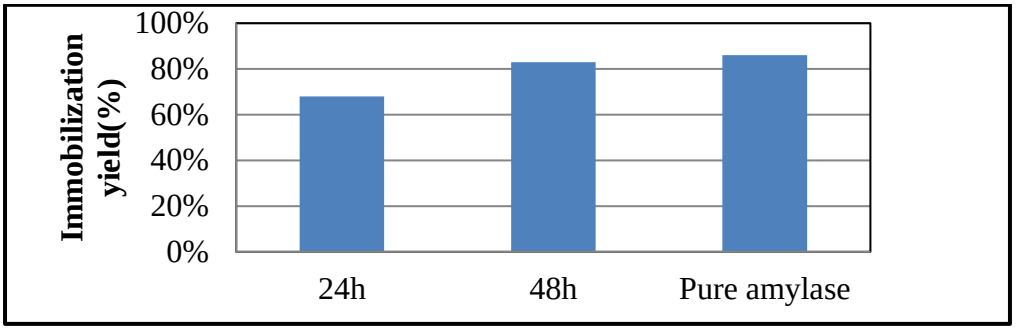

Figure 2: Enzyme activity given by isolate AP7 after 24 & 48 hours and pure amylase

C. Results of Curing Time

Hardness of calcium alginate beads depends upon the time required for the gel set [15]. For AP7 and pure α-amylase 120 minutes gave highest immobilization yield for both enzymes. After 120 minutes immobilization yield was not found to increase so that maximum curing time can be done for 120 minutes is sufficient. Prolonged curing of the beads with calcium chloride solution did not improve the immobilization yield. It might be due to constant hardness of the calcium alginate beads observed after 120min curing time [15]. At lower curing time, the beads were very fragile resulting in more leakage and hence giving very low percent immobilization yield.

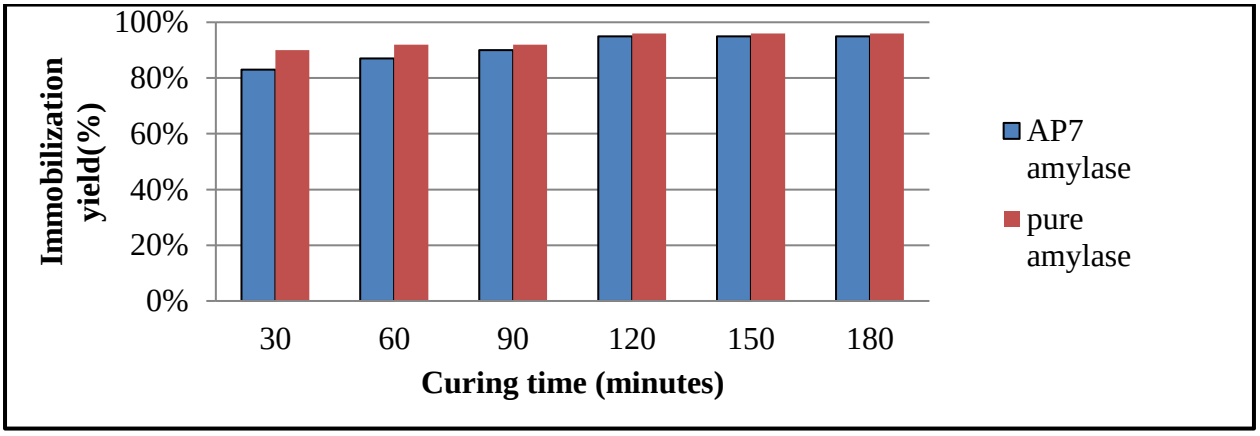

Figure 3: Effect on immobilization yield of α-amylase at various curing time

D. Results of sodium alginate concentration

It has been reported that the immobilization yield of enzyme depends on concentration of sodium alginate [15], the porosity of the calcium alginate beads depend upon the alginate type and the gelling agent concentration [20]. Highest 86% immobilization yield was resulted with 3% w/v sodium alginate concentration for AP7 enzyme and 75% immobilization yield with 4% w/v sodium alginate pure α-amylase.

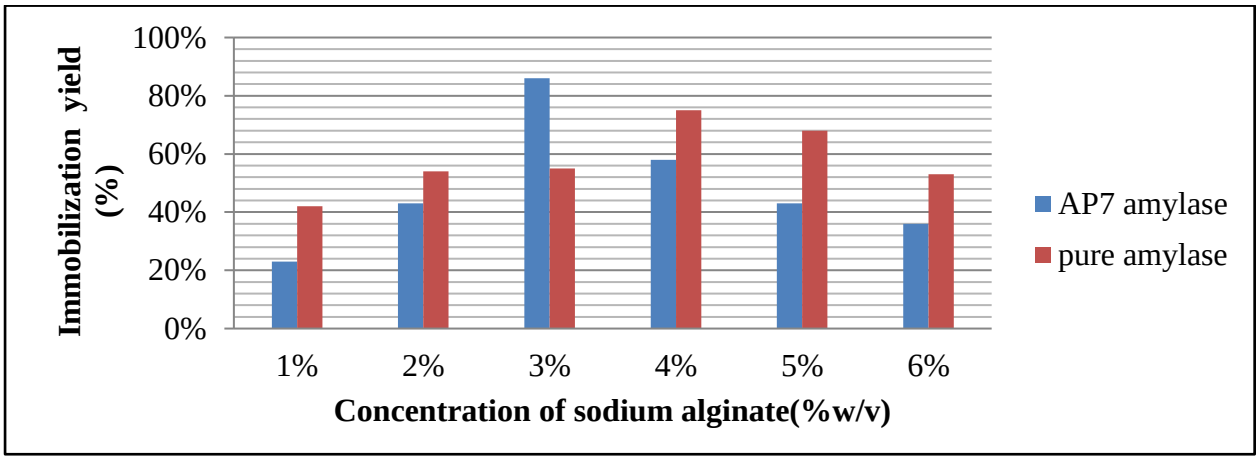

Figure 4:Effect on immobilization yield of α-amylase with various sodium alginate concentrations

E. Results of calcium chloride concentration

0.5M to 1M calcium chloride concentration tested. 1M calcium chloride gives highest immobilization yield. Beyond 1M yield was decreased. A decrease in the relative protease activity with increase in calcium chloride concentration has been reported [21, 22].

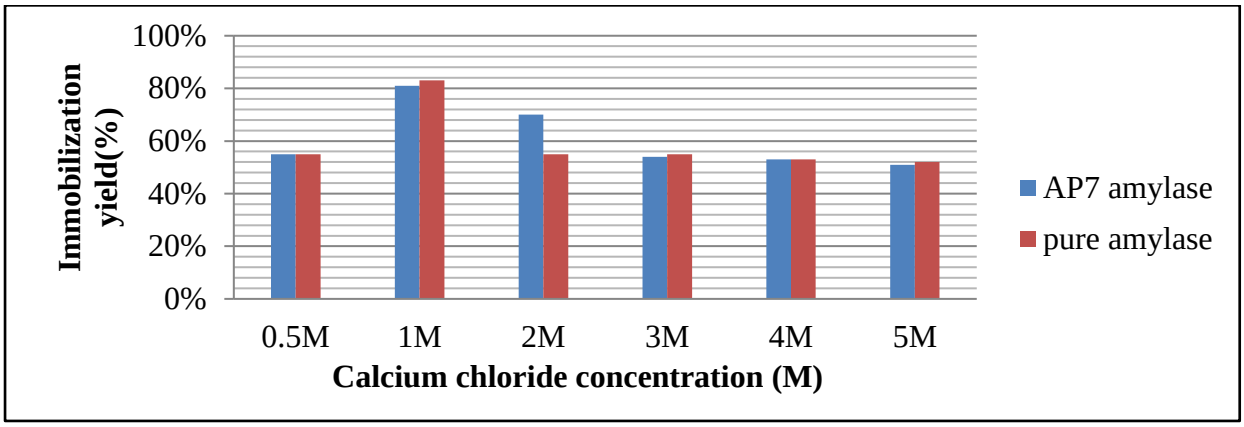

Figure 5: Effect on immobilization yield of α-amylase with various calcium chloride concentrations

F. Effect of pH for both free and immobilized enzyme

Immobilization usually results in shift of optimum pH due to conformational changes in enzymes. The effect of pH on activity of both free and immobilized enzyme of AP7 and pure α-amylase was tested. Optimum pH found was 6.6 for free pure amylase and 7.8 for immobilized pure amylase, optimum pH 7 for AP7 enzyme.

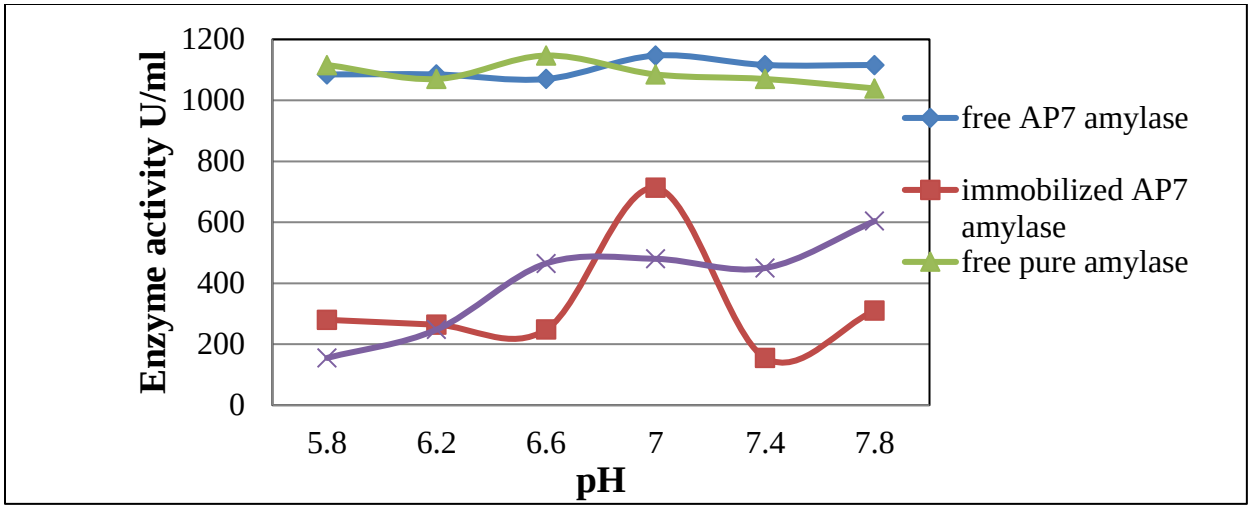

Figure 6: Effect of pH on enzyme activity of immobilized and free α-amylase

G.Effect of immobilized and free α-amylase at various Temperature

The activity of enzyme is strongly dependent on temperatures. The activity of enzyme increased with temperature and maximum activity was observed at 60˚C for both immobilized enzyme. For both free enzymes optimum activity observed at 70˚C.It was found that immobilized enzyme have more temperature resistance than free enzyme [23, 15].

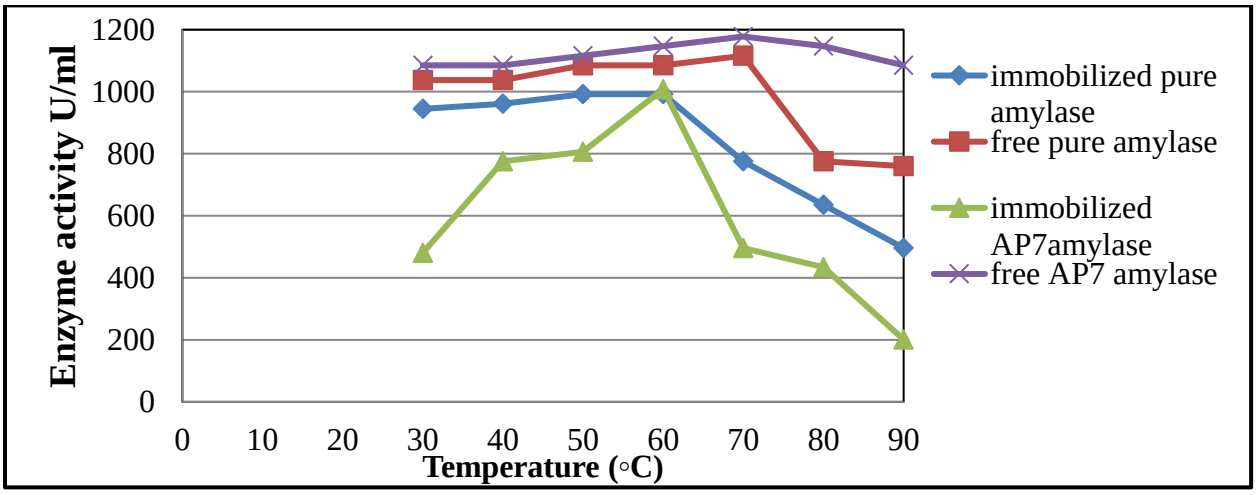

Figure 7: Enzyme activity of immobilized and free α-amylase at various temperatures

H. Effect of bead size

In the system of enzyme immobilization by entrapment, substrate has to be transported from the bulk solution to the outer surface of the matrix and then to the inner part of matrix. So both the intra particular diffusion and the external mass transfer should be taken into consideration. The intra particle mass transfer depends on the size of the bead which has significant effect on the rate of starch hydrolysis [15]. From the various bead size tested (1.5, 2.0, 2.6) mm it was found that bead size 1.5mm was showing highest activity for both AP7 and pure α-amylase.

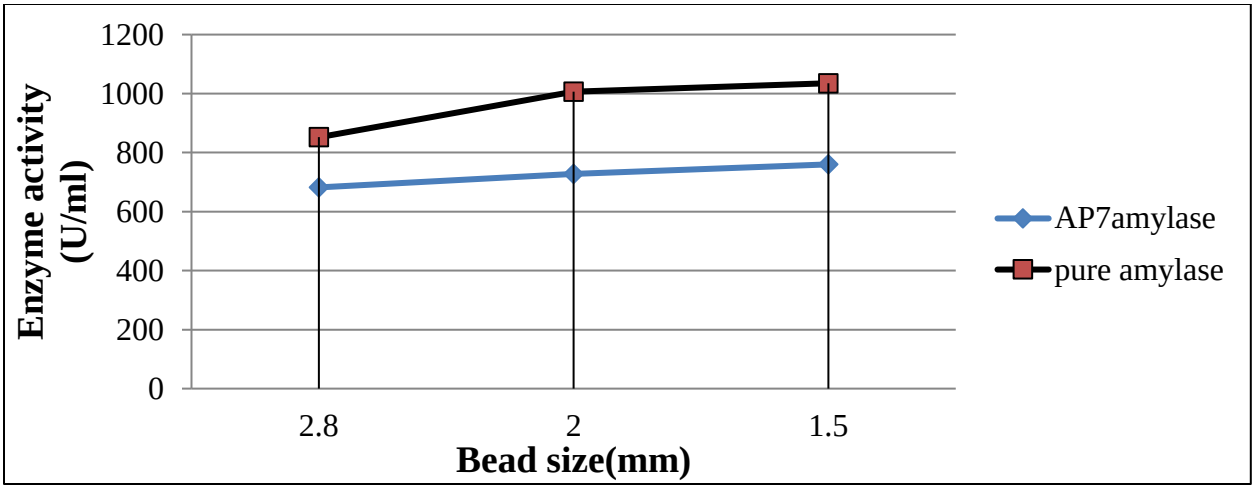

Figure 8: Effect of bead size

I. Thermal stability of α-amylase

After exposing the beads to 60°C for 60 minutes immobilized enzyme shows 36% relative activity for AP7 enzyme and 50% relative activity for pure α-amylase as compare to original activity. Immobilized enzyme should remain activate for longer period because industrial demand excessive temperature.

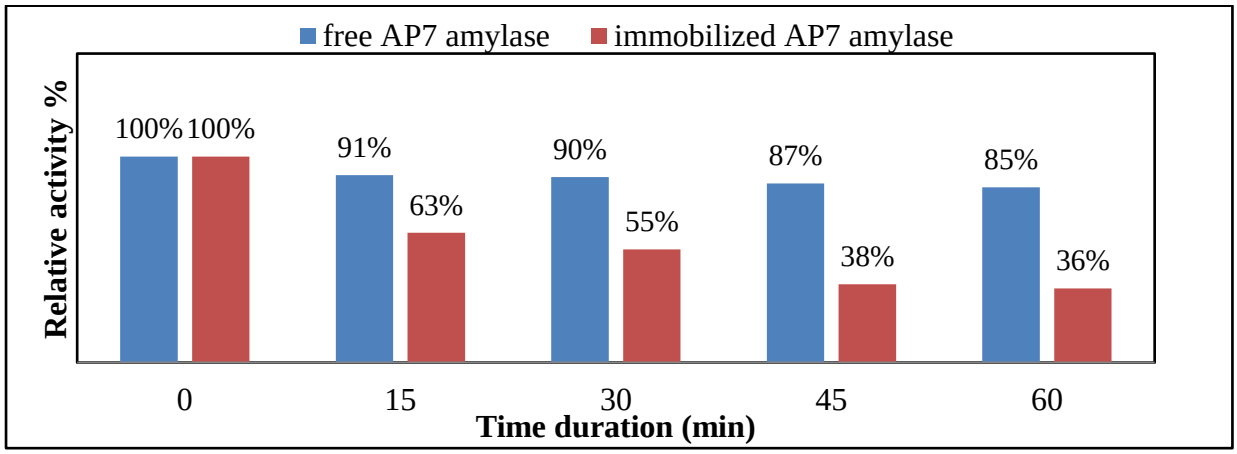

Figure 9: Results of thermal stability study of AP7 enzyme

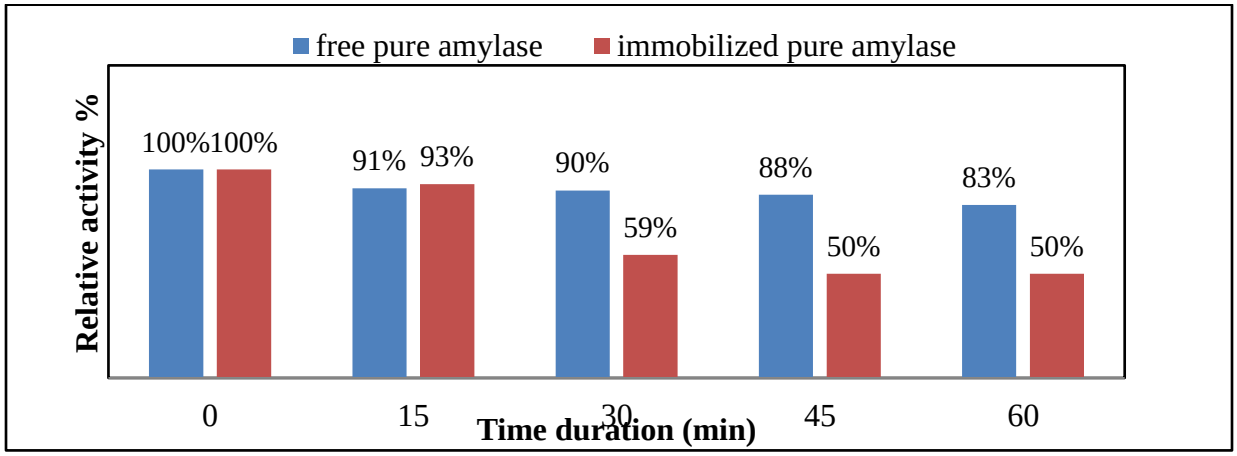

Figure 10: Results of thermal stability study of lab α-amylase

J. Reusability study of immobilized α-amylase

The reusability of immobilized both AP7 and lab α-amylase was studied up to 3 cycles. 83% relative activity of AP7 immobilized and 86% for immobilized lab enzyme retained after 3 cycle.

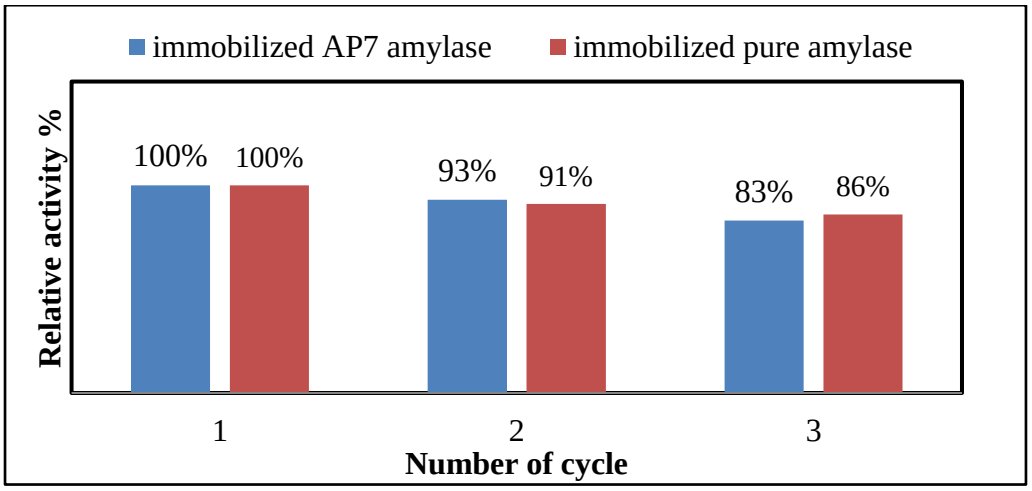

Figure 11: Results of Reusability study of immobilized enzyme

K. Storage stability

The purpose of enzyme immobilization is to increase shelf life and storage stability. So, immobilization enzyme should be active for longer period of time in storage conditions. AP7 immobilized enzyme shows 76% relative activity and 81% relative activity for immobilized pure α-amylase as compare to original activity.

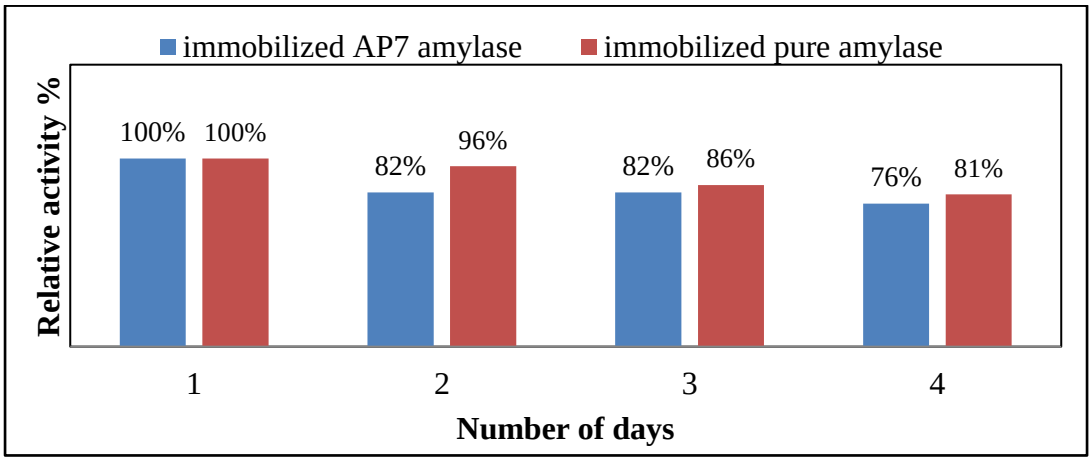

Figure 12: Results of Storage stability study of immobilized enzyme

L. Kinetic analysis

Kinetic parameters of both free and immobilized enzyme for AP7 and pure α-amylase were measured. The rate of reaction when the enzyme is saturated with substrate is the maximum rate of reaction, Vmax. The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for the substrate. After immobilization, Km and Vmax vale was increased that shows substrate affinity of α-amylase decreased.

Table2. Values of Km and Vmax

	Michealis menton plot		Line weaver burk plot	
Enzyme	Km	V_{max} (µmoles /min)	Km	V_{max} (µmoles /min)
Free AP7 α-amylase	650	68	474.23	76.92
Immobilized AP7 α-amylase	1000	55	1043.15	52.63
Free pure α-amylase	700	72	395.69	76.92
Immobilized pure α-amylase	900	57	851	55.55

M. Application in stain removal by free and immobilized enzyme

α- amylase has wide application in detergent industry as they have potential for better stain removal. It was analysed by treating cotton cloth with food gravy. Results of applications shows that better stain removal is observed by free amylase + detergent and immobilized amylase + detergent than detergent and enzyme (free or immobilized) alone. Efficiency of enzyme checked in stain removing using various type of washing treatment on cloth pieces.

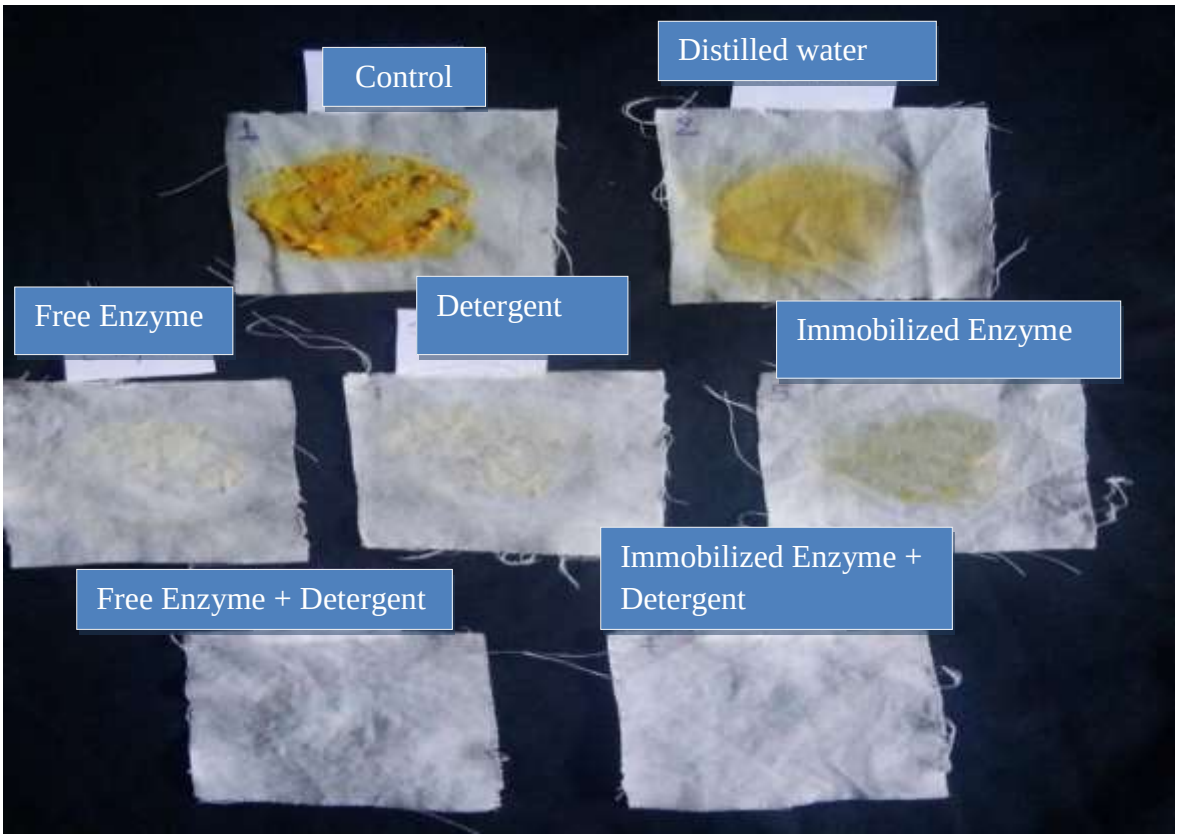

Figure 13: Stain removal by various combination of detergent and pure α-amylase

Figure 14: Stain removal by various combination of detergent and AP7 enzyme

CONCLUSION

Immobilization of α-amylase was successfully done by entrapment calcium alginate beads. Higher percentage of immobilization can be achieved by 120 min curing time, 3% sodium alginate, 1M calcium chloride, 7 pH, 60˚C temperature, 1.5mm bead size for immobilized AP7 enzyme. For pureα-amylase higher percentage of immobilization can be achieved by 120 min curing time, 4% sodium alginate, 1M calcium chloride, 7.8 pH, 60˚C temperature, 1.5mm bead size. Optimum pH for free AP7 enzyme and free pureα-amylase is 7pH and 6.6pH respectively. Optimum temperature for free AP7 enzyme and pureα-amylase is 70C˚ for both.Kinetic study suggest that immobilized AP7 enzyme have *Km* value 1000µg & 1043.15µg and *Vmax* value 55 µM/min & 52.63 µM/min as per MM plot and L-B plot respectively. Immobilized pure α-amylase have *Km* value 900µg % 851µg and *Vmax* 57 µM/min & 55.55µM/min as per MM plot and L-B plot respectively. For free AP7 enzyme have *Km* value 650 µg & 474.23 µg and *Vmax* value 68 µM/min & 76.92µM/min as per MM plot and L-B plot respectively. Free pure α-amylase have *Km* value 700µg & 395.69µg and *Vmax* value 72 µM/min & 76.92µM/min as per MM plot and L-B plot respectively. Both immobilized enzyme found to be thermally stable activate at 60˚C for longer period of time, reusable even after 3 cycle, stable during storing condition.Both free and immobilized enzyme when mixed with detergent shows efficient stain removal on stain cloth.

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