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Hydrolytic Response of Beta-Amylase to Selected Starches when Adsorptively Immobilized on Agarose Gel

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Abstract

Immobilization has been an effective tool commonly employed to maintain and/or improve the activity of industrially important enzymes and for ease of recovery and re-use. The present study was aimed at evaluating the effect of temperature and pH on immobilized β-amylase, its pH and temperature stability and the effects of different substrates and metal salts on the enzyme. Enzyme activity and protein concentration were also determined before and after immobilization. Immobilization was carried out on agarose gel support using adsorption method. The enzyme had its optimal activity at pH 4.5 and temperature 60 ºC with percentage relative activity of 100% each. The temperature and pH also had effect on the stability of the enzyme. The enzyme was more stable at pH 4.5 and temperature 60 ºC as it gave its optimum activity at these points while it also exhibited some measure of stability at other pH and temperatures. The results of the substrates specificity and effect of metal salts showed that immobilized β-amylase acted better on potato starch compared to other substrates. Also, Fe^{2+} had the highest inhibitory effect on the enzyme compared to other metal salts while other metals showed non-inhibitory effects.

Keywords: *Immobilization, β-amylase, hydrolysis, activity, selected starches*

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Introduction

Enzymes are biological substances or macromolecules that are produced by a living organism which act as catalysts to bring about specific biochemical reactions (Enujiugha, 2020). These are like the chemical catalysts in a chemical reaction which help to accelerate the biological or biochemical reactions inside as well as outside the cell. These are generally known as "Biocatalysts" (Gupta *et al*., 2010). Enzymes are highly efficient, which can increase reaction rates by 100 million to 10 billion times faster than any normal chemical reaction. The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Various carbohydrases, primarily amylases and cellulases, used in industries such as starch, textile, detergent, and baking industries represent the second largest group of enzymes. Most enzymes are much larger than the substrates they act on, and only a small portion of the enzyme (around 2-4 amino acids) is directly involved in catalysis (Gupta *et al*., 2010). However, most enzymes are relatively unstable, their costs of isolation are still high, and it is technically very difficult to recover the active enzyme, when used in solution, from the reaction mixture after use (Abu *et al*., 2022a, b). This has led to the introduction of immobilization. The term "immobilized enzymes" 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" (Shipra et al., 2011). The introduction of immobilized catalysts has, in some cases, greatly improved both the technical performance of the industrial processes and their economy. Quite often when an enzyme is immobilized, its operational stability is improved (Batista-Viera and Brena, 2013). 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). Immobilization is important to maintain constant environmental conditions in order to protect the enzyme against changes in pH, temperature and ionic strength (Mansour and Dawoud, 2003).

Amylases are enzymes of great importance in the food industry (Enujiugha *et al*., 2002; Abu et al., 2014). This has therefore made them the subject of intense application as well as basic research. Amylases have been reported to occur in microorganisms, plants and animals (Gupta *et al.*, 2010).

Ekunsanmi (2002) described three kinds of amylases based on their ability to breakdown amylose. They include alpha amylase, beta amylase and amyloglucosidase. Amylases have potential application in a number of industrial processes such as in the food, textiles, paper industries, bread making, glucose and fructose syrups, detergents, fuel ethanol from starches, fruit juices, alcoholic beverages, sweeteners (Gupta et al,. 2010), digestive aid and spot remover in dry cleaning. Bacterial α-amylases are now also used in areas of clinical, medicinal, and analytical chemistry. The most widely used thermostable enzymes are the amylases in the starch industry (Octavio *et al.*, 2000). Beta amylase (EC 3.2.1.2) is another form of amylase synthesized by bacteria, fungi, and plants (Abu et al., 2014). β-amylase catalyzes the hydrolysis of the second α-1, 4 glycosidic bond, working from the non-reducing end, cleaving of two glucose units (maltose) at a time (Morrison et al., 1993).

Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time. Several natural and synthetic supports have been assessed for their efficiency for enzyme immobilization. The immobilization of enzymes has proven particularly valuable, because it has allowed enzymes to be easily reused multiple times for the same reactions with longer half-lives and less degradation and has provided a straight forward method of controlling reaction rate as well as reaction start and stop times. It has also helped to prevent the contamination of the substrate with enzymes, protein or other compounds (Spahn and Minteer, 2008). Adsorption involves the enzyme being physically adsorbed onto the support material (polymer matrix). It involves bathing the support in an aqueous solution containing the enzyme ensuring that it penetrates the pores of the solids. Typically, the volume of the aqueous solution used is just enough to wet all the support particles. Excess water is then removed by drying at low pressure and room temperature or by lyophilization (Mansour and Dawoud, 2003). Adsorption/carrier-binding method uses water-insoluble carriers such as polysaccharide derivatives, synthetic polymers and glass.

Cocoyam is an important crop only in warm, humid forest areas because of its need for high annual rainfall and a long wet season. Taro (*Colocassia esculenta*) is a tropical tuber crop largely produced for its underground corms and consumed in tropical areas of the world. Taro is also rich in gums (mucilage) and up to 9.1% crude taro mucilage has been extracted from taro corms (Oluwamukomi and Akinsola, 2015). Because of the small sizes of its starch granules, taro is highly digestible, and it a very good source of starch for domestic and industrial applications (Lawal, 2004). The high carbohydrate content of cocoyam makes it a highly under-utilized tuber when compared to cassava and potato in terms of industrial applications. Cocoyam starch had been extensively studied (Lawal, 2004). Despite their nutritional, industrial and health importance, cocoyam has not gained sufficient research attention to enhance its potential. As such these tubers have a poor position on the food security profile of countries.

Potato is a very accommodating and adaptable plant and will produce well even without ideal soil and growing conditions (Abiodun and Enujiugha, 2021; Abu et al., 2022b). The potato starch has a higher viscosity than wheat and maize starches. It can be used as a binding agent in ice-cream, biscuits, cake mixes and dough (Abu et al., 2022a). This study is primarily designed to expose β-amylase to immobilization as this will help to increase the utilization of some tuber crops such as cocoyam and potato that have been categorized as underutilized or abandoned and also to reduce post-harvest losses of the tubers. It is also important to enhance the production of diverse products from underutilized tubers.

Materials and Methods

Source of Materials: The β-amylase enzyme was purchased from British Scientific laboratory, Lagos state, Nigeria. Agarose support was gotten from Pascal laboratory, Akure, Ondo state, Nigeria. The various reagents and chemicals used were obtained from the Department of Food Science and Technology and Biochemistry (Enzymology) Laboratory in Federal University of Technology, Akure. All other chemicals and reagents used for the analysis were of analytical grade.

Enzyme activity: Beta- amylase activity was estimated by the 3, 5 Dinitrosalicylic acid (DNSA) method of Bernfield (1955). The activity of extracellular amylase was estimated by determining the amount of reducing sugars released from starch. Enzyme (β-amylase) solution was prepared by dissolving 2mg/ml of the enzyme in 1ml of acetate buffer. Starch solution was prepared from 1% (w/v) soluble starch in distilled water. 100µl of the enzyme extract, 100 µl of 1% starch solution were added in test tube and the mixture was incubated at 30ºC for 15 minutes. 100µl of DNSA was added to terminate the reaction and the reaction mixture was boiled at 100ºC for 5 minutes. The amount of reducing sugars in the final mixture was read using a spectrophotometer at 540 nm One unit of enzyme activity (U) was defined as the amount of the enzyme liberating one µ mole of reducing sugars as glucose/min (Singh, 2014).

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.

Enzyme immobilization: 10ml of enzyme solution was prepared in acetate buffer of pH 4.5. It was then divided into two stocks, 5ml each. 100µl was taken from one stock and 400µl of buffer was added. This mixture is used to determine the enzyme activity and protein concentration. Prior to immobilization, agarose gel was properly washed with distilled water. 1g of the agarose gel was suspended into 10ml of 50mM acetate buffer at pH 4.5 containing 0.5g of β-amylase and stirred gently for twelve hours at cold condition (Nwagu *et al*., 2011). After stirring it was decanted and the gel was collected by filtration. The filtrate was then freeze-dried and the dried support was redissolved in acetate buffer and filtered to obtain the immobilized enzyme solution which was tested for enzyme activity and protein concentration.

Effect of temperature on activity: The effect of different temperatures on the immobilized enzyme was monitored. This was done by dispensing 200µl of the enzyme and 200µl of the substrate (soluble starch) into a test-tube in which the enzyme

activity was monitored at varied temperature of 30ºc to 90ºC. The absorbance was then read at 540nm (Abu et al., 2014).

Effect of pH on activity: 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 15 min. After which the absorbance was read at 540nm (Abu et al., 2014).

Thermal stability of enzyme: The thermal stability of the enzyme was determined by incubating 1ml of the pooled enzyme at varying temperatures between 30ºC to 90ºC without the substrate for two hours. At 30 min intervals, aliquot of 200µl of the incubated enzymes were assayed for residual activity (Ojo and Ajele, 2011).

pH stability of enzyme: Aliquots of 1ml enzyme solution were mixed with 1 ml of the buffer at room temperature for 2 h. At thirty minutes interval, aliquot of 200µl from the mixture was assayed for residual activity under standard assay conditions. The procedure was repeated for various pH ranging 4 -11 (Ojo and Ajele, 2011).

Effects of metal salts: The effect of metal salts was carried out by mixing 100µl of each salt solution to 300µl of enzyme solution. The blank contained 100µl salt and 100µl buffer. The mixture was incubated for 5min at room temperature. A 200µl of the mixture was withdrawn and assay according to standard assay procedure. A control was also prepared containing only the assay buffer (acetate buffer) and the same procedure was repeated.

Effects of different substrates: The amylase activity was assayed by measuring the reducing sugar released during the reaction, using complex polysaccharide substrates (corn starch, cassava starch, Irish potato starch and cocoyam starch) according to the method of (Abu *et al*., 2014) . The reaction mixture contained 200µl of 1% solution of the substrates separately prepared in 50mM sodium acetate buffer of pH (4.5) and 200µl of enzyme solution.

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

Results and Discussion

Protein and Enzyme Activity before and after Immobilization

The results presented in Table 1 demonstrate the protein concentration and activity of β-amylase before and after immobilization. It was observed that there was an increase in protein activity from 0.203 of un-immobilized β-amylase to 0.204 of immobilized β-amylase. Also, there was an increase in the activity of β-amylase from 0.00031(un-immobilized) to 0.0036 (immobilized). This relatively shows that immobilization has a more productive effect on the enzyme compared to its natural state. Tavano et al., (2013), gave a result that corresponds to the result stated above, where the β-amylase immobilized on agarose support gave an increased activity when compared with free enzyme.

Effect of Temperature on Immobilized β-amylase

The optimum temperature for immobilized B-amylase is shown on Figure 1. This was recorded as 60ºC. There was a decline in relative activity at temperature 70ºC and 80ºC and afterwards the relative activity increased at 90ºC. This implies that immobilized β- amylase is more effective when incubated at 60ºC. This is in agreement with Tavano *et al*., (2013) which also recorded optimum enzyme activity of immobilized β-amylase at temperature 60ºC.

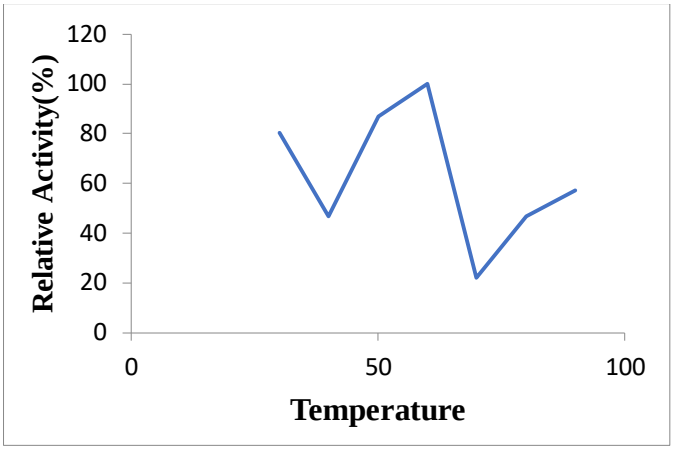

Figure 1: Effect of Temperature on the Relative Activity of Immobilized β-amylase.

Effects of pH in Immobilized β-amylase: The effect of various pH on immobilized β-amylase on incubation for 15minutes at 30ºC is shown on figure 2 The curve presented shows that the optimum relative activity was observed at pH 4.5 which is acidic. The immobilized enzyme was found to be active in the acidic pH range of 4.5-5.0 and had an optimum relative activity (100%) at pH 5.0. There was a decline in the activity of enzyme from slightly acidic pH 6.0-6.8 but again experiencing a slight increment at neutral pH. Even though the bio-catalysis environment for free and immobilized enzyme was different, the results tallied with Kumar et al. (2012), reporting about a slight increase in the pH of the immobilized enzyme from the pH 5.5 for free β-amylase.

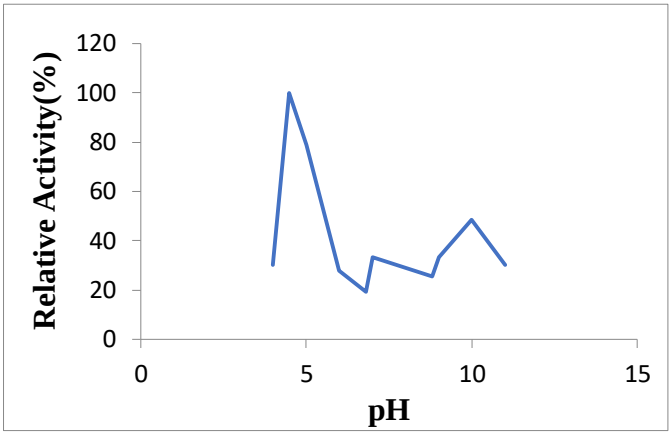

Figure 2: Effect of pH on the Relative Activity of Immobilized β-amylase

Table 1: Enzyme Activity and Protein Concentration before and after Immobilization.

Parameters	Before immobilization	After immobilization
Enzyme activity	0.00036	0.0031
Protein concentration	0.203	0.204

Effect of Temperature on stability of Immobilized β-amylase

The thermal stability of β-amylase at various temperatures from 30ºC to 90ºC is shown in Figure 3. At 60ºC, there was initially a decrease in activity after incubating for 30minutes. The relative activity began to rise gradually as the time lasted one hour. After another 30minutes, there was a slight decrease in the activity and on reaching two hours, the enzyme gave its optimum relative activity. Also, at 70ºC, between 0 to 30minutes there was an increase in the relative activity of immobilized β-amylase which later reduced after one hour at 90 minutes. There was a rise in the activity after 90minutes at 120 minutes. This result agrees with the result recorded by Palmieri et al., (1994) which recorded enzyme stability at temperature range of 50ºC-60ºC.

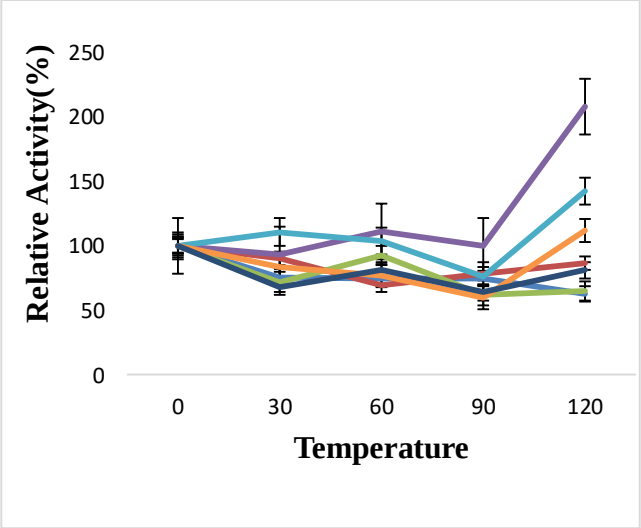

Figure 3: Effect of temperature on the stability of immobilized β-amylase

Effect of pH on stability of Immobilized β-amylase

Figure 4. shows the effect of pH on the stability of immobilized β-amylase. At pH 6.8, the enzyme incubated at 30ºC for two hours showed a slight increase in activity after 30 minutes which began to decrease after 30minutes till it lasted for 90 minutes. After 90minutes, there was a slight increase in its activity. The immobilized enzyme showed optimum relative activity at pH 4.5 on incubation at 30minutes. Afterwards, a slight decrease was observed at 90minutes of incubation. After 90 minutes, there was an increase in the activity of the enzyme. This reveals that at pH 4.5 and 6.8, immobilized β-amylase has its optimum performance on incubation for 30minutes and 120minutes. This goes along with the result shown by Kumar et al., (2012) in which recorded an optimum activity of pH 4.5-6.0.

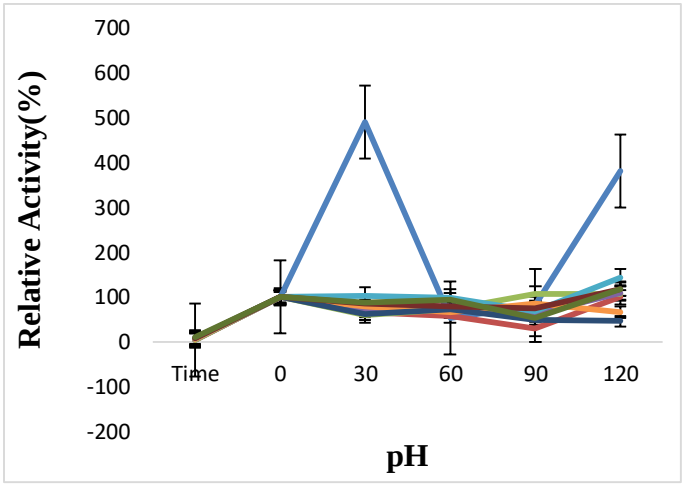

Figure 4: Effect of pH on the stability of immobilized β-amylase

Effects of Metal Salts on Immobilized β-amylase

Metallic cofactors are essential factors in the regulation of enzyme activity. Metal ions present in food substances can either enhance or inhibit amylase activity as well as the rate of digestion. Figure 5 shows the effects of various metal ions on the relative activity of immobilized β-amylase which shows that Tris salt inactivated the activity of the enzyme while Fe^{2+}, Na^{+} and Zn^{2+} enhanced the activity of the immobilized enzyme. The graph also shows that Fe^{2+} has the highest activity on the enzyme, showing that the presence of Fe^{2+} in food substances does not have an inhibitory effect on the enzyme. This result contradicts the result recorded by Titilayo and Azeez, (2012) which recorded enhanced activity with Na^{+} and Ca^{2+}.

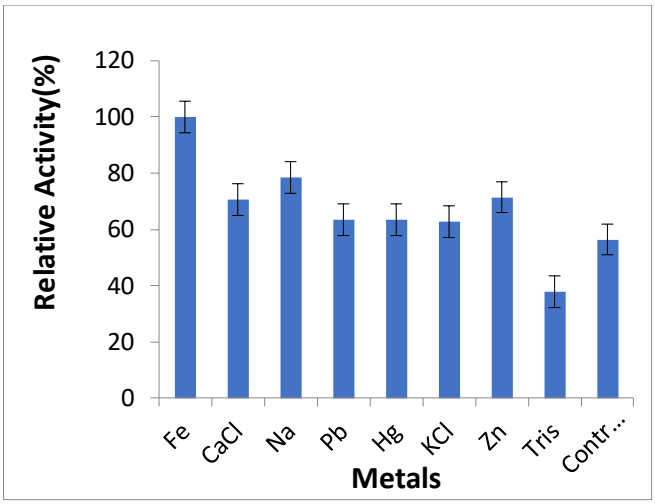

Figure 5: Effects of metal salts on immobilized β-amylase

Effects of Different Carbohydrate Substrate on β-amylase. Figure 6 shows the substrate specificity of immobilized β-amylase. Different carbohydrate substrates at 1% concentration each were used and it was discovered that Irish potato starch has the maximum relative activity and was followed by Corn starch. Cocoyam starch gave a residual activity of 87.5% of the control (Corn starch). Cassava starch gave the minimum relative activity of 53.1% of the control. From these results, it can be concluded that immobilized β-amylase performs better on Irish potato with a higher activity.

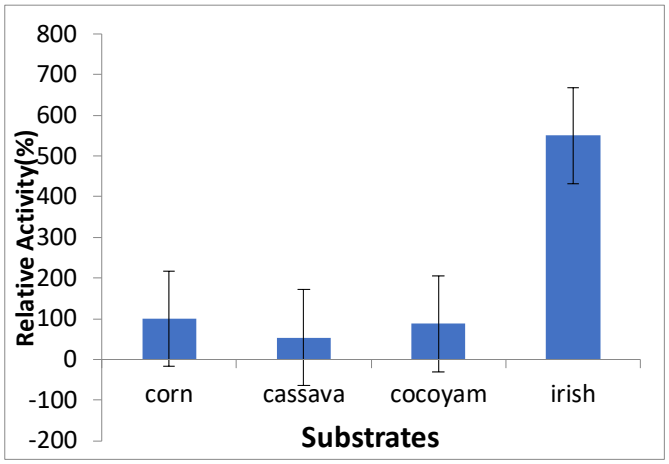

Figure 6: Effects of Different Carbohydrates substrates on Immobilized β-amylase.

Conclusion

The result from this study has shown that β-amylase can be successfully immobilized using agarose as surport.The immobilization of β-amylase has led to the improvement of its activity and stability especially on incubation within 30 minutes and 120 minutes when compared with unimmobilized enzyme. It has also shown that immobilized β-amylase has a better effect on irish potato starch than other starch substrates and also that Fe^{2*} has a high inhibitory effect on the immobilized β-amylase.

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