ENTRAPMENT OF β-AMYLASE ON SILICA SOL-GEL FOR ENHANCED ACTIVITY ON SELECTED STARCHES

Abu, T.F.A[1]**, Enujiugha,** V.N**, Odumosu,** E.

[1]Department of Food Science and Technology, Federal University of Technology, Akure, Ondo State
Corresponding author email: **topab2001@gmail.com.**

Abstract

Entrapment method of enzyme immobilization was applied to β-amylase on silica sol-gel and the effect of pH, temperature and metal ions on the activity of the immobilized β-amylase was investigated. Also, the effectiveness of the immobilized enzyme in the hydrolysis of selected starches was examined. Immobilization increased protein concentration from1.263-1.311 mg/ml and enzyme activity from4.8×10^{-3} to 6.1 ×10^{-4} Mm/min. The optimum pH and temperature was found to be 7 and 40^{o}C while the enzyme retained more than 70% of its activity almost 2 h incubation at different pH. The temperature stability revealed that the enzyme was stable at 70^{o}C and retained more than 60% to 80% of its activity at that level. Enzyme activity was activated and enhanced in the presence of KCl, Na^{+} and EDTA respectively while other metal ions such as Fe^{2+}, CaCl, Pb^{2+}, Hg^{2+} and Zn^{2+}showed only slight increases in the enzyme activity. Also the immobilized enzyme exhibited increased hydrolysis of carbohydrate substrates with the enzyme exhibiting more activity on substrates such as cassava starch, potato starch and cocoyam againstcorn starch (control). The study showed that immobilization of β-amylase on silica sol-gel improved the industrial application potential of the enzyme.

Key words: Immobilization, Entrapment, β-Amylase, Starch hydrolysis, Activity

Received: 06.03.2022 Reviewed: 06.04.2022 Accepted: 07.04.2022

INTRODUCTION

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 immobilization (Kragl, 1996). Several methods of immobilization exist which include; adsorption, covalent cross linking and entrapment. Entrapment method for immobilization consists of the physical trapping of the active components into a film, gel, fibre or coating (Bickerstaff, 1991). It involves the occlusion of the enzymes within natural or synthetic polymeric networks with a permeable membrane allowing the substrates and the products to pass, but it retains the enzyme inside the network (Bernfeld and Wan, 1963). The advantage of entrapment technique of enzyme immobilization is that it is fast, cheap and mild conditions are required for reaction process. Kennedy (1987) also stated that entrapment is the most preferable method because it prevents excessive loss of enzyme activity, increases its stability in microenvironment of matrix and protects enzyme from microbial contamination. Its limitation is in mass transfer. Increased stability, easy recovery, easy separation of reactant and product, repeated or continuous use of a single batch of enzyme (Raviyan *et al.*, 2003) which ultimately results in saving the enzyme, labour and overhead costs are some of the advantages of enzyme immobilization. Although the best method of immobilization differs from enzyme to enzyme, from application to application, from carrier to carrier, and from peculiarities of specific applications to others, yet the criteria for assessing the robustness of immobilized enzymes remain the same (Cao, 2005; Mateo *et al.*, 2007).Enzymes particularly amylases are employed in biotechnological applications in starch processing industries for the hydrolysis of polysaccharides such as starch into simpler sugar constituents (Mubarak, 2005). Other industrial applications such as production of ethanol and high-fructose corn syrup, detergents, resizing of textiles, modified

starches, hydrolysis of oil-field drilling fluids and paper recycling are also inclusive (Sumathi *et al*., 1995).

Potato is a tuber rich in starch about (8-28%) but with only about 1-4% protein (Brown, 2007) containing 25% amylose and 73% amylopectin and high phosphate content(Anon, 1985). 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.

MATERIALS AND METHODS

Source of Materials and instrumentation

Potato tubers were purchased from Oba market Akure, Ondo State, Nigeria and the starch was obtained by peeling the tuber, mashing, sieving and drying the slurry. Purified β-amylase was purchased at British Scientific Laboratory Lagos State and Silica from Pascal Scientific Laboratory Akure, Ondo State. 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), Shaking incubator (Stuart U.K), Water bath (Gallen Kamp) and UV/Visible spectrophotometer.

Enzyme Immobilization:five grams **(**5g) silica particles support was weighted, washed in distilled water and filtered. Subsequently, 2g of the washed silica particles was dissolved in an already prepared 10ml of enzyme-buffer mixture (0.05g/ml) and stirred constantly for 24h in an ice condition.

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 and 100 µl of 1% starch solution were added into 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 concentration: The amount of protein in the enzyme sample was estimated by the method of Lowry et al (1951) using bovine serum albumin as standard. The assay was performed by adding 2.1 ml of the alkaline copper reagent which was freshly prepared by mixing 2% Na_2CO_3 in 0.1 M NaOH / 1% $CuSO_4$.$5H_2O$ / 1% Na, K tartrate.$4H_2O$ (98:1:1, by volume) to 0.2 ml of the test sample in a test tube. The mixture was vortexed and allowed to stand for 10 min after which 0.5 ml of diluted Folin-Ciocalteu colour reagent was added. The resulting reaction mixture was vortexed and allowed to stand at room temperature for an hour after which the absorbance of the mixture was read at 590 nm against a reagent blank. The blank was made up of 0.2 ml of distilled water and appropriate volume of the diluents and colour reagent. When the reagents were separately prepared, 0.2 ml of the enzyme was pipetted into test tube. Approximately 1.56 ml of reagent C {Reagent A: 2% sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH) was used.

Effect of temperature on activity: The effect of different temperatures on the activity of 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 assayed 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, 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 excel and the utilization of standard error calculation.

RESULTS AND DISCUSSION

Enzyme assay of free and immobilized β-amylase: The protein concentration of β-amylase increased after immobilization from 1.263mg/ml to 1.311mg/ml. From Table 1, it can be seen that the protein concentration increased after immobilization of the enzyme while enzyme activity decreased from 4.8×10^{-3} to 6.1 $\times10^{-4}$ Mm/minwhich may be significantly influenced by the silica sol-gel entrapping the enzyme (Hwang *et al.*, 2012).

Effect of pH on β- amylase activity: The enzyme was assayed at pH ranging from 4.0-11.0 at 30ºC and its relative activity is presented on Figure1. The enzyme was found to be slightly active in the acidic pH of 4.5 (about 70%), had optimal activity at pH 7 while there was reduced activity of enzyme at pH 5, 6 and 8 but a slight increase in its activity at pH 9. This report differs from free β-amylase optimal activity at pH 5 reported by Ojo and Ajele.(2011). 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.

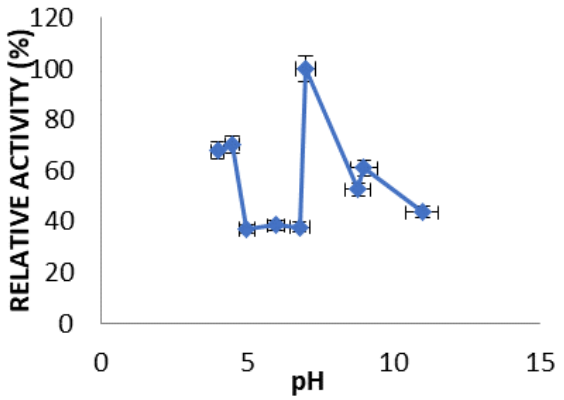

Figure 1. Effect of pH on β-amylase activity

Effect of temperature on β- amylase activity: The effect of temperature on activity of the enzyme is shown on Figure 2. The activity of β- amylase at 30ºC was (44.46%) while it increased at 40ºC to its optimum activity (79.25%), declined to 72.54% and 32.91% at 50 and 60ºC and increased slightly at 70ºC

with 42.28%. The enzyme retained 42.28% of its activity at 70ºC.This is at variance with Tavano *et al*., (2013) which also recorded optimum enzyme activity of immobilized β-amylase at temperature of 60ºC.

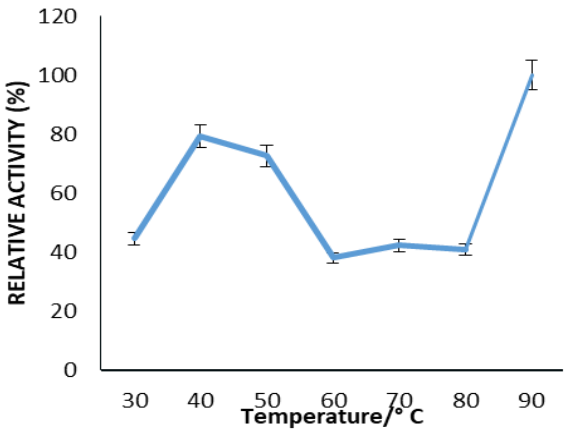

Figure 2. Effect of temperature on β-amylase activity

Effect of pH on β- 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 shown on Figure 3. At 60 min of incubation, it was found 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 but thereafter activity gradually declined. The results is similar to partially purified amylase from *Heliodiaptomus viduus* with pH stability in the ranges reported in the present resultby (Dutta et al., 2006).

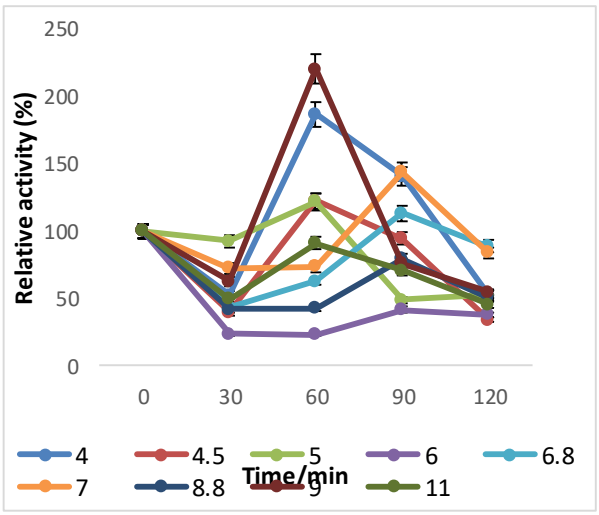

Figure 3. Effect of pH on β-amylase stability

Effect of temperature on β- amylase stability
The effect of temperature on the thermostability of β- amylase at various temperatures is shown on Figure 4.

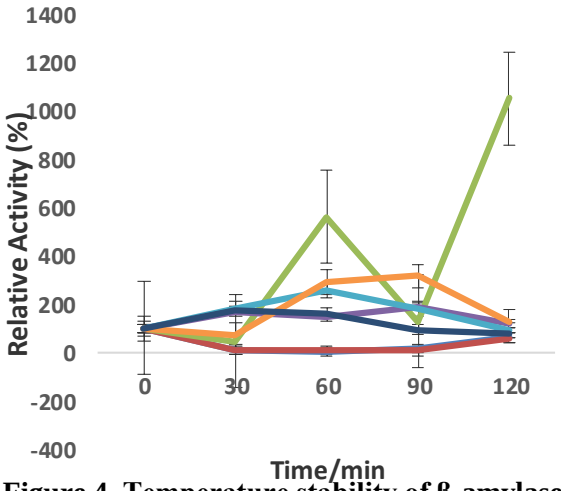

Figure 4. Temperature stability of β-amylase

The relative residual activities were 562 and 291.80 % at 50 and 80ºC after 60min of incubation. Also after 90min of incubation, 316.39% of the enzyme relative activity was retained at 80ºC after which there was a decline in residual activity. This research work reveals the thermostability of immobilized β-amylase with an optimum activity at 40ºC in comparison with potato leaf beta-amylase and other raw starch digesting enzymes as reported by Bartholomew *et al*. (2000). The optimum temperature stability of immobilized beta-amylase varied from 50 to 80ºC after an hour and half of incubation.The β-amylase from *B. subtilis* retained 60% of its activity at 80ºC corresponding to what Abu *et al*., (2014)reported for β-amylase purified from *Bacillus subtilis*.

Effect of metal ions on β-amylase activity
The effect of metal ions on β-amylase activity as shown in Figure 5 indicate that the enzyme was relatively active in the presence of KCl, Na ions and EDTA retaining activity of 946.56, 847.32 and 694.65% respectively above its normal activity (control) while other metal ions such as Fe^{2+}, CaCl, Pb^{2+}, Hg^{2+} and Zn^{2+}increased the enzyme activity slightly above 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. While K^+ activated the enzyme at different concentrations tallying with (Sarowar et al., 2012) and Ca^{2+}almost had no effect or negligible increase on the activity of the enzyme, NaCland EDTA enhanced the activity of the β – amylase, concurring with Sarowar et al., (2012) reports of β-amylase from Radish (*Raphanus sativus* L.) root.

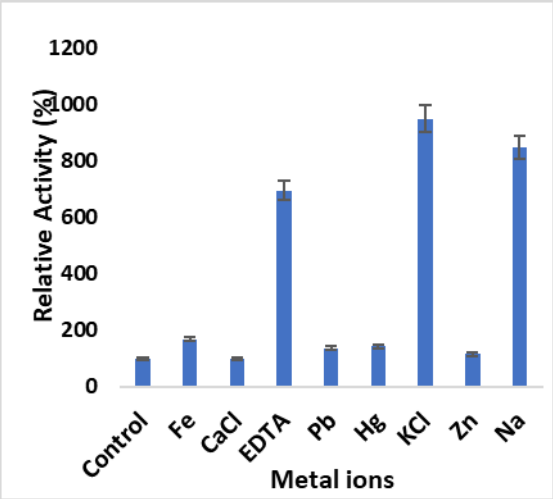

Fig. 5 Effect of metal ions on β-amylase activity

Effect of substrates on β-amylase activity

Affinity for different carbohydrate substrates by the enzyme is shown in Figure 6.

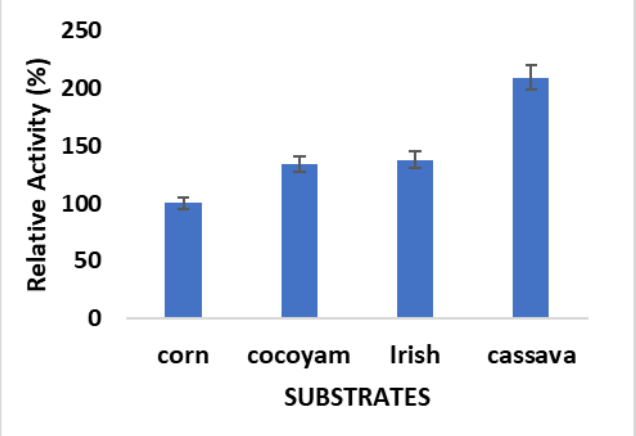

Figure 6. Effect of substrates on β-amylase activity

Cassava starch hydrolysis by the enzyme was high reaching residual activity levels of 209.01% followed by Irish Potato starch 137.70%, Cocoyam 133.60% 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 indicate that the immobilized enzyme can hydrolyze their starch effectively with high retention of residual activity.

TABLE 1 Enzyme assay of β-amylase before and after immobilization

Parameters	Before immobilization µg/ml	After immobilization µg/ml
Protein conc	**1.263**	**1.311**
Enzyme activity	**0.0048**	**0.00061**

CONCLUSION

Enzyme immobilization has a lot of advantages in achieving a better yield of catalytic reactions in any biological system and in industrial production of food products involving the use of enzyme to hydrolyse the system. In comparison with the free enzymes, immobilized β-amylase in silica sol gel has increased specificity, stability and improved activity with regard to pH, temperature, metallic ions, and some selected substrates.

RESEARCH HIGHLIGHTS

- The research made use of silica sol –gel for immobilization of beta amylase
- Some metal ions like EDTA had an enhancing property on the activity of the immobilised enzyme
- Various selected carbohydrate substrate was acted upon by the enzyme

REFERENCES

[1] Abu T.F.A, Enujiugha V.N, Sanni D.M and Bamidele O.S (2014**).** Purification and Characterisation of β-Amylase from *Bacillus Subtilis* Isolated from Fermented African LocustBean (*Parkia Biglobosa*) Seeds, *International Journal of Life Sciences Biotechnology and Pharma Research*, 3(4) ISSN 2250-3137

[2] Ajele, J.O., (1997). Some physicochemical properties of soyabean β-amylase. Nig. J. Biochem. Mol. Biol., 12: 61-66.

[3] Anon. International potato center. *Annual Report*, Lima, Peru, (1985), 23.

[4] Babu, R., A. Roy, Y. Gupta and M. Gupta, (1977). Fungi associated with deteriorating seeds of Cannabis of sativa. Curr. Sci., 46: 719-720.

[5] Bartholomew Okolo, N., S. Francis Ire, I. Lewis Ezeogu, U. Chukwudi Anyanwu and J.C. Frederick Odibo, (2000). Purification and some properties of a novel raw starch-digestingamylase from *Aspergillus carbonarius*. J. Sci. Food Agric., 81: 329-336
[6] Bernfeld P (1955). Amylase, α and β. Methods Enzymol. 1:149-1589
[7] Bernfeld P. and Wan J (1963). Antigens and enzymes made insoluble by entrapping them into the lattices of synthetic polymers Science, 142, 678-679.
[8] Bickerstaff, G.F (1991).*Enzymes in Industry and Medicine*, Cambridge University Press, UK.
[9] Cao L., (2005) "Immobilised enzymes: science or art?" *CurrentOpinionn in Chemical Biology*, vol. 9, no. 2, pp. 217–226.
[10] Hwang, E. T., Lee, B., Zhang, M., Jun, S. H., Shim, J., Lee, J., Kim, J., and Bock Gu, M., (2012) Green Chem. 14, 1884-1887.
[11] Kennedy JF, Enzyme technology In Biotechnology; Kennedy J.F,Cabral J.M S, Eds, VCH Publ.- Verlagsgesellschaft mbH: Weinheim, Germany, 1987: 7a
[12] Kragl, U., Immobilized enzymes and membrane reactor, in *Industrial Enzymology*, Godfrey, T. andWet, S., Eds., Macmillan Press, London, 1996.
[13] Lowry, O.H., Rosebrough N.J Farr A.L Randall R.J (1951). Protein measurement with the Folin phenol reagent. J. Bio. Chem., 193: 265-275.
[14] Mateo C, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan, and R. Fernandez-Lafuente, (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques, *Enzymeand Microbial Technology*, vol. 40, no. 6, pp. 1451–1463..
[15] Mubarak AE (2005) Nutritional composition and antinutritional factors of mung bean seeds (*Phaseolus aureus*) as affected by some home traditional processes. Food Chem., 89 (4): 489-495.
[16] Ojo O.O and Ajele O.J (2011). Isolation, Purification, Characterization and the Possible Involvement of Histidine and Cysteine in the Catalytic Mechanism of Beta-amylase Sourced from Cassava (*Manihot esculenta* Crantz) Peel, Pakistan Journal of Nutrition. 10 (9): 823-830
[17] Raviyan P, Tang J and BA Rasco, (2003). Thermal stability of alpha-amylase from Aspergillus oryzae entrapped in polyacrylamide gel, J. Argic. Food Chem, 51(18): 5462-5466.
[18] Sarowar J, Shaela P, Shariar S, DevSharma R and Habibur R (2012), "Effect ofMetal Ions, Chelating Agent and SHReagentson Radish (*Raphanus sativus* L.)Root β-Amylase", *J. Stress Physio. &Biochem*., Vol. 8, No. 3, pp. 180-188.
[19] Sumathi A, Malleshi NG and Rao SV (1995) Elaboration of amylase activity and changes in past viscosity of some common Indian legumes during germination. Plant Foods Hum.Nutr., 47: 341-347.
[20] Tavano O.L, Pessela BCC, Goulart AJ, Fernandez-Lafuente R, Guisan JM, MontiR. (2008): Stabilization of an Amylase from Neurospora crassa by Immobilization onHighly Activated Supports. *Food Biotechnol* , 22:262–75.