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Saccharification of Starch by Gelatin Immobilized Beet Root Amylase (*Beta vulgaris*)

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ABSTRACT

Amylase has been purified to apparent homogeneity from beet root (*Beta vulgaris*) by means of ammonium salt precipitation (65% w/v), heat treatment at 50 ± 1°C and gel filtration (Sephadex G-100) in 50 mM acetate buffer at 0°C. The purified enzyme showed 24.7% fold purification with 56.7% recovery. The specific activity of the purified enzyme was calculated to be 2343 ± 250 U/mg protein. The purified enzyme was immobilized on 8% bone gelatin membrane using 0.1% glutaraldehyde as the coupling agent. The pH optimum for immobilized and soluble enzyme was found to be 5.6 and 4.6 respectively. Immobilized enzyme showed temperature optima at 50°C while it was found to be 30°C for the soluble beet root amylase. There was no change in the K_m value (2.12 mM) on immobilization while the V_{max} values were found to be 0.25 mM/min and 0.416 mM/min for soluble and immobilized enzyme respectively. The storage stability of gelatin immobilized amylase was found to be of 60 days with 50% residual activity at 4°C. 85% hydrolysis of 10% starch solution was obtained in a doubled chambered plastic analytical column developed with gelatin immobilized amylase.

Key words: Gelatin immobilized amylase, Saccharification, Glucose syrup, *Beta vulgaris*

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INTRODUCTION

Conventional process technology methods used in industrial and analytical field are being replaced by biotechnological techniques in last few decades. Enzymes are ubiquitous magician and bioactive molecules of the biological world, catalyzing one substance into a material that is substantially different. Some of the disadvantage associated with use of enzyme as a catalyst is high product and substrate inhibition, and one time usability [1]. Immobilization helps in overcoming the drawbacks and thus preferred over native ones. Immobilized enzyme has found a wider application in food, analytical, pharmaceutical and biotechnology industry. Covalent binding is very effective method of immobilization if the amino acid involved in the covalent binding is not a part of active site. Covalently immobilized enzyme has following merits: negligible leaching, easy contact with the substrate as immobilized enzyme is present on the surface and enhanced thermo stability [2].

Amylases have found a great deal of use in food, paper, textile, baking and fermentation industries. Alpha amylase (1, 4 α –D- glucan glucanohydrolase) is a metallo-glycoprotein which require calcium ion for its activity.

In present work, amylase was purified to apparent homogeneity. Purified enzyme was immobilized on gelatin membrane via covalent attachment method employing glutaraldehyde as the coupling agent. With the help of immobilized system, a column set up was prepared. This column was explored for its potentiality in saccharification of starch in order to go for glucose syrup production.

MATERIALS AND METHODS

Chemicals:

Beet root was purchased from local market while bone gelatin, soluble starch, dinitro salicylic acid, folin reagent, sodium hydroxide, sodium carbonate, copper sulfate, sodium potassium tartrate, bovine serum albumin, glucose, potassium iodide, iodine, glutaraldehyde, sephadex G-25 and G-100, ammonium sulfate were purchased from HiMedia and Merck, India.

Extraction of amylase:

Beet root (*Beta vulgaris*) was employed for extraction of amylase for present work. Thoroughly washed fresh beet roots purchased from local market were peeled and grinded in acetate buffer (50 mM, pH 4.6, 1:4 w/v) under chilled conditions. The homogenate was filtered through muslin cloth and filtrate was subjected to centrifugation at 10,000 g for 15 min at 4^{o}C. The supernatant was checked for enzyme activity as described by Okolo et al. [3]. Protein estimation was done by Lowry et al. [4].

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Purification of amylase:

Supernatant was treated with different concentrations of ammonium sulfate (20-80% w/v). The resultant precipitate was centrifuged at 21,000 g for 20 min at 0-4^{o}C and dissolved in minimum volume of acetate buffer (50 mM, pH 4.6) and was treated to a desalting column made up of G-25. The enzyme preparation obtained after salt precipitation was heat treated at 50 ± 0.5^{o}C, centrifuged at 10,000 g for 10 min at 4^{o}C and loaded on sephadex G-100 column. The height and diameter of the column was 45 cm X 1.5 cm respectively. 2 ml fractions were collected with a flow rate of 2 ml / 8-10 min. The fractions were checked for protein and enzyme activity. Tubes showing reasonable enzyme activity were pooled together and used as partially purified source of enzyme.

Enzyme immobilization:

Purified enzyme was immobilized on 8% gelatin membrane by covalent attachment method of immobilization using 0.1% glutaraldehyde.

Membrane casting:

Bone gelatin of varying concentration (2-12%) was prepared by heating required amount of gelatin in distilled water in a boiling water bath for 30 min. The clear solution of gelatin was uniformly casted on plastic paper of 10 X 10 cm dimension at low temperature to obtain gelatin membrane. These casted membranes were allowed to air dry at room temperature for 72 hrs. The dried membrane of uniform thickness was explored for its immobilization efficiency.

Glutaraldehyde treatment:

A piece of dimension 0.5 X 0.5 cm of dry gelatin membrane was placed in acetate buffer (50 mM, pH 4.6) for an hrs and then treated with varying concentration of glutaraldehyde (0.05-0.5%) for 2 hrs. The excess of glutaraldehyde solution was decanted and the membrane was thoroughly washed with acetate buffer. Glutaraldehyde treated gelatin membranes were then allowed to interact with 0.2 ml of purified amylase preparation (0.172 mg protein, 403 U activity) for 45 min. The excess of enzyme solution was decanted and the membrane was washed with acetate buffer. The membrane was then checked for enzyme activity.

Activity Assay for soluble and immobilized amylase:

Enzyme activity was determined as described by Okolo et al. [3] with some modifications. 0.2 ml purified enzyme was added to a tube containing 1 ml soluble starch (1%) and 0.8 ml acetate buffer (pH 4.6, 50 mM). The tube was incubated for 30 min at 30^{o}C. Then 0.5 ml of dinitro-salicylic acid was added and the tube was heated for 10 min in boiling water bath. Final volume was made up to 5 ml and absorbance was checked spectrophotometrically at 545 nm [5].

A piece of gelatin membrane immobilized amylase (0.5 X 0.5 cm) was added to a tube containing 1 ml of soluble starch (1%) and 1 ml acetate buffer (50 mM, pH 4.6) followed by incubation for half an hour at 30^{o}C. On completion of incubation period, the membrane was recovered from the reaction mixture, thoroughly washed with buffer and stored for reuse. 0.5 ml dinitro- salicylic acid reagent was added to the reaction mixture and heated for 10 min in boiling water bath. Total volume was made up to 5 ml by addition of distilled water and absorbance of resultant colored solution was checked spectrophotometrically at 545 nm.

Steady State Kinetics:

The comparative kinetic study of free and immobilized amylase was done. The pH and temperature optima of the free and immobilized amylase were determined by varying the pH between 2.0 to 8.0 and temperature between 0^{o}C to 100^{o}C under standard assay condition. For each pH and temperature value tested, fresh piece of membrane was employed. K_m and V_{max} were calculated by Lineweaver-Burk plot by varying the substrate concentration from 0.5 mg/ml to 10 mg/ml.

Operational and storage stability:

Operational stability of the gelatin membrane immobilized enzyme was checked in batch process. The reusability of immobilized amylase was checked for seven cycles with a time interval of 12 hrs. The membrane was stored in wet condition in buffer system for three months. The activity in a fresh piece of membrane was checked initially for 15 days regularly, followed by activity evaluation at alternative days by the above mentioned assay method.

Column Preparation:

A plastic column of 50 ml capacity was developed by employing two plastic syringes. The mid of the plastic column was supplement with gelatin immobilized amylase supported on a nylon cloth base. The gelatin membrane acted as a semi permeable septum partitioning the column in two chambers. The upper chamber of the column was treated as the substrate loading segment while the lower chamber used for collection of hydrolyzed product. The column was loaded with the 10% starch solution and hydrolyzed product was collected in the lower chamber. The solution collected in the lower chamber was checked for presence of glucose by DNSA method and percent starch hydrolysis was estimated by Willstater method.

RESULTS AND DISCUSSION

Purification of amylase:

Result for purification of beet root amylase has been summarized in Table 1. The result shows that 24.7 fold purified amylase with 56.7 percent recovery was obtained. The specific activity was determined to be 2343 ± 50 U mg/protein. Amylase isolated from hyperthermophilic archaeon thermococcus profundus DT5432 was reported to have specific

activity of 1143 U/mg [6]. While a lower specific activity of 367.16 U/mg was also reported for amylase isolated from Thermus sp.[7]. Purification of amylase from A. flavus var. columnaris by ammonium salt precipitation resulted in specific activity of 3670.51 (units/mg protein/ml) and purification folds 5.66 times [8]. A 40 fold purification of amylase with a high with specific activity of 4900 U/mg was reported from irradiated wheat [9]. Purification of α-amylase from A. flavus, S-7 by a process of ammonium sulfate precipitation at 80% saturation and sephadex G-200 column chromatography resulting in a purified enzyme with specific activity of 28.6 (units/mg protein/ml) and 6.7 purification folds [10]. Recently researchers purified alpha amylase by ammonium sulfate precipitation, dialysis, sephadex G-100 and DEAE Sepharose CL-6B column chromatography resulted in an enzyme with specific activity of 154.2 units/ml/mg protein with 38.5 fold purification [11].

Table1: Beet root amylase purification summary at each purification step employed

Steps	Vol. (ml)	Activity (U/ml)	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	% Recovery
Crude	100	355	35500	375	94.6	---	100
Salt precipitated	50	670	33500	35. 6	941	9.94	94.3
Heat treatment	15	1710	25650	15.8	1623.4	17.1	72.2
Gel filtration	10	2015	20150	8.6	2343	24.7	56.7

Enzyme immobilization:

Mode of immobilization employed in present study was covalent attachment method. Gelatin membranes of less than 8% were very thin and fragile. Whereas membranes with high gelatin percent were difficult to obtain in uniform thickness as well as such membranes turn out to be brittle after drying process. 8% gelatin membrane was found to be the best suited one for our studies. Therefore 8% gelatin membranes were used for the further immobilization experiments. Fig. 1 gives a view of membrane casting. Immobilization of organophosphate hydrolase on 5% gelatin pad reported to give 40 mg of protein out of 50 mg to be absorbed or linked covalently [12]. According to reports 10% concentration of gelatin was found to be suitable for maximum entrapment of enzyme [13].

Glutaraldehyde treatment:

Glutaraldehyde is one of the favorite choices of covalent agent for the immobilization procedure. For the present study, maximum immobilization was obtained with 0.1% of glutaraldehyde. In some reports [14], amylase was immobilized by means of covalent coupling to 0.5% glutaraldehyde pretreated colloids and produced an active dynamic membrane with an activity of 59.8 U/g wet gel while in other, 2.5% glutaraldehyde solution was used for immobilization of amylase on affixed glass beads [15].

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Fig.1. Gelatin membrane casting on nylon piece (transparent) under low temperature condition.

Steady State Kinetics:

Variation of enzyme activity as a function of temperature is shown in Fig.2. The temperature optima were found to be 50ºC and 30ºC for immobilized and soluble enzyme respectively. Optimum temperature of 35ºC for soluble and 60ºC for immobilized amylase has been reported in literature [16]. A shift toward higher temperature was found when polyacrylamide was used for entrapment of amylase [17]. It is suggested that an increase in temperature optima could result from a lower temperature in the gel microenvironment compared to the bulk solution [18]. At 100ºC, complete deactivation of enzyme was recorded. Fig.3 shows relative activity of enzyme at different pH values in both, soluble and immobilized state. For immobilized enzyme, optimum activity was obtained at pH 5.6 while for soluble enzyme, it was 4.6. Similar pH values have also been reported for amylase immobilized on polyacrylamide, DEAE- cellulose and AS alumina [19]. The lineweaver – Burk plot (Fig. 4) was used for determination of K_m and V_{max} value at varying substrate concentration. Gelatin immobilized amylase revealed an apparent K_m value of 2.12 mM which was similar to that of soluble enzymes. The V_{max} value was reported as 0.25 mM/min for soluble enzyme and 0.416 mM/min for immobilized enzyme. Alumina immobilized amylase was also reported to have a K_m of 4.67×10^{-4} mol/ml and V_{max} of 0.99×10^{-4} mol/ml/min [20]. A decrease in K_m (for starch) and V_{max} has also been reported in literature [15].

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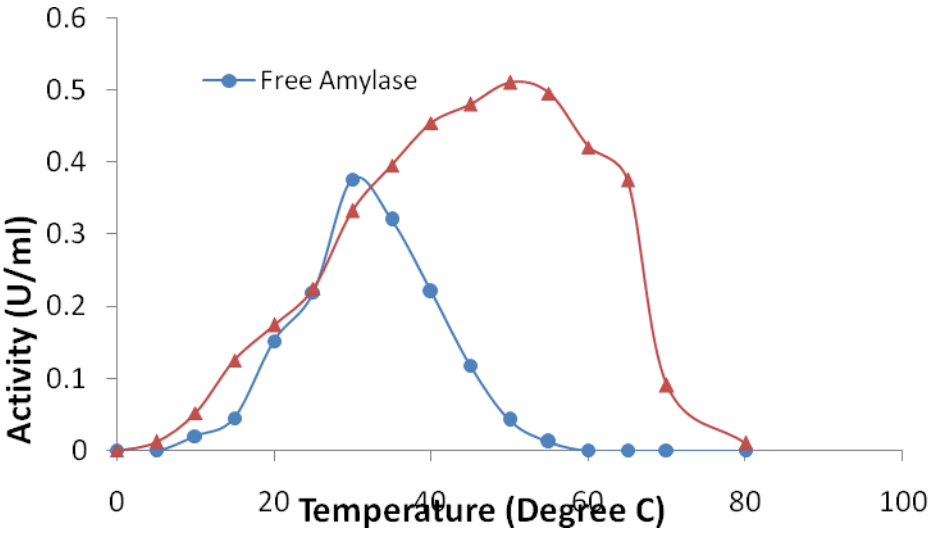

Fig 2: Effect of temperature on the activity of soluble and immobilized amylase.

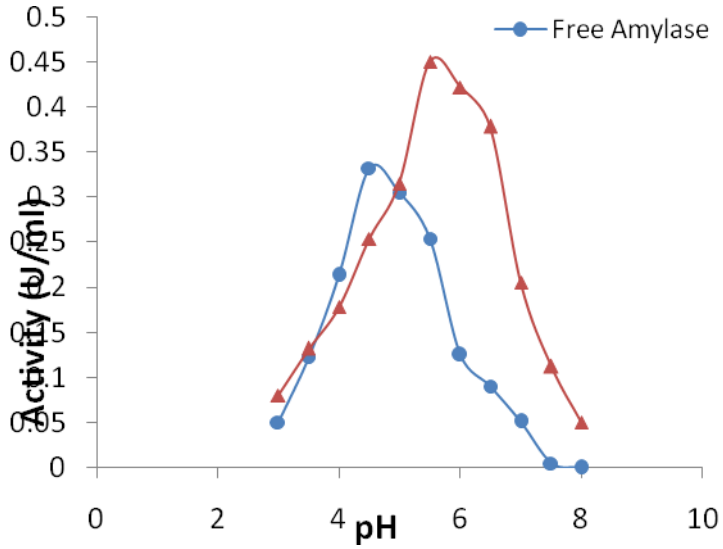

Fig 3: pH kinetics of soluble and immobilized amylase

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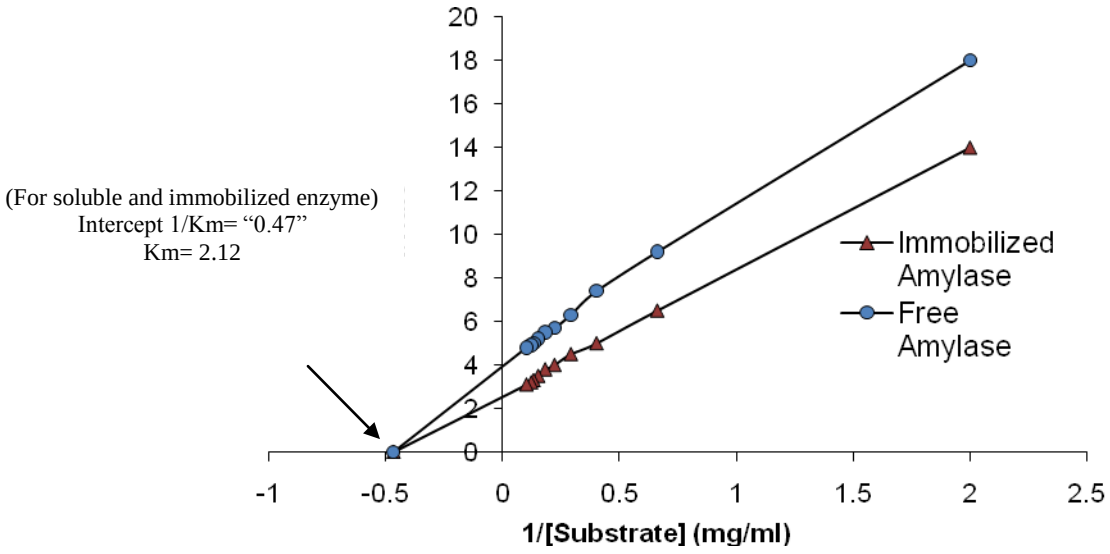

Fig.4. Double reciprocal plot for determination of Km and Vmax values of free and immobilized amylase under standard assay conditions as specified.

Operational and storage stability:

The reusability of column containing immobilized amylase was found to be fairly good as after five uses, the residual activity was determined to be 50%. The shelf life of enzyme was found to be increased to 60 days when stored at 4ºC. In the literature, 24.5% and 52.5% of initial immobilized amylase activity values was reported for carbodiimide and thionyl chloride activated poly (methyl methacrylate-acrylic acid) microspheres respectively, after storage for one month [21]. In some cases, initially the immobilized enzyme showed higher activity than that of free enzyme mainly due to higher enzyme concentration in the membrane structure, and then the apparent activity decreased gradually [22]. However, it can be regenerated by switching pH which causes contraction/expansion of the structure. Covalently bound amylase on glass beads was stable up to 5 days only and lost 20% of activity in 25 days [23].

Column Preparation:

The column prepared showed a high efficiency of converting starch to glucose. As shown in Fig. 5, initially the column was loaded with 10% starch solution in upper chamber. After an interval of 12 hrs, the product collected in lower column was checked for glucose by DNSA method. Within 12 hrs, 85% starch hydrolysis was monitored. A 90% conversion was given by silica immobilized amylase after 24 hrs of operation [24] while 85% hydrolysis of raw potato starch was performed by free enzyme [25]. Free and DEAE-cellulose immobilized enzyme activated with chloride compounds when used in the saccharification process gave 84% and 56% conversion of starch to D-glucose, respectively [26]**.** On comparing the hydrolytic efficiency of free and immobilized enzyme on soluble and CR starches, more efficient hydrolysis of starch was reported by immobilized enzyme than soluble one [27].

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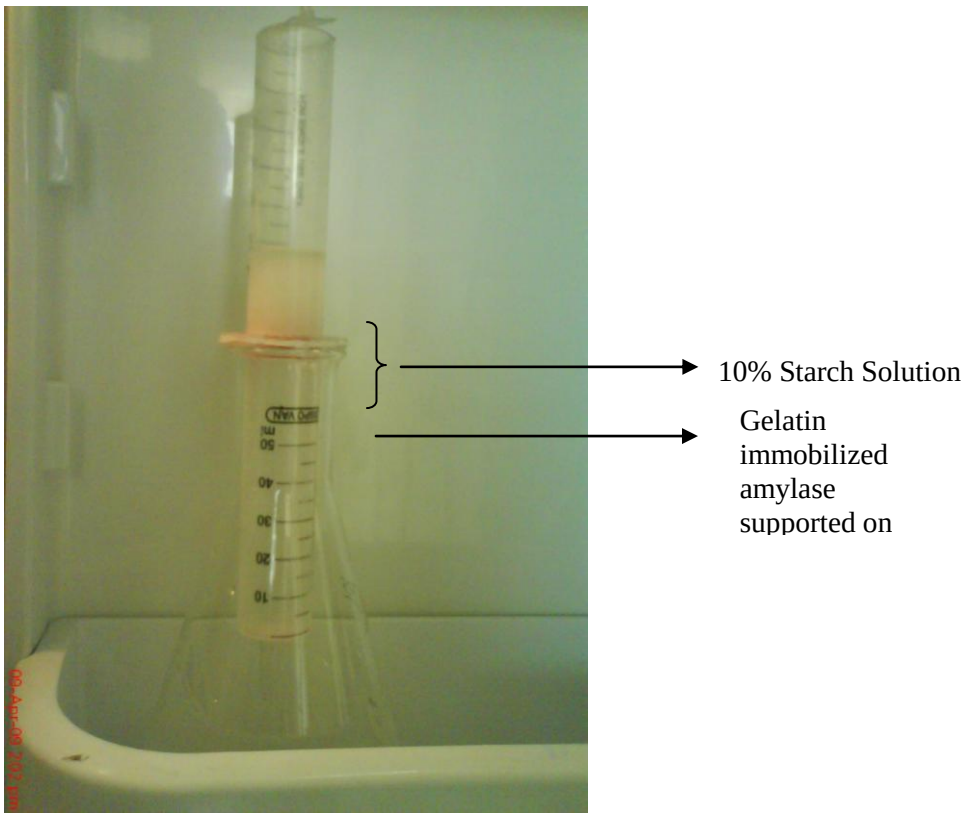

Fig.5. Column for the production of glucose from starch by gelatin immobilized amylase. The upper syringe contain 10% starch solution which is converted by immobilized amylase, placed in the mid of column supported by a nylon cloth. The hydrolyzed product is collected in the lower column.

CONCLUSION

In this study, enzyme amylase has been purified from beet root (*Beta vulgaris*) upto apparent homogeneity which was further used starch liquefaction in a plastic column. 85% starch hydrolysis was obtained with a time interval of 12 hrs. The immobilization induces no changes in K_m value while its V_{max} value increases to double. Also the immobilization method provides thermal stability to the enzyme, to be used at varying parameters. The shelf life of immobilized amylase was found to be 60 days with 50% residual activity. From the data, it is concluded that the enzyme was immobilized efficiently which further facilitate the production of glucose syrup with high yield. The developed column for starch hydrolysis also offers a possibility for application in industrial processes.

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