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Immobilization of α-Amylase by Entrapment Method and its Comparative Study with Free α-Amylase.

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Abstract:** This study aimed to immobilize α-Amylase in calcium alginate beads. Various immobilization conditions such as different sodium alginate concentrations, calcium chloride concentrations, curing times were studied and optimized. Combination of matrix such as starch with sodium alginate were studied. Optimization of starch concentration, reaction time, pH, temperature were carried out for free and immobilized α-Amylase. Effect of bead size in rate of starch hydrolysis, reusability, storage stability, thermal stability of immobilized α-Amylase was investigated. Efficiency of free and immobilized α-Amylase in stain removal was also checked. Studies showed that 3% w/v sodium alginate, 1 M calcium chloride, 150 minutes curing time and 1% w: w starch + sodium alginate mixture gives highest %immobilization yield. 1% w/v starch, 5 minutes reaction time found to be optimum for both free and immobilized enzyme. Optimum pH for free and immobilized α-Amylase is 5.8 & 6.8 respectively. Optimum temperature pH for free and immobilized α-Amylase is 70°c & 60°c respectively. Kinetic analysis shows increased value for immobilized α-Amylase when compare to free form. From the different bead size 1.5 mm bead size gave higher rate. Immobilized α-Amylase is reusable even after 8 cycle, stable in storage condition showing activity after 10 cycle, thermally stable for 60 minutes at 60°c with decreased relative activity. Both free and immobilized α-Amylase shows efficient stain removal when mixed with detergent when compared to enzyme or detergent alone.
Keywords:** Immobilization, Entrapment, Calcium alginate beads, α-Amylase.

I. INTRODUCTION

The α -amylase (EC 3.2.1.1) enzyme which hydrolyzes starch to malto oligosaccharide is of great importance in present day biotechnology with applications ranging from food, baking, brewing, fermentation, detergent applications, textile desizing, paper industries, etc. [1, 2]. Inspite of broader uses of enzyme their application is restricted by many problems. Apart from high isolation and purification costs, the main problems are their fragility, sensitivity to harsh environmental conditions results in limited operational lifetime of enzymes and difficulty in their recovery in the active form after the process for reuse. [3-6]. The term *immobilized enzyme* refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" [7]. Enzyme immobilization is advantageous as it eases enzyme recovery from product, enzyme reuse and process scaling up. Moreover, it decreases costs and waste generation [8, 9].

Different approaches for Enzyme immobilization are: Adsorption, covalent binding , cross linking, entrapment or encapsulation. Each immobilization method has its advantages and disadvantages since it is generally accompanied by changes in enzymatic activity, optimum pH, temperature and stability. Depending on the type of support and type of immobilization process, these changes vary to a large extent. [10].Method of immobilization should not alter the enzyme structure and should not hinder the enzyme catalysis reactions with its substrate. Immobilization by Entrapment method fulfills these requirements. In entrapment method enzymes are occluded in the synthetic or natural polymeric networks, it is a permeable membrane which allows the substrates and the products to pass, but it retains the enzyme inside the network, the entrapment can be achieved by the gel, fibre entrapping and microencapsulation [11]. The advantage of entrapment of enzyme immobilization is fast, cheap and mild conditions required for reaction process. The disadvantage is that limitation in mass transfer. The support matrix protects the enzymes from microbial contamination, proteins and enzymes in the micro Environment [12]. Natural polymers like cellulose, dextrans, agar, agarose, alginate, and chitin are widely used as matrix material. One of the frequently used is "alginate" usually in form of beads. [13]. This system is beneficial as it is nontoxic, simple, cheap and offering good mechanical strength, high porosity for substrate and product diffusion, and simple procedural part for immobilization [14]. Alginate is a natural anionic polysaccharide obtained by extraction from marine brown algae. Alginate is a linear binary copolymer consisting of (1, 4) β-d-mannuronic acid (M) and α-l guluronic acid (G) residues. Different types of alginate are selected for each application on the basis of the molecular weight and the

relative composition of mannuronic and guluronic acids. For example, the thickening function (viscosity property) depends mainly on the molecular weight of the polymer; whereas, gelation (affinity for cation) is closely related to the guluronic acid content. Thus, high guluronic acid content results in a stronger gel. [15]. Alginate can be either water soluble or insoluble depending on the type of the associated salt. The salts of sodium, other alkali metals, and ammonia are soluble, whereas the salts of polyvalent cations, e.g., calcium, are water insoluble, with the exception of magnesium.Polyvalent cations bind to the polymer whenever there are two neighboring guluronic acid residues. Thus, polyvalent cations are responsible forthe cross-linking of both different polymer molecules and different parts of the same polymer chain. The process of gelation, simply the exchange of calcium ions for sodium ions, is carried out under relatively mild conditions.

$$2\ Na\ (Alginate) + Ca^{++} \text{-------} > Ca\ (Alginate)_2 + 2\ Na^{+}$$

Because the method is based on the availability of guluronic acid residues, the molecular permeability does not depend on the immobilization conditions. Rather, the pore size is controlled by the choice of the starting material[15].The ionically linked calcium alginate gel structure is thermostable over the range of 0-100ºC; therefore heating will not liquefy the gel[16].

II. MATERIALS AND METHODS

α –amylase was procured from Himedia (Mumbai, India). Sodium alginate, calcium chloride, starch, DNSA and other regent were purchased from LOBA chemie (Mumbai,India). All the reagents and chemical were of analytical grade.

A. Preparation of Enzyme Solution.

α –amylase (freeze-dried) is dissolved in 0.1 M sodium phosphate buffer (pH 7.0). Concentration of enzyme solution is 1mg/ml. enzyme solution is stored at 4°c.

B. Enzyme Assay

Amount of reducing sugar released was measured by following DNSA (3,5 dinitrosalicylic 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. Enzyme U/ml was calculated by following equation: α - amylase activity (U/mL) = $\Delta E \times Vf / \Delta t \times \sum \times Vs \times d$

where,ΔE = Absorbance at 540 nm, Vf = Final volume of assay system, Vs = Volume (mL) of α-amylase used, Δt = Time of hydrolysis, Σ = Extinction coefficient ,d = Diameter of cuvette (1 cm for standard cuvette).

C. Immobilization of Enzyme

1 ml of α-amylase enzyme solution (concentration: 1mg/ml) is added in 9 ml of sodium alginate solution of defined concentration. The total volume of matrix and enzyme mixture being 10ml. Mix this solution well. The mixture was taken into a burette or syringe, and beads were formed by dropping the solution drop wise into 50 ml of calcium chloride solution of define concentration with gentle stirring. Allow the beads for hardening in calcium chloride solution for defined time period. After that the formed beads were recovered by filtration and dried. Beads are then added in 100 ml of starch solution of known concentration and reacted for predetermine time and is subjected to enzyme activity determination. The filtered calcium chloride solution was collected for enzyme activity determination. Calculate immobilized yield. [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, B is the activity of remaining enzyme in filtered calcium chloride solution.

D. Optimization of Immobilization Parameters.

Various sodium alginate concentration (1%-7% w/v), calcium chloride concentration (0.5M – 5M), curing time (30 mins – 180 mins), mixture of starch+sodium alginate (1:1,w:w; 1% - 6%) were studied to achieve higher % immobilization yield and were optimized.

E. Optimization of Substrate Concentration, Reaction Time, PH, Temperature for free and Immobilized α-amylase .

Different concentration of starch (0.1% - 1% w/v), enzyme & substrate reaction time (2minutes - 7 minutes) were studied for both free and immobilized enzyme and enzyme activity (U/ml) were calculated. To optimize the pH, enzyme activity was determined in the pH range of 3-9 using 0.1M buffers (pH 3-5, sodium acetate buffer; pH 6.8, sodium phosphate buffer; pH 7.5-9.5, Tris-HCl buffer).starch is dissolved in buffers of respective pH. Free and immobilized enzyme is allowed to react with the starch. Effect of pH is checked by enzyme assay and enzyme activity (U/ml) is calculated. To optimize the temperature for free and immobilized α-amylase, enzyme activities were performed in the temperature range of 20-80°C and enzyme activity were checked by enzyme assay and enzyme activity (U/ml) were calculated.

F. Effect of Bead size in Rate of starch Hydrolysis.

Alginate gel beads were prepared in optimized conditions and concentration. Drop sodium alginate solution with α-amylase (the total volume of matrix and enzyme mixture being 10ml) through three gauges of hypodermic needles (18, 22, 26 gauge) into Calcium chloride solution. The resulting beads were of 2.8, 2.0, and 1.5 mm diameter respectively. And by using burette; bead size of 3.5 mm diameter was obtained. Beads of respective sizes was added to 100 ml of 1 %(w/v) starch solution. Aliquots were withdrawn after every 5 min and checked for amount of maltose produced.

G. Kinetic Analysis.

Kinetic parameters of free and immobilized α-amylase were estimated by measuring initial reaction rates using different starch concentrations in the range of 0.1-1.0 mg/ml. Km, Vmax values of free and immobilized α-amylase were calculated using Michaelis-Menten curve.

H. Reusability of Immobilized α-amylase.

Alginate beads are formed under optimized condition.and its enzymatic activity is checked. Then beads are recollected and washed properly, air dried. And again transferred to next fresh reaction batch after drying. These steps are repeated several time. Enzyme assay is performed for each cycle .Relative enzymatic activity is calculated for each cycle by considering results of 1st cycle as 100%.

I. Storage stability of immobilized α-amylase.

Alginate beads of α-amylase is formed under optimized conditions; and its enzyme activity is checked. After that the beads are recollected from reaction mixture, washed properly with deionized water and air dried. Then beads are stored at 4°C in deionized water until next use. After each 24 hour that same beads were again suspended in fresh reaction system for enzyme activity assay. Repetition of these steps for checking the efficiency of storage stability of immobilized enzyme. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%.

J. Thermal Stability of immobilized α-amylase.

Beads are exposed to 60°c for various time period (0 min, 15min, 30 min, 45 min, 60 min) Then beads are allow to react with starch . Enzyme assay is performed .Residual enzymatic activity of immobilized enzyme is checked for each time exposure. Results of enzymatic activity at 0 min (without exposing beads to 60°c) considered as 100%.

K. Application of Free and immobilized enzyme in Stain Removal.

Clean white cotton cloth is cut into pieces of 10 × 10 cm². All pieces are stain equally with food gravy and were completely dried. The commercially available detergent powder was dissolved in deionized water to the concentration of 1% w/v and was boiled for 15 min to inactivate the enzymes that could be part of their formulation. A cloth piece with dried stain is left untreated and considered as a “control”. Other cloth pieces treated seperetly in flasks in shaking condition at 37°C. The following sets are prepared and studied:

1) Flask with 100 ml of deionized water and stained cloth.
2) Flask with 98 ml of deionized water + stained cloth + 2ml of commercial detergent (1% w/v solution).
3) Flask with 98 ml of deionized water + stain cloth + 2 ml of free α-amylase solution.
4) Flask with 96 ml of deionized water + stain cloth + of 2ml of commercial detergent (1% w/v) + 2 ml of free α-amylase solution
5) Flask with 100 ml of deionized water + stain cloth + beads of 2 ml α-amylase.

III.RESULTS AND DISCUSSION

A. Optimizing Sodium Alginate Concentration.

Studies reported that immobilization yield is controlled by sodium alginate concentration [13]. Pore size of beads based on the alginate type and the gelling agent concentration [20]. Various concentrations of sodium alginate ranging from (1% - 7%) were used for preparation of calcium alginate beads in order to vary the relative degree of cross linking which would create different pore size. Highest 71.82 % immobilization yield (fig - 1) was resulted with 3% w/v sodium alginate concentration. With lower sodium alginate concentration pore size formed in beads are larger so that enzyme may leak out from matrix which leads to lower immobilization yield [13]. Increased sodium alginate reduces the pore size which create barrier for substrate diffusion into the matrix. Reduced pore size reduces enzyme leakage from matrix but some initial leakage of the enzyme molecule is certain to occur so 100% immobilization yield could not be attained [21].

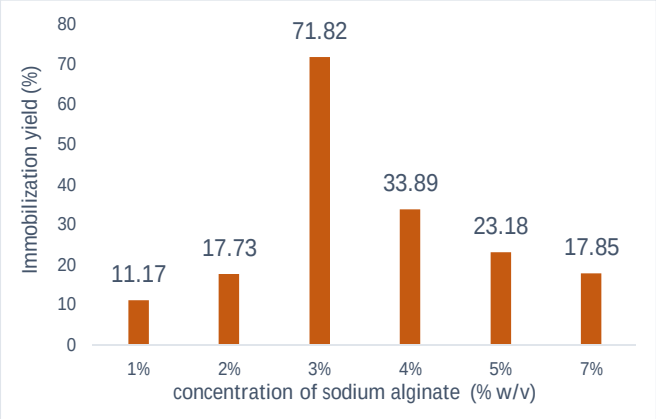


Fig. 1 Immobilization yield of α-amylase with various sodium alginate concentration

B. Optimizing Calcium Chloride Concentration.

Concentration of calcium chloride ranging 0.5M to 5M was tested. Among these (fig - 2) 1M calcium chloride gives highest immobilization yield (61.53%) of α-amylase. Beyond 1M immobilization yield was decreased. A decrease protease activity with higher calcium chloride concentration is reported in studies [22, 23].

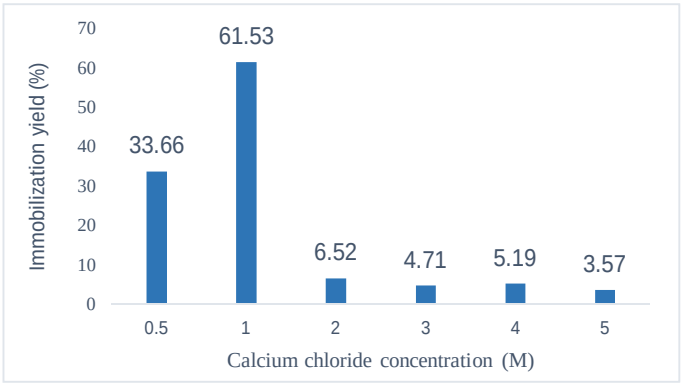


Fig.2 Immobilization yield of α-amylase with various calcium chloride concentration

C. Optimizing Curing Time.

Hardness of the calcium alginate beads depends upon time required for the gel to set [13]. The treatment of the beads in a calcium chloride solution for 150 minutes gave highest immobilization yield of 80.50 %(fig 3). Higher immobilized yield at 150 minutes might be due to formation of optimal pore size that can prevent enzyme leakage from matrix. After 150 minutes no significant increase in immobilization yield as constant leakage of α-amylase above 150 minutes curing time. At lower curing time, lower % immobilized yield was obtained as beads formed were fragile and unable to form enough small pore size that can held enzyme within the matrix; leads to enzyme leakage. Beads formed under optimized condition is shown in fig-4.

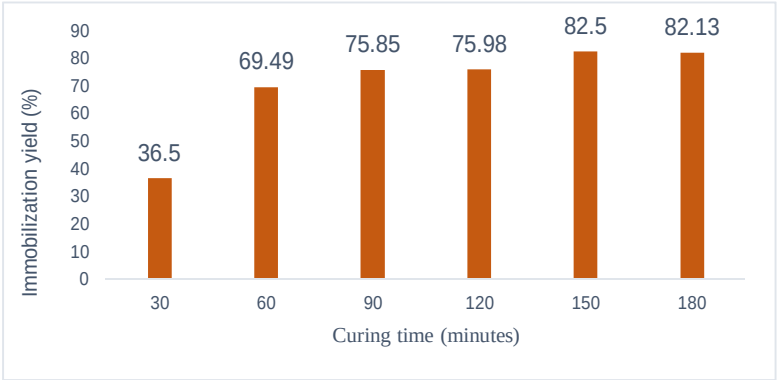


Fig.3 Immobilization yield of α-amylase with various curing time

Fig.4 beads of immobilized α-amylase under optimized condition.

D. Immobilization of α-amylase in mixture of matrix: starch + sodium alginate.

Different concentration of starch and sodium alginate (1:1; w: w) ranging (1%-6%) were tested. Among them 1% starch + sodium alginate giving the highest immobilized yield (80.83%) (fig-5).this shows that combination of natural polymer for immobilization could give better results.

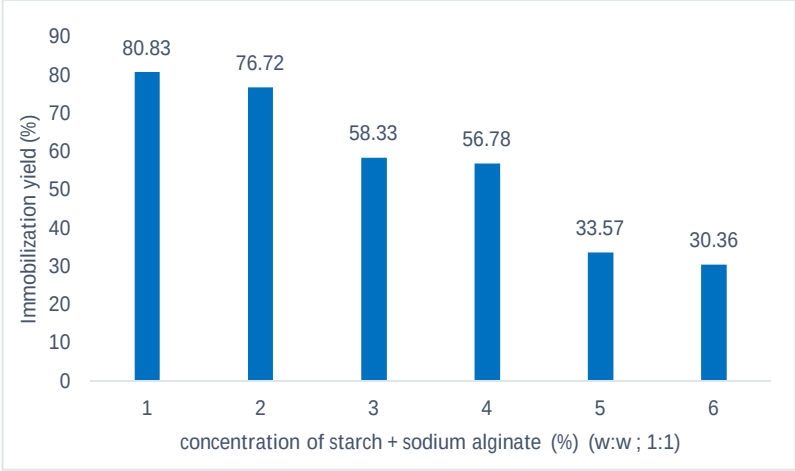
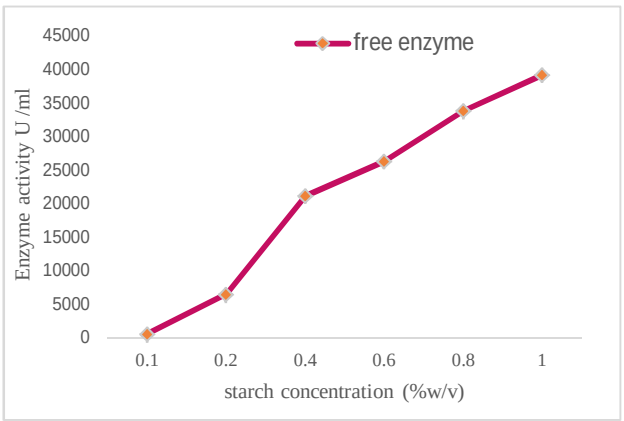


Fig.5 Immobilization yield of α-amylase with various starch+sodium alginate concentration

E. Optimizing Starch Concentration for Both free and immobilized α-amylase.

Starch concentration ranging from 0.1 % to 1.0 % were tested. Enzyme activities for both free and immobilized enzyme gradually increase with increased concentration of starch . 1%w/v starch concentration is found to be optimum for both free and immobilized enzyme giving highest enzyme activity of 39120 U/ml and 2736 U/ml respectively (fig-6).



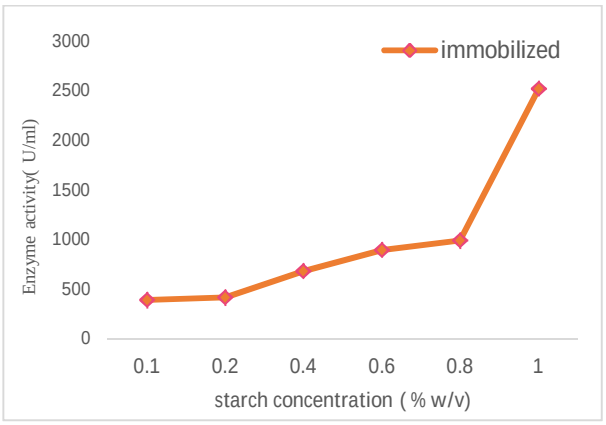


Fig.6 enzyme activity of free and immobilized α-amylase with various starch concentration.

F. Optimizing reaction time for both free and immobilized α-amylase.

Studies shown that as reaction time of enzyme and substrate increases the enzymatic activity of both free and immobilized α-amylase was also increase. Optimum reaction time found to be 5 minutes (fig-7). After 5 minutes the activity was found to be stable due to enzyme-substrate saturation condition achieved. Thus even after longer reaction time no significant increase in enzyme activity.

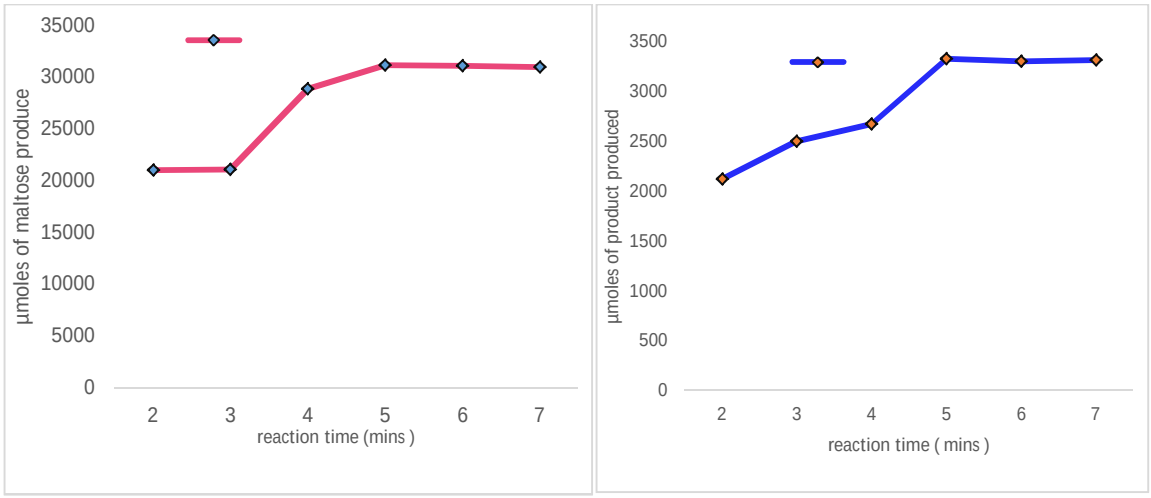


Fig.7 amount of maltose produced by free and immobilized α-amylase with various reaction time

G. Optimizing pH & Temperature for both free and immobilized α-amylase.

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 free and immobilized α-amylase is given in Optimum pH values were 5.8 and 6.8 for free and immobilized α-amylase (fig-8). Change in optimum pH of immobilized α-amylase due to conformational changes in enzymes; change in acidic and basic amino acid side chain ionization in the microenvironment around the active site [24].During α-amylase immobilization, similar shift in the optimum pH towards acidic direction was observed [25].

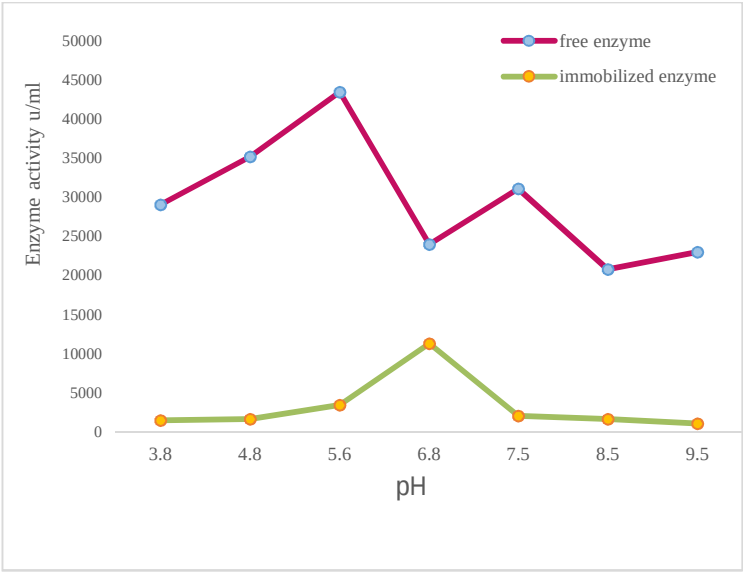


Fig.8 Enzyme activity of immobilized and free α-amylase at various pH

The activity of enzyme is also strongly dependent on temperature. The activity of free α-amylase increased with temperature and maximum activity was observed at 70°C. The optimum temperature of α-amylase was shifted to 60°C after immobilization in calcium alginate beads(fig-9).It was found that immobilized enzyme have more temperature resistance than free enzyme[13,26].

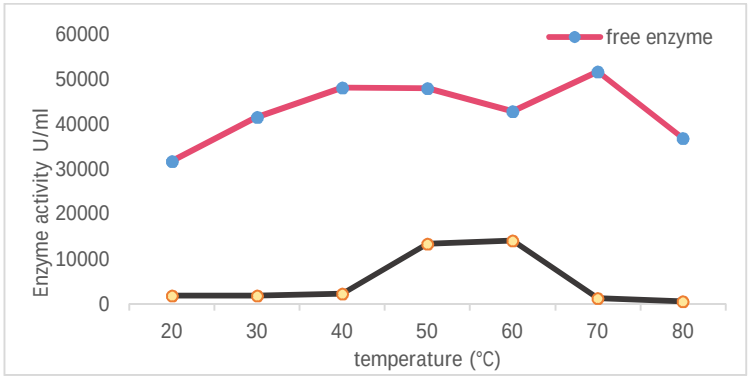


Fig.9 Enzyme activity of immobilized and free α-amylase at various temperature.

H. Effect of Bead size on rate of hydrolysis.

Among the various bead size tested (1.5, 2.0, 2.8, 3.5)mm it was found that bead size of 1.5 mm was showing highest result in terms of µmoles of maltose produce(fig-10). 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 intraparticular diffusion and the external mass transfer should be taken into consideration. However it is assumed that greater contribution is from the intra particle mass transfer. The intraparticle mass transfer depends on size of the bead which has significant effect on the rate of starch hydrolysis [13]. Different size of beads (in terms of diameter) were measured by using vernier caliper.

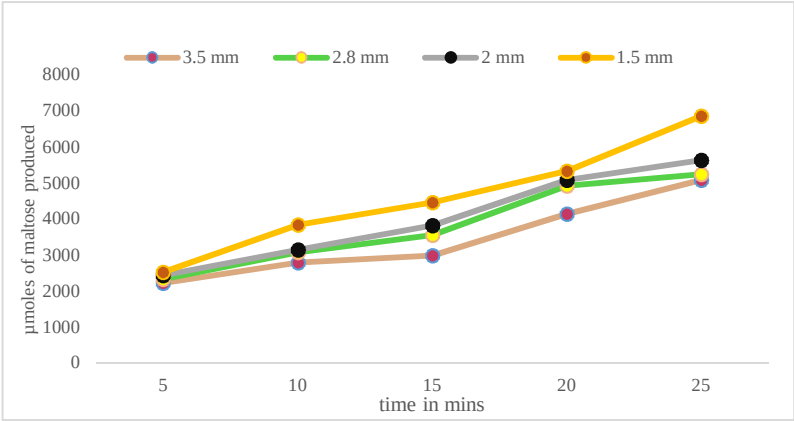


Fig.10 Effect of bead size on starch hydrolysis

I. Kinetic Analysis.

Kinetic parameters of both free and immobilized 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 its substrate. This is usually expressed as the *Km* (Michaelis constant) of the enzyme, an inverse measure of affinity. For practical purposes, *Km* is the concentration of substrate which permits the enzyme to achieve half *Vmax*. An enzyme with a high Km has a low affinity for its substrate, and requires a greater concentration of substrate to achieve *Vmax*."For both forms of α-amylase, Michaelis-Menten type & double reciprocal (line weaver burk plot) plot kinetic behavior was observed. Values of *Km* and *Vmax* for free and immobilized enzyme are described in Table-1. After immobilization, *Km* and *Vmax* value was increased that shows substrate affinity of α-amylase was decreased. This might be due to the lower accessibility of the substrate to the active site of the immobilized enzyme. Similar results were found in other studies[27-29].

Table 1 values of *Km* and *Vmax*

Enzyme	Michealis menten plot		Line weaver burk plot	
	Km (µg)	*Vmax*(µmoles/min)	*Km*(µg)	*Vmax*(µmoles/min)
Free	636.40	202	1098.65	44.05
immobilized	647.33	226	1874.06	156.25

J. Reusability of Immobilized α-amylase.

Immobilization provides excess to reusable enzyme preparation and also it gives a fruitful factor by reducing the industrial cost. The reusability of immobilized α-amylase was studied up to 8 cycles. As shown in Fig.11, 44.61% relative activity of immobilized α-amylase retained after 8 cycle.

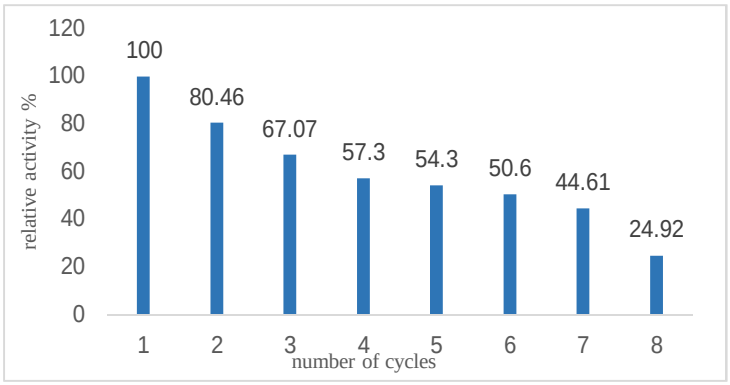


Fig.11 Reusability of immobilized *α* -amylase

K. Storage Stability of Immobilized α-amylase.

The main purpose of immobilization of enzyme is to uplift its shelf life and storage stability. To add to this, immobilized enzyme should be capable for being active over longer period of time in storing conditions. However, as illustrated in fig.12 immobilized α-amylase shows 48.19 % relative activity as compare to original activity.

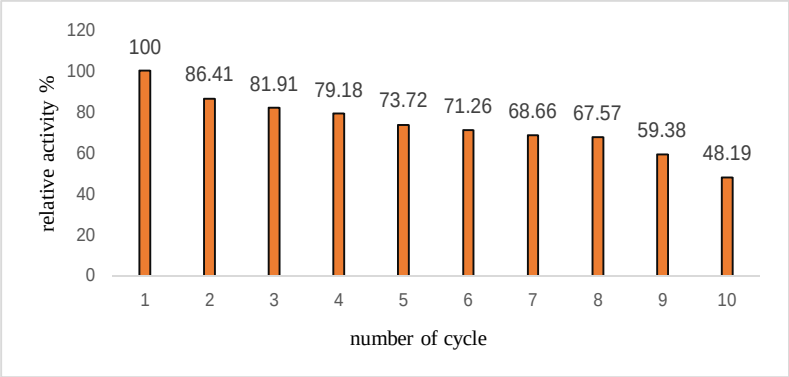


Fig.12 Storage stability of immobilized *α* –amylase

L. Thermal Stability of Immobilized α-amylase.

Immobilized enzyme should remain activate for longer period of time at higher temperature because industrial processes demands excessive temperature. As it displays data in fig.13 immobilized α-amylase shows 40% relative activity as compare to original activity after exposing the beads to 60°c for 60 minutes.

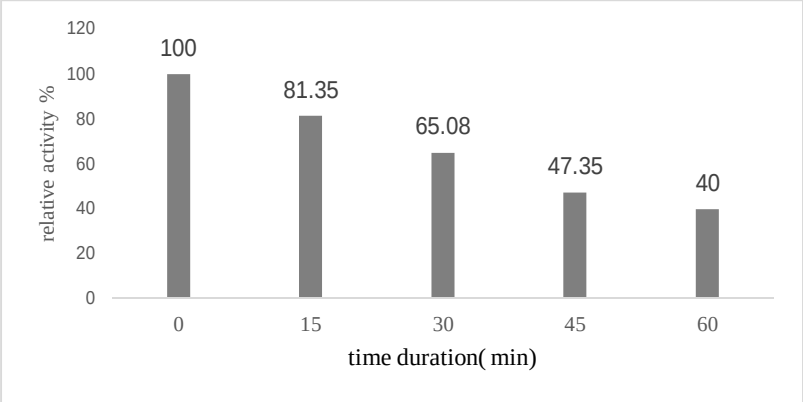


Fig.13 Thermal stability of immobilized *α* –amylas

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M. Stain Removal by Free and Immobilized α-amylase.

α-amylase has wide application in detergent industry as they have potential for better stain removal. This was analysed by treating cotton fabric with food gravy. Result shows that better stain removal is observed by free enzyme + detergent and immobilized enzyme + detergent than detergent and enzyme (free or immobilized) alone. But mixture of free α-amylase + detergent remove stain more efficiently and effectively than immobilized α-amylase + detergent. This might be due to free α-amylase can make contact more effectively with substrate in stain than immobilized α-amylase. Stain removing efficiency of various type of washing treament on cloth pieces is shown in figure 14.

(a) Stain cloth as control (b) washed with deioned water (c) washed with free enzyme

(d) washed with detergent (e) washed with immobilized enzyme (f) washed with free enzyme + detergent

(g) washed with immobilized enzyme + detergent

Fig.14 Stain removal by various combination of detergent and Enzyme

IV. CONCLUSIONS

Immobilization of α-amylase was successfully done by entrapment in calcium alginate beads. Higher percentage (upto 80%) of immobilization can be achieved by 3% w/v sodium alginate, 1M calcium chloride concentration, 150 min curing time, 60ºc temperature, 6.8 pH and 1.5 mm bead size, 1% w/v starch concentration as substrate and 5 minutes of reaction time. 1% w/v starch and sodium alginate matrix also gives good immobilized yield. Optimum pH for free α-amylase is 5.8 and optimum temperature is 70ºc.Kinetic study suggest immobilized α-amylase have *Km* value 647.33 µg & 1874.06 µg and *Vmax* value 226 µmoles/min & 156.25 µmoles/min as per MM Plot and LB plot respectively. For free α-amylase have *Km* value 636.40 µg & 1098.65 µg and *Vmax* value 202 µmoles/min & 44.05 µmoles/min as per MM Plot and LB plot respectively. Studies shown that immobilized α-amylase found to be thermaly stable & activate at 60ºc for longer period of time (about 1 hour), reusable even after 8 cycles, stable during storing condition and found to be active even after 10 cycles. Both free and immobilized enzyme when mixed with detergent shows efficient stain removal on stained cloth.

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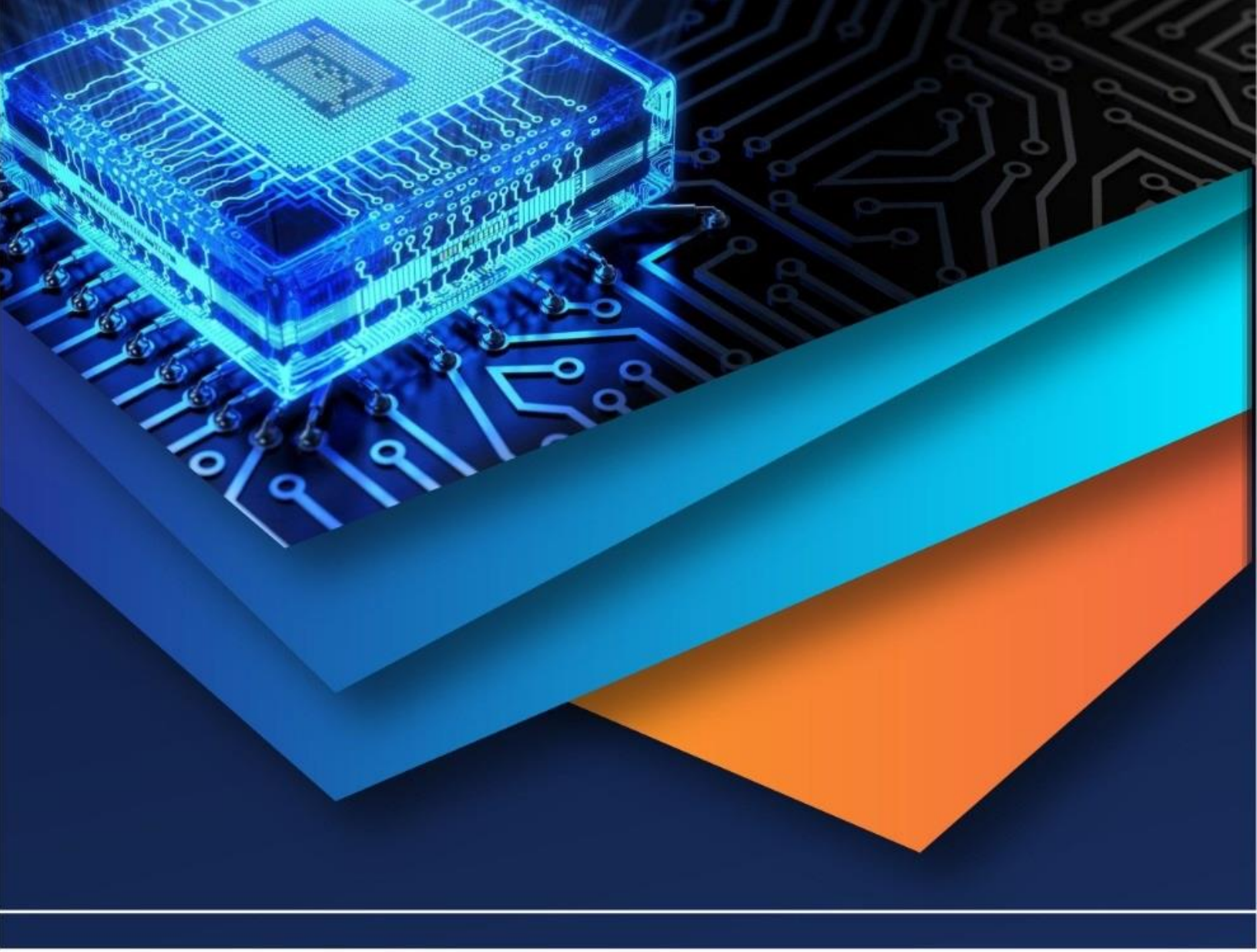

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