ADSORPTIVE IMMOBILIZATION OF ALPHA AMYLASE ON CHITOSAN BEADS AND THE EFFECT ON POTATO STARCH HYDROLYSIS

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Abstract

Alpha amylase was immobilized on chitosan beads using adsorptive technique and the activity of the alpha amylase before and after immobilization was determined. The study further characterized the immobilized alpha-amylase and it was found that optimal temperature and pH of activity were between 50^{o}C -60^{o}C and pH 7-8 while the enzyme exhibited more activity on corn starch while exhibiting varying lesser degrees of activity on cassava, cocoyam and irish potato starch substrates. The effect of metal ions revealed that Ca+ and Pb2+ had no effect on the enzyme in relation to the control while Fe2+, Hg, K+, Zn+ and EDTA all inhibited the activity of the enzyme. The temperature and pH stability further revealed that the immobilized enzyme was more stable at 40^{o}C and pH 7 respectively.

Key words: Immobilization, Enzymes, Amylase, Adsorption

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INTRODUCTION

Enzymes are biological catalysts that speed up the rate of specific biochemical reactions. Microorganisms have been the principal source of many different enzymes for years, which were identified after extensive research and currently find their main uses in industrial applications (Aquino *et al*., 2003).Proteases and amylases are the most prominent enzymes among the industrially important enzymes since they are widely utilized in brewing, detergent and food industries. There is difference in the properties of amylases produced by various bacterial strains with reference to temperature, pH etc. The use of enzymes in industrial sector is increasing due to the increase of food, beverage, textile, leather and paper industries (Yandri *et al*., 2010).

Source of enzymes includes plants, animals and microbes and those from microbial source are mostly preferred since they are easy to handle.

Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes (Bernfeld, 1955). The physical localization of enzymes within a space of a material (support) with the retention of its catalytic properties to enable its continuous use is termed enzymes immobilization (Kragl, 1996). There are several methods of immobilization which include adsorption, crosslinking and entrapment. Immobilized enzymes offer several advantages, including repeated use of the enzyme, ease of product separation, improvement of enzyme stability, and continuous operation in packed-bed reactors (Abdel-Naby*et al.*, 1999).

Chitosan (poly-β (1-4)-2-amino-2-deoxy-D-glucose], a cationic biopolymer, is a de-acetylated derivative from chitin, the second most abundant polysaccharide in nature next to cellulose **(**Amorim*et al.*, 2003**)**. Commercially available chitosan is obtained from crustacean and has been used in a wide variety of applications. Its membrane has several uses including food processing, protein purification, and skin replacement technology **(**Muzzarelli, 1983**)**. Recently, chitosan has attracted great attention, which has been reported to be a promising polymer in medical and biotechnology areas. Chitosan is known as an ideal support material for enzyme

immobilization because of its many characteristics like hydrophilicity, biocompatibility, and low biodegradability **(**Martino *et al..,* 1995), form versatility (powder, gel beads, fibers, capsules and membranes) high permeability toward water, good adhesion **(**Noda *et al..,* 2001) and high affinity towards proteins. In addition to these, chitosan is an inexpensive, inert, non-toxic, high mechanical strength support, and is thus attractive for enzyme immobilization (Kumar, 2000 and Ibrahim *et al.,* 2002). Also, the amino groups of chitosan facilitate the immobilization of enzyme, either by physical or chemical reaction (Desai *et al..,* 2006).

Potato is grown in more than 100 countries, under temperate, subtropical and tropical conditions. It is essentially a "cool weather crop", with temperature being the main limiting factor to production: tuber growth is sharply inhibited in temperatures below 10°C and above 30°C, while optimum yields are obtained where mean daily temperatures are in the 18 to 20°C range. Potato is a very accommodating and adaptable plant and will produce well even without ideal soil and growing conditions.

The objective of this study is to check the activity of free and immobilize α- amylase on hydrolysis of potato starch compared with other starch substrates, determine the optimum pH, temperature, effect of heavy metals.

MATERIALS AND METHODS

Source of Materials: The freshly harvested potatoes were purchased from Oba market, Akure, Ondo State, Nigeria. The various reagents and Equipment used for analysis were obtained from the Department of Food Science and Technology and Biochemistry (Enzymology) laboratories in the Federal University of Technology, Akure. The equipment used were weighing balance (Ohaus-Adventuer SC), pH meter (Mettlermp 220), Refrigerated centrifuge (Beckman model IJ-6), and Shaking incubator (Stuart U.K), Magnetic stirrer, Water bath (Gallen Kamp) and UV/Visible spectrophotometer. All the reagents and chemicals used in the analysis were of analytical grade.

Extraction of Starch from Substrate: TheIrish potato was obtained from Oba market, Akure, Ondo State, Nigeria. It was peeled, immersed in water for 4 h and grinded. A muslin cloth was used to separate the starch. The starch was allowed to settle, and the water decanted from the starch to get the starch slurry. The starch slurry was dried at 55^{o}C for 6 h. After drying, the starch was kept in a cool and dry place before it was used for analysis.

Protein determination: Protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard. To determine the protein concentration, 20µl was taken from the enzyme stock and dispensed into a test-tube. 780µl of distilled water was added. Afterwards, 200ml of Bradford's reagent was added and allowed to stand for 20 minutes and then read at 595nm.

Immobilization of enzymes: 10 ml of chitosan which contains 30mg/ ml of phosphate buffer 7 was prepared. The chitosan solution was mixed with 5mg/ml of alpha amylase enzyme (10ml of support, 5ml of enzyme). It was continuously stirred gently for 16hrs in a dark room. It was filtered and rinsed leaving the residue in the filter paper which can later be re-used.

Amylase Activity: Alpha- amylase activity was estimated by the 3, 5 Dinitrosalicylic acid (DNSA) method of (Bernfield, 1955). It measures the increase in the reducing power of the digests in the reaction between starch and the enzyme. Appropriately diluted 200µml of enzyme was added to 200µml of 1% (w/v) soluble starch which was dissolved in appropriate buffer solution (phosphate buffer, pH 7). The reaction mixture was made in triplicates. The reaction tubes were incubated at 30°C temperature for 15 minutes. Then 2ml of colour reagent (DNSA) was added to the reaction mixture and placed in boiling water bath (Gallenkamp) for 5 mins. The tubes were allowed to cool at room temperature. After which 2ml of distilled water was further added to the cooled tubes and absorbance at 540nm was measured using spectrophotometer

Control tube consisted of 200µml buffer solution plus 200µml soluble starch solution.

The assay was also carried out as explained above. All assays were done in triplicates. One unit of alpha amylase activity is defined as the amount of enzyme required to produce one micromole of glucose from starch under the assay condition.

Effect of temperature: The determination of temperature on enzyme activity was carried out by incubating the starch substrate and enzyme solution for 15mins at varying temperatures (30^{o}C to 90^{o}C) (Abu et al 2014).

Effect of temperature on Alpha-amylase stability: The thermal stability of the enzyme was determined by incubating about 4 ml of the pooled enzyme fractions at temperatures ranging from 30^{o}C to 90^{o}C without the substrate for 2 hours. At intervals of 30 mins, aliquot of 1 ml of the incubated enzyme was assayed for residual activity, 200uml of DNSA was added and was boiled at 100°C for 5mins and 2000uml of distilled water was added to measure absorbance at 540 nm (Abu et al 2014)

Effect of pH: Various buffers were prepared at varying pH values ranging from (4.0-11.0) and 200µl each was dispensed into test tubes along with 200µl of substrate (soluble starch) and incubated for 15minutes. After which the absorbance was read at 540nm (Yang *et al*., 1996) and (Rehman *et al.,* 2011).

Effect of pH on stability of the immobilized enzyme: The stability of purified enzyme was determined by measuring the residual activity of the enzyme being incubated for a specific period at different pH (4.0-11.0) values at temperature 30^{o}C based on the method applied by (Yang *et al*.,1996) and (Rehman *et al.,* 2011). This was determined by mixing aliquots of 2 ml enzyme with 2 ml buffer solution earlier described. The mixture was incubated 30^{o}C for two hours. At 30mins interval, aliquot of 1 ml from the mixture was assayed for residual activity under standard assay condition except that each buffer solution was used to prepare 1% soluble starch used as substrate in assaying the enzyme activity, 200µml of DNSA was added and was boiled at 100°C for 5mins and 2000µml of distilled water was added to measure absorbance at 540 nm

Effects of metal salts: A stock solution of 0.01M of $HgCl_2$ and EDTA were prepared. Two milliliter of each salt solution was mixed with 200uml of enzyme solution. The mixture was incubated for 15mins at30°C. 1ml of the mixture was withdrawn and assay according to standard assay procedure. A stock solution of 0.01M of each salt was prepared. The salts used were $CaCl_2$, Fe^{2+}, Hg, Zn^{2+} and KCl_2 .Two milliliter of salts solution was mixed with 200uml of enzyme solution. And the same procedure for heavy metals was followed (Ojo and Ajele 2011).

Alpha Amylase assay using different substrates: The amylase activity was also assayed by measuring the reducing sugar released during the reaction, using complex polysaccharide substrates (corn starch, cassava starch, potato starch and cocoyam starch) according to the method described by Abu *et al*., (2014). The reaction mixture contained 200uml of 1% solution of the substrates separately prepared in 200uml phosphate buffer of pH (7.0) and 200uml of enzyme solution, 200uml of DNSA was added and was boiled at 100°C for 5mins and 2000uml of distilled water was added to measure absorbance at 540 nm

Statistical analysis: The effects of various catalytic and physicochemical parameters on the enzyme activity were evaluated using Microsoft excel.

RESULTS AND DISCUSSION

Effect of temperature on α- amylase activity: The effect of temperature on activity of the enzyme is shown on Figure 2. The upper temperature for enzyme activity is governed by the limits of enzyme stability. As well-known, immobilized enzyme is more resistant to heat and denaturing agents than the free form **(**Ohtsuka *et al.,* 1984). The activity of α-amylase at 30^{o}C was (67.69%) while there was a marginal decrease at 40^{o}C to (49.23%). It achieved stability and optimum activity

(77.53%) and (83.03%) at 50ºC and 60ºC before decreasing sharply at 70ºC with (28.92%).

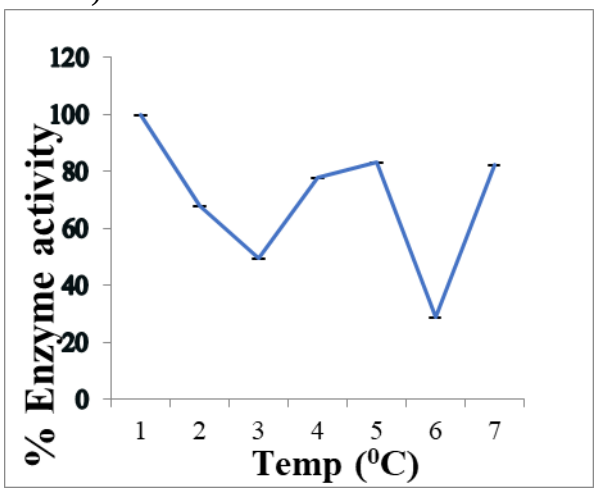

Figure 1. Effect of temperature on immobilized alpha amylase

This is in agreement with Enass (2009) which also recorded optimum enzyme activity of immobilized α-amylase at temperature 50-60ºC while also recording free α-amylase optimal activity at 40ºCA similar increase in the temperature optima had been found for the immobilized α-amylase by Yoshida *et al*.(1989), β-amylase (Emi *et al.*, 1990).

Effect of pH on α- amylase activity: Free enzyme has a different optimum pH than that of the immobilized enzyme on a solid matrix. This difference depends on the surface and residual charges on the solid matrix and the nature of the enzyme-bound pH value in the immediate vicinity of the enzyme activity. A change in the optimum pH normally accompanies the insolubilization of enzymes, depending upon the polymer used as support. Since the enzyme activity is markedly influenced by the environmental conditions, especially pH, the behavior of enzyme immobilization is useful for understanding the structure-function relationships of enzyme proteins. The enzyme was assayed at pH ranging from 4.0-11.0 at 30 ºC and its relative activity is presented on Figure 3. The enzyme was found to be slightly active in the acidic pH of 4.5 (about 78%), optimal activity at pH 7-8 while there was reduced activity of enzyme at pH 5 and 6 but a slight increase in its activity at pH 9. This report tallies with free and immobilized α-amylase optimal activity at pH 7-7.5 reported by Enass, (2009) and was consistent with the previous immobilization processes (De Cordt *et al.*, 1993).This may be due to the immobilization carried out on the enzyme. The immobilized enzyme activity vary from acidic to slightly alkaline environment which is similar to soybeans beta-amylase with pH 4.5 (Ajele, 1997), rice beta-amylase activity at pH of 5.5-6.5(Babu *et al*. 1977) while the optimum pH of 6-9 was reported by Bartholomew *et al*. (2000) for raw potatoes beta-amylase

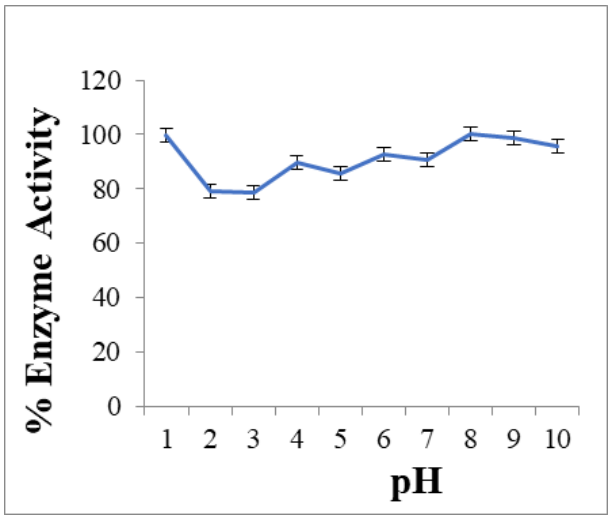

Figure 2. Effect of pH on immobilized alpha amylase

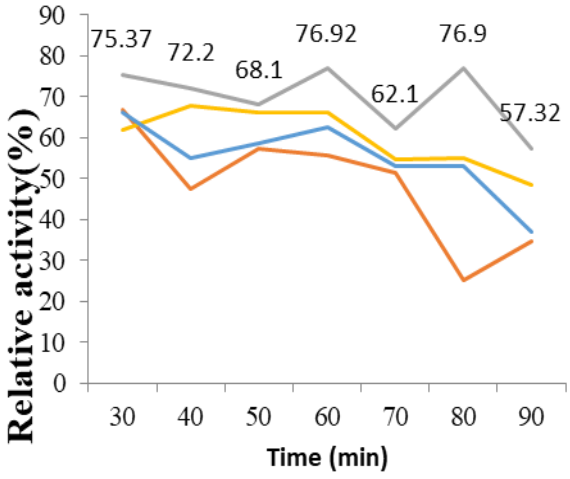

Figure 3. Thermal stability on immobilized alpha amylase

Effect of temperature on α- amylase stability

The effect of temperature on the thermostability of α- amylase at various temperatures is shown in Figure 4. The relative residual activity of 76.92 and 76.9 % at 60 and 80 °C after 60min of incubation. Also, after 90min of incubation, 57.32% of the enzyme relative activity was retained at 80 °C after which there was a decline in residual activity. This research work reveals the thermostability of immobilized α-amylase with an optimum activity at 40°C. The optimum temperature stability of immobilized beta-amylase varies from 50 and 80°C after an hour and half of incubation. The α-amylase retained 60% of its activity at 80°C corresponding to Abu *et al.*, (2014*)* reported for β-amylase purified from *Bacillus subtilis.* The temperature profile of the immobilized enzymes was much broader at higher temperatures demonstrating the effectiveness of the polymer protecting the enzymes. Also, the immobilized enzymes (natural polymer and synthetic polymer) showed higher thermal stability.

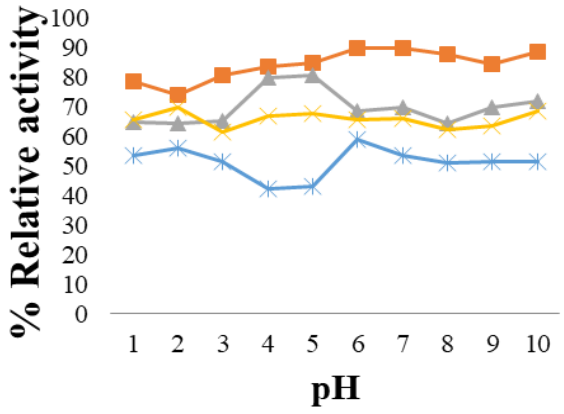

Figure 4. pH stability on immobilized alpha amylase

Effect of pH on α- amylase stability

The effect of pH on the residual activity of the immobilized enzyme at pH 4.0-11.0 against time of incubation at room temperature is illustrated in Figure 5. At about 60 min of incubation, it was examined that the enzyme retained 219.85%, 121.61%, 121.61% and 186.65% relative activity at pH 9, 5, 4.5 and 4 respectively. The enzyme was relatively active for almost 2 h at pH 7 but thereafter activity gradually declined. The results is similar to partially purified amylase from *Heliodiaptomus viduus* by (Dutta et al., 2006) with pH stability in the ranges reported in the result above.

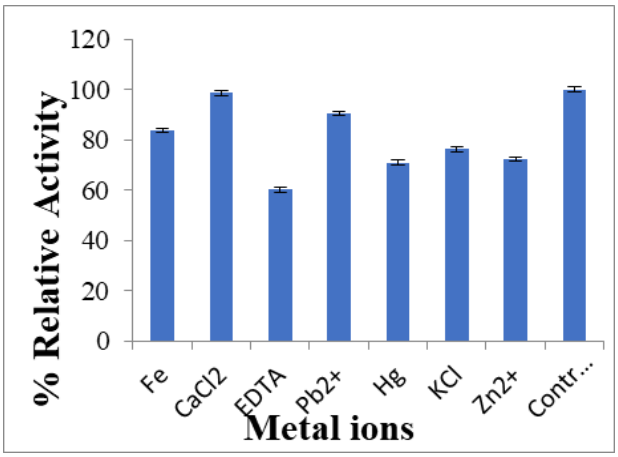

Figure 5. Effect of metal ions on immobilized alpha amylase

Effect of metal ions on α-amylase activity

The effect of metal ions on α-amylase activity as shown in figure 5 indicate that the enzyme was relatively active in the presence of KCl, Ca ions and EDTA retaining activity even though they were still inhibited below the control while other metal ions such as Fe^{2+}, Pb^{2+}, Hg^{2+} and Zn^{2+}reduced the enzyme activity slightly below the control. Inhibition or enhancement of amylase activity can be enabled due tothe presence of specific metallic ions along with food content and their rate of hydrolysis concurring with Sarowar et al., (2012) reports of β-amylase from Radish (*Raphanus sativus* L.) root.

Effect of substrates on β-amylase activity

Affinity for different carbohydrate substrates by the enzyme is shown in figure 6. Cassava starch hydrolysis by the enzyme was high reaching residual activity levels of 98.01% followed by Irish Potato starch 57.70%, Cocoyam 43.60% in relation to Corn starch (control). The effect of carbohydrate substrates (cassava, Irish potato, cocoyam and corn starch) on the hydrolytic activity of the enzyme varies but indicate that the immobilized enzyme can hydrolyze their starch effectively with high retention of residual activity.

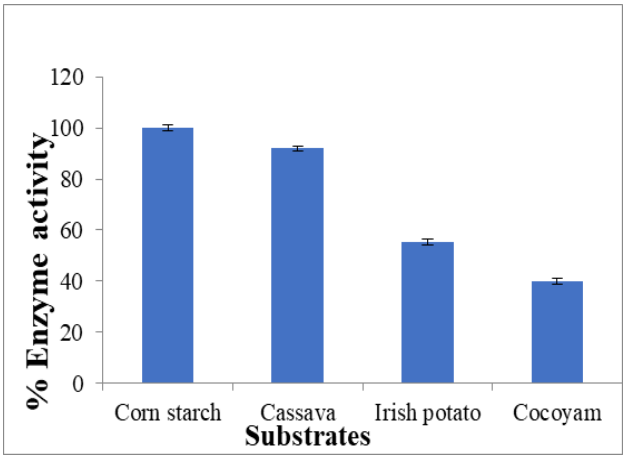

Figure 6. Effect of substrates on immobilized alpha amylase

Table 1: Immobilization Table showing the result of enzyme and protein concentration beforeand after immobilization

Determinants	Before Immobilization	After Immobilization
Protein concentration (ml)	0.021	0.0068
Enzyme Assay (µg/mm)	0.0045	0.0087

CONCLUSION

The result from the study has shown that Alpha-amylase can be immobilized on polymeric support resulting in varied ability of the enzyme. Comparing the effect on the various characteristics like effect of temperature, substrates, thermal stability and pH stability, effect of metal ions and substrates on free and immobilized alpha-amylase, it was observed that the immobilized enzyme had an improved activity than the free enzyme. The immobilized enzymes on polymeric support is prepared for purpose of repeated use and the possibilities of continuous reaction system. One of the most important properties is the temperature and pH stability when they are used in some medical and industrial applications. The immobilization of the enzymes improves this property as well as many other properties.

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