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Immobilization of *Vigna Mungo* β-amylase onto NaCl and $NaNO_3$ treated woven *Bombyx* Mori silk fabrics

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Abstract

Vigna Mungo β-amylase was immobilized onto activated *Bombyx mori* silk fabric by enrichment with chlorination and treated with sodium nitrate ($NaNO_3$) via glutaraldehyde coupling. The immobilization of *Vigna Mungo* β-amylase onto activated *Bombyx mori* silk fabrics was excellent by having 72-84% of retention of enzyme activity. The immobilization optimum conditions such as pH, reaction time, substrate concentration, $CaCl_2$ concentration and temperature were studied. Thermal stability of the enzyme was improved after immobilization which was 72°C as compared to free enzyme (40°C). The optimum substrate concentration and effect of $CaCl_2$ concentration was also carried out. In addition, the immobilized enzymes had good storage stability and reusability by maintaining 60-70% of its activity after 90-120 days

INTRODUCTION

β-amylase (3.2.1.2.) (alternative names: 1,4-α-D-glucanmaltohydrolase; glycogenase; saccharogen amylase) is also synthesized by bacteria, fungi and plants. Working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second α-1,4glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruits, β-amylase breaks starch into maltose, resulting in the sweet flavor of ripe fruit. Many microbes also produce amylase to degrade extracellular starches [1]. Scutellar epithelium has also been indicated as the dominant site of the enzyme formation in germinating seeds [2]. Silk fabric is an important textile stock due to its unique tensile strength and elasticity, good thermal stability, hygroscopic and microbial resistance. Silk biomaterials are biocompatible and can be chemically modified through amino acid side chains to alter surface properties for immobilizing the cellular growth factors. Woven *Bombyx mori* silk fabric has excellent properties in diffusivity of substrates, mechanical strength, and handlings. Fibrous silk has a large surface area, high mechanical strength and good compatibility which are advantageous to the use as a support for the enzyme immobilization [3,4, 5]. However several methods of immobilization could be used but all are expensive methods as it required tedious techniques for activation of silk fabric and required costly equipments as well as commercial enzyme. But, in this work, we describe the immobilization of β-amylase which is extracted from *Vigna Mungo* onto aminated chlorinated woven *Bombyx mori* silk fabric through covalent coupling with glutraraldehyde method [6]. However several methods of immobilization could be used but all are expensive methods as it required tedious techniques for activation of silk fabric and required costly equipments as well as commercial enzyme. This present study was based on characterization of β-Amylase extracted from *Vigna Mungo* which was immobilized on charged silk fabrics as compared to free enzyme. And it was found that the enzyme was more thermally stable at higher temperature after immobilization.

MATERIALS AND METHODS

Bombyx mori silk were chlorinated with NaCl solution (chlorine content, 3%) and then treated with sodium nitrate and glutraldehyde according to the procedure reported previously [7, 12]. Amylase was extracted and purified from *Vigna radiata.* Glutraldehyde (GA), sodium nitrate and NaCl were of analytical grade.

Extraction of β-Amylase from Vigna Mungo

Vigna Mungo seeds were soaked in distilled water for 2-3 days for sprouting. 500mg of sprouted *Vigna Mungo* seeds were crushed in pestle mortar in solution mixture of 5ml of 0.05M phosphate buffer (pH5) and 1ml of 0.5M NaCl. Then, centrifuged it at 8000rpm for 15 min at 4°C. Supernatant was collected which was contained enzyme. Enzyme activity of the crude enzyme extract was taken by dintrosalicylic acid method.

Activation of Silk fabrics and Immobilization of *Vigna Mungo* β-Amylase

Silk fabric (*Bombyx mori*) was cut into 2 x2 cm long pieces and dipped in 2ml of 3% NaCl (Silk 1) as well as in 2ml of 3% NaCl along with 10mg of $NaNO_3$ (Silk 2) for half an hour for incubation. After the incubation, fabrics were activated and washed with 0.1M phosphate buffer (pH -7). The activated fabrics were then dipped in 3-5ml of 10% Glutaraldehyde solution and were incubated at 37°C for 1 hour. The charged fabrics were then washed with double distilled water for 4-5 hours at the interval of 30 minutes. 5ml of the enzyme extract was poured onto the charged fabric and incubated at 37°C for 24 hours. Fabrics were then washed with 1M KCl for 2 hours at 37°C. Fabrics (Silk 1 & Silk 2) were then stored in 0.1M KCl at 4°C. Enzyme activity of the immobilized enzyme on both NaCl (Silk 1) as well as $NaNO_3$ (Silk 2) charged silk fabric was done by dinitrosalicylic acid method.

Enzyme Assay

Amylase activity was measured spectrophotometrically by incubating immobilized enzyme strips (glutaraldehyde treated woven *Bombyx mori* silk fabric) with 2.0 ml of 3,5-dinitrosalicylic acid at 37°C for 2 minutes by detecting the concentration of maltose(reducing group), liberated from starch on enzymatic activity of β-amylase which was read at 570nm [8]. One unit of enzymatic activity is defined as the amount of enzyme that produces µmol/min of maltose.

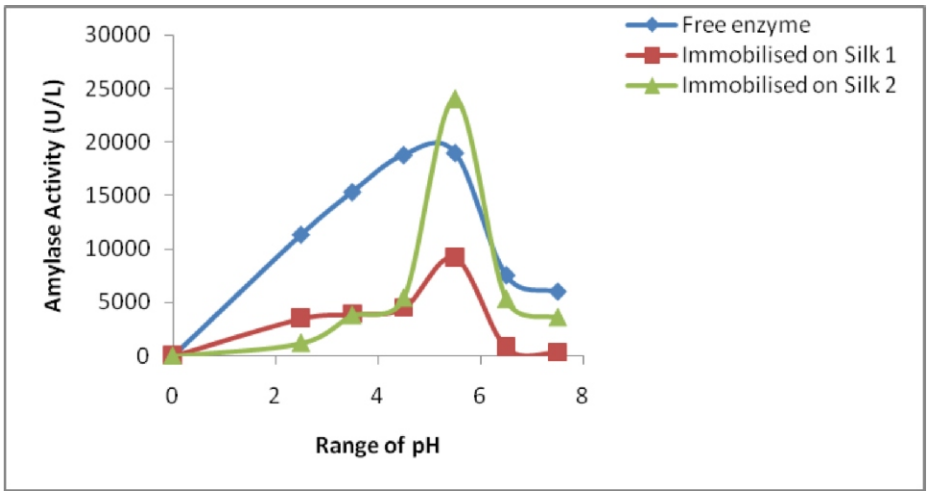

Fig. 1 : Effect of pH on free and immobilized *Vigna Mungo* β-amylase chlorinated (Silk fabric 1) and sodium nitrate treated (Silk fabric 2) silk fabrics

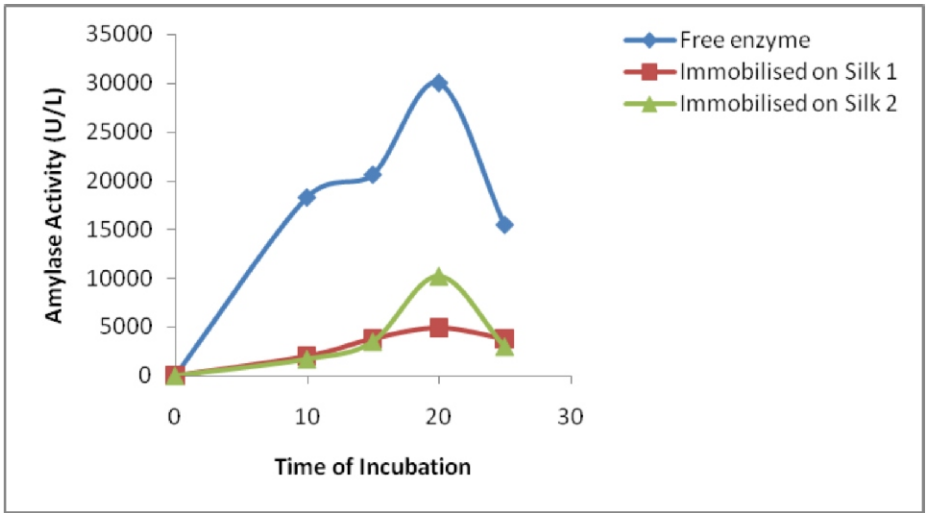

Fig. 2 : Effect of time of incubation on free and immobilized *Vigna Mungo* β-amylase chlorinated (Silk fabric 1) and sodium nitrate treated silk fabrics

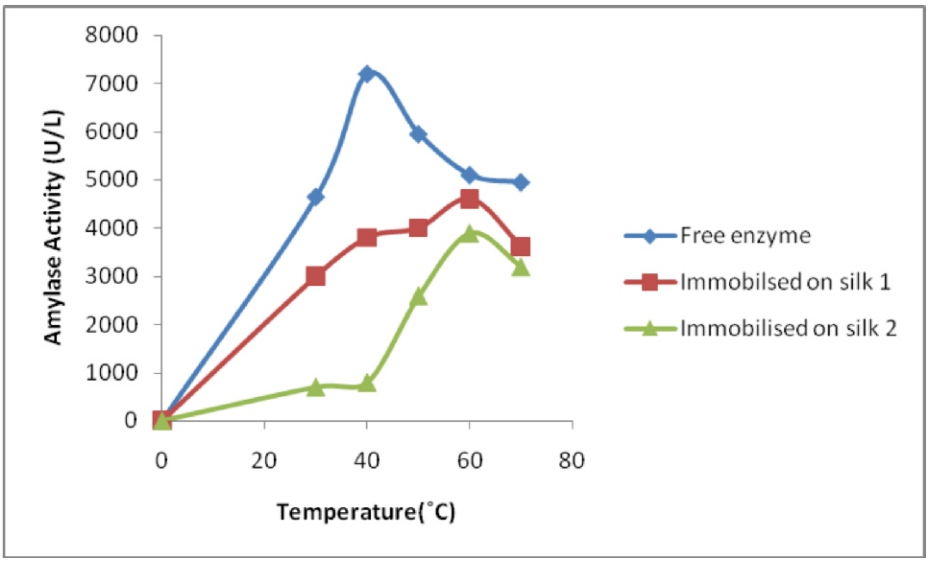

Fig. 3 : Effect of temperature on free and immobilized *Vigna Mungo* β-amylase chlorinated (Silk 1) and sodium nitrate treated (Silk 2) silk fabrics

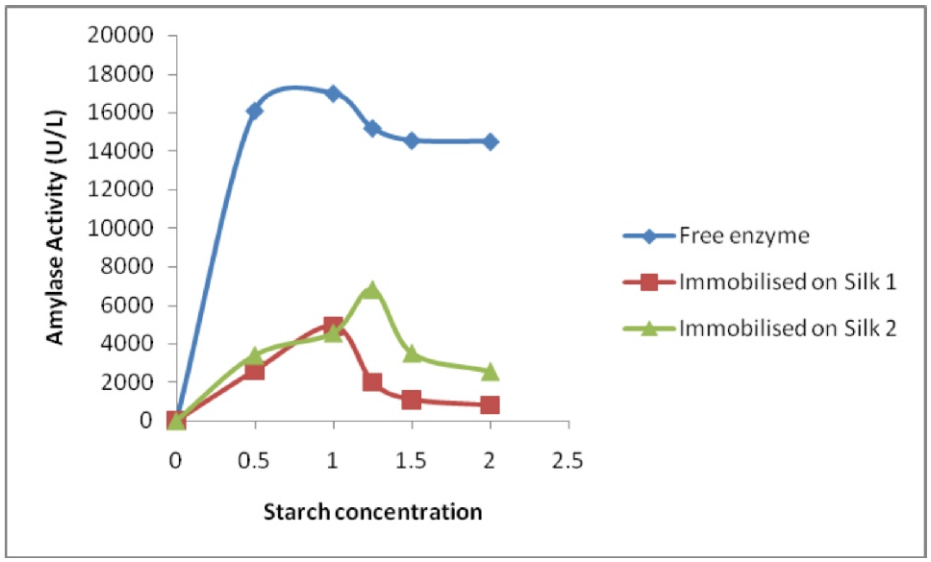

Fig. 4 : Effect of starch concentration on free and immobilized *Vigna Mungo* β-amylase chlorinated (Silk 1) and sodium nitrate treated (Silk 2) silk fabrics

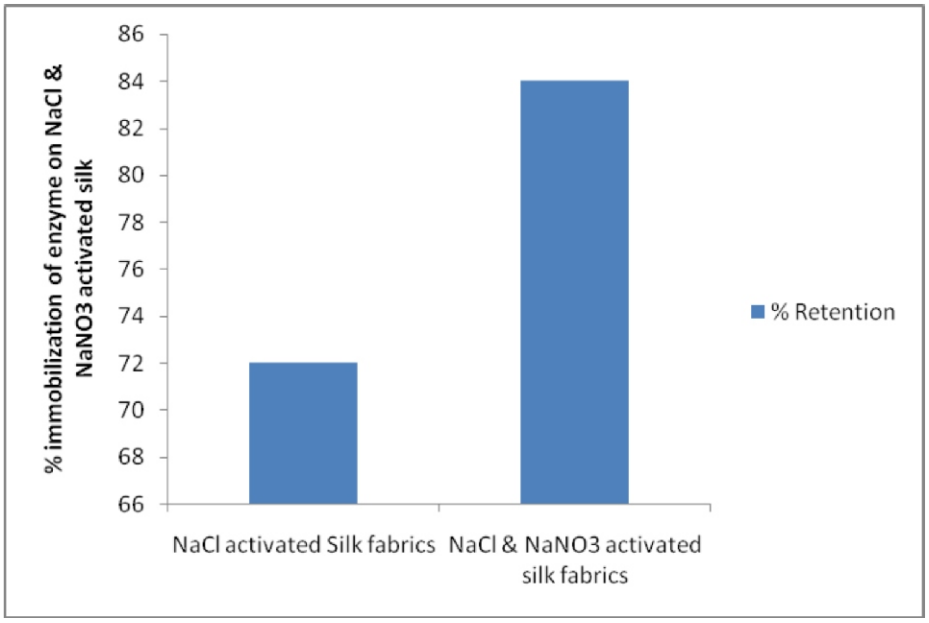

Fig. 5 : % of immobilization of *Vigna Mungo* β-Amylase onto NaCl and NaNO3 activated Silk fabrics.

% Retention of enzyme activity

The enzyme bound to sodium nitrate-treated chlorinated woven *Bombyx mori* silk fabric was estimated by determining the residual specific activity from solution of enzyme during immobilization by determining its specific activity by dinitrosalicyclic acid method which was determined as follows:

$$\% \text{ Retention} = \frac{\text{Specific activity of immobilized enzyme}}{\text{Specific activity of free enzyme}} X\ 100$$

Study of kinetic parameters

The free and immobilized enzymes (Silk 1 and Silk 2) were characterized for its various kinetic properties i.e. effect of pH, temperature, incubation time, $CaCl_2$ concentration and substrate concentration.

Effect of pH

The effect of pH on activity of free and immobilized enzymes was studied by performing the enzyme assay at different pH using acetate buffer, phosphate buffer and carbonate buffer (pH 2.5-10.5), the optimum pH of the enzyme was determined by incubating the enzyme with varying buffer solution and then carried out the enzyme activity by dinitrosalicyclic acid method.

Effect of incubation time

The effect of time on the activity of free and immobilized enzymes was studied by performing the enzyme assay at different time (5min-25min), with an interval of 5 min. The optimum time

was determined by incubating the enzyme at varying incubation time and then carrying out the enzyme activity by dinitrosalicyclic acid method.

Effect of temperature

Optimal temperature needed for free and immobilized enzymes for maximal activity was estimated by incubating the reaction mixture for 15 minutes at different temperature (20°C-80°C). The optimum temperature of enzyme was determined by incubating the enzyme solution at temperature in the range of 20°C- 80°C and estimating the enzyme activity by dinitrosalicyclic acid method.

Effect of substrate concentration

Optimal substrate concentration needed for maximal enzyme activity for free and immobilized enzymes which were estimated by incubating the reaction mixture at different concentrations of starch solution (0.25% - 1.75%) and enzyme activity was carried out by dinitrosalicyclic acid method.

Effect of $CaCl_2$

The effect of $CaCl^2$ on activity of the enzyme was studied by performing the enzyme assay at different $CaCl^2$ concentrations (2%-8%), the optimum enzyme activity was determined by incubating the enzyme mixture with varying $CaCl^2$ concentrations and then carried out the enzyme activity by dinitrosalicyclic acid method.

RESULTS & DISSUSSION

% Retention of enzyme activity

Our present study was reported 72-84% retention of enzyme activity of activated woven *Bombyx mori* silk fabrics (Silk1 & Silk 2) which showed that maximum activity and stable binding of amylase due to having good conformational stability too (Fig 5).

Effects of pH

The pH of the reaction mixture was varied from 2.5 to 10.5 as shown in Fig 1. A distinct peak corresponding to pH 5.5 was obtained which was similar to free enzyme and earlier reports [10 & 12]. There was no change in pH after immobilization.

Effect of incubation time

The reaction mixture of free and immobilized enzymes was incubated for varied time intervals from 5 to 25 minutes. The incubation time of 20 minutes was found to be optimum (Fig 2). Our present study was showed that incubation time of immobilized enzymes was similar to free enzyme (20min) as well as comparable to earlier report which was 15 min [9,12].

Effect of temperature

Optimum temperature of immobilized enzymes was determined by various temperatures from 20°C to 80°C. The enzyme was found to show optimum temperature of maximum activity at 60°C as shown in Fig 3. The present study was showed maximal thermal stability at 72°C as compared to free enzyme (40°C) and comparable to earlier reports [7, 12].

Effect of Substrate Concentration

The starch concentration was varied from 0.25 to 1.75 as shown in Fig 4. There was no change in substrate concentration after immobilization and quite close to free enzyme (1-1.25%).

Effect of $CaCl_2$

The Ca^{2+} is a divalent cation and it is extremely important in maintaining the stress conditions in plant seeds caused due to sodium ions. Divalent cations are affected the overall activity of the enzyme involved in different physiology of plants. The reaction mixture of free and immobilized enzymes was incubated for varied $CaCl_2$ concentration (2-8%). $CaCl_2$ concentration was 6% which was found to be optimum to increase the activity of free and immobilized enzymes as well as pretty similar to previous findings [13].

CONCLUSION

Amylases are one of the most widely used enzymes required for the preparation of fermented foods. Apart from food and starch industries, in which demand for them is increasing continuously, they are also used in various other industries such as paper and pulp, textile, detergents, food & pharmaceuticals industries [13]. There was no change in pH, time of incubation, starch concentration and effect of $CaCl_2$ in case of free and immobilized amylase onto activated silk fabrics (Silk1 & Silk 2). However, the immobilized *Vigna Mungo* β-amylase onto activated silk fabrics was showed thermal stability at higher temperatures at 60ºC which was only 40ºC for free enzyme. After the immobilization, the enzymes had increased storage stability and reusability for 90-120 days when stored at 4ºC.

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