International Digital Organization for Scientific Research ISSN: 2579-0811
IDOSR JOURNAL OF BIOCHEMISTRY, BIOTECHNOLOGY AND ALLIED FIELDS 4(2): 86-94, 2019.

Evaluation of temperature, total protein and pH of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *E. coli*

Amasiorah V.I[1]., Oyeleke S. B[2]. Egwim E.C[2]. and Nna E[3].

[1]Department of Genetics and Genomics and Bioinformatics, National Biotechnology Development Agency (NABDA), Abuja, Nigeria.
[2]Department of Microbiology Federal University of Technology, Minna, Niger State, Nigeria.
[3]Safety Molecular and Pathology Laboratory (SMPL) Enugu, Nigeria.

ABSTRACT

This research evaluated the temperature, total protein and pH of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *E. coli.* The enzyme analysis showed 0.63µg/ml/seconds and 0.66µg/ml/seconds activity for the native and cloned amylase respectively. The native enzyme has a specific activity of 0.088 µg/ml/second/mg protein while that of the cloned was established as 0.102 µg/ml/second/mg protein. Both the native and cloned amylase shared an optimum temperature of 70°C though with different activities of 0.72µg/ml/seconds and 0.77µg/ml/seconds respectively. While a pH peak of 5.0 with activity of 1.06µg/ml/sec was recorded with the native amylase, two peaks (pH 5 and 7) were revealed by the cloned amylase enzyme with activities of 1.09µg/ml/sec and 1.08µg/ml/sec respectively. The cloned enzyme had a better activity and specific activity than the native. More so, it can be produced in larger quantity than the native by as much as six times compared to the native enzyme.
Keywords: Amylase, temperature, total protein, pH, native and cloned.

INTRODUCTION

The enzymes from microbes are favoured to those from both plants and animal sources since they are relatively inexpensive to manufacture, more convenient, dependable, predictable [1] and above all, they have short generation time.

For optimum commercial production of enzymes such as amylase, genetic engineering of the relevant microorganisms has become necessary and the common practice has been biotransformation [2]. Genetic transformation (biotransformation) is a practice by which cells that are competent such e.g. *Escherichia coli* (*E. coli*) absorb fragments of DNA via their cell walls and getting changed or transformed in the process [3].

Ordinarily, a characteristic cloning exercise may involve the cutting of the plasmid DNA conferred with a specific marker gene e.g. an antibiotic resistance and the gDNA with a particular restriction enzyme called endonuclease at only one position [4]. Subsequently, the target gene is amplified using a primer set in a process commonly referred to as polymerase chain reaction (PCR). Since the plasmid and the gene were cut with the same endonuclease, they possess opposite though sticky ends that permits them to fasten together [5].

Amylases are hydrolases that breakdown or hydrolyse starch mainly maltose and non-fermentable [6]. It catalyses the hydrolysis of starch molecules liberating diverse products, including dextrins and progressively, small glucose polymers [7], [8], [9]. Amylase has been used in various ways [10], [11]. Amylase is a key raw material in the fermentation industries namely Food, Brewing and Beverages [12], [13]. These enzymes are of utmost significant in biotechnology [14].

Amylase is an essential raw material in the food, beverage and other industries. Till date our local industries scattered across the federation still import the enzyme. It is estimated that Nigeria loses over five hundred million naira (₦200m) in foreign exchange annually in view of enzyme importation [15]. Expectedly, this amount has continued to increase due to increase in demand for amylase driven by-products and its over-head cost. The use of grains, the so-called malting or sprouting of grains [16] has continued to take its tolls on Nigerians as grains meant for food are used for amylase production in malting. This calls for the use of rDNA technology otherwise called genetic engineering or biotransformation. Meanwhile inexpensive and freely accessible agricultural wastes such as cassava peels [17], yam peels and grain dumps which currently are a form of hazard to the management of waste (solid) may be a rich source of amylolytic bacteria. Biotransformation method will not only eliminate the afore-mentioned lapses but also provides very efficient enzymes [18] for industries. In essence, this research work tries to examine and take advantage of some of these environmental and economic challenges by looking at the temperature, total protein and pH of the native amylase and cloned amylase.

Aim and Objectives

To evaluate temperature, total protein and pH of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *E. coli*

The objectives include:

To evaluate temperature of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *E. coli.*

To evaluate total protein of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *E. coli.*

To evaluate pH of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *E. coli.*

MATERIALS AND METHODS

Collection of Sample

The sample used for the isolation of *Bacillus subtilis* was soil. Five hundred grams (500g) of sample soil was collected from a cassava waste dumpsite in Minna, Niger State (Plate I) and taken to the Microbiology and Biotechnology Department of National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja for isolation and analysis. Subsequently, the isolated organism was taken to Safety Molecular and Pathology Laboratory, University of Nigeria Nsukka (UNN), Enugu Campus, Enugu State, for the biotransformation segment of the research work.

Preparation and Inoculation

Five grams (5g) of the soil sample was placed in a flask containing 45 ml of sterilised Ringer's solution and shaken to formulate a stock mix. The stock mix was placed onto a water bath maintained at 90°C for 1 hour to encourage the formation of spores as well as eliminating other unwanted microorganisms in the sample. The sample was serially diluted in six test tubes (from $10^{-1}$to 10^{-6}) in a Biohazard safety chamber (BSC) and the 10^{-6} dilution was used afterwards. The serial dilutions was achieved by adding one mililitre (1 ml) of the stock mix serially to 9 ml of Ringer's solution, placed in each of the six test tubes arranged on a test tube rack. The nutrient agar medium (100 ml) was prepared in agreement with the instructions of the manufacturers. The 100ml nutrient agar was supplemented with one gram of soluble starch in a conical flask to yield 1% starch nutrient agar. The medium was heated over an electric heater to enable the agar dissolve completely. With the aid of a syringe, 19 ml of the medium was dispensed in five slant bottles and autoclaved at 121°C for 15 minutes. Earlier on, the glass Petri dishes were washed, autoclaved at 121°C for 15 minutes and dried in a hot chamber oven at 180°C for one hour. The 19 ml agar-medium in the slant bottles was allowed to cool before inoculation with 1ml of the diluted soil sample from the 10^{-6} dilution. The already cooled inoculated media (though not gelled) in the slant bottles were emptied into the sterile glass Petri dish

and allowed to cool further in the BSC. The medium with the inoculated soil sample was incubated at 36.8°C in the Memmert incubator along with the control. The control consists of 1% starch nutrient agar only without the soil sample. Growth occurred after 24 hours.

Screening and Identification

The isolate was morphologically and biochemically characterized and identified up to genus level in accordance with the provisions of Determinative Manual of Bacteriology (Bergey's): identification flow chart (citation needed). The isolate was screened by sub culturing on fresh media (streak culture method); from 1% starch nutrient agar to another, 1% starch nutrient agar to 6.5% NaCl agar, 1% starch nutrient agar to Simmon citrate agar (SCA). Three plates were made for each medium including the control. From the Gram's stain result further inter media culture inoculation was made by streaking on the three media with the aid of a sterilised wire loop followed by incubation at 36.8°C. The morphology was reconciled by comparing the physical appearance with that of American Society of Microbiology (ASM) and subsequently a smear was made and viewed under a light microscope for Gram's stain. Further sub culture was made from the colony where positive result was obtained during the Gram's stain. Finally, the following biochemical tests; Gram stain, spore staining, starch hydrolysis, catalase, citrate utilization, 6.5% NaCl bacterial culture medium growth and incubation at 55°C were performed.

Gram Stain

Procedure: A smear of the isolate was made by placing a drop of normal saline on the slide. Then, some of the sample was collected aseptically with the aid of the wire loop, mixed with the normal saline and fixed on the burner. It was kept for about 30minutes to cool and dry before staining with crystal violet solution (the primary dye) and kept for 60 seconds. It was washed with tap water making sure that all the excess water was drained off to avoid diluting the mordant. Subsequently, it was treated with Gram's iodine solution for 30 seconds and gently washed with tap water. It was decolourised with alcohol, which was added drop wise on the tilted slide until all free colours (blue) were removed. Finally, the stuff was washed by gently dropping tap water on the slide. Cautiously, the slide was flooded with safranin (counter stain) for 60 seconds. The smear was viewed under a light microscope in an oil immersion. The Gram stain was positive with purple colour and the cells rod-shaped.

Spore Stain

Procedure: The spore stain was carried out according to Wirtz's method (modified). One drop of normal saline was placed on a clean slide. The culture (over 24 hours) was picked aseptically from the petri dish with the aid of a wire loop, mixed with normal saline and fixed. The smear was flooded with Malachite green and brought to steaming. It was allowed to remain for 3 minutes, washed with tap water before applying safranin solution. The stain was kept on the bench for 30 seconds, washed with water, dried and examined under oil immersion. The stain was positive with light green colour.

Extraction and Recovery of the Native and Cloned Amylase (Crude Amylase)

Procedure:

With the aid of a sterile wire loop, ten colonies each of the organisms (*Bacillus subtilis* and Transformed *E. coli*) were collected from the 24 hour culture, placed in two separate 250ml-Erlenmeyer flasks containing 1% starch broth and allowed to stand for 24 hours at 37°C.

The flasks were positioned in a shaker incubator set at 120 rpm at 30°C.

The extracellular enzyme solutions were obtained by centrifugation at 5000 rpm for 20 minutes using a high speed centrifuge.

The supernatant obtained was collected and tested as protein and crude enzyme.

Estimation of Total Protein of the Native and Cloned Amylase by BIURET Method

Procedure: The tubes (8) were labelled as blank, crude, purified and standard for each of the amylase. One point five

millilitre (1.5 ml) of the Biuret reagent was placed in each of the tubes. Then 0.025 ml (25 µl) each of the crude, purified and standards (Bovine albumin) respectively, was placed in the tubes as labelled and mixed thoroughly by several inversions. However, in blank 25 µl of the water was added instead. The tubes kept for ten minutes at 26°C (room temperature) and optical density read with the aid of a Biophotometer set at 550 nm. The Biophotometer was zeroed (initialised) with the reagent blank.

Partial Purification of the Native and Cloned Amylase

Procedure: The two organisms were propagated at 37°C for 24 hours in two separate 250ml-Erlenmeyer flasks containing 1% starch broth. The flasks were placed in a shaker incubator, set at 30°C and 120 rpm. Then, the mixture was spinned for 15 minutes at 10,000 rpm and the supernatant gathered as the foundation of crude amylase. Partial purification involves the addition of a solution of sixty five percent (w/v) sodium sulphate to the spelt out supernatant, spinning at 10,000 rpm and suspension of the pellet in 0.005 M Na_2HPO4 (pH 6.0). The partially purified amylase was used for further analysis of the enzyme.

Effect of Temperature on Native and Cloned Amylase

Procedure: The optimum temperature of the enzyme was approximated by examining activity of the amylase at different ranges of temperature of 20, 30, 40, 50, 60, 70, 80, 90 and100°C for 30 minutes. The protocol was as in effect of increasing starch on the enzyme activity however, each temperature range has its own control or sample blank.

Effect of pH on the Native and Cloned Amylase

Procedure: The optimum pH for the activity was determined by examining the activity among pH ranges; 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10 and 11. The assay was carried out using one percent starch in 0.05 M Na_2HPO4 buffer solution. The variation in pH was achieved by gradual addition of hydrochloric acid (0.1N) and 0.1N sodium hydroxide to attain acidity and alkalinity respectively. The method was as in effects of increasing starch concentration except that each pH has its own control or blank.

RESULTS AND DISCUSSIONS

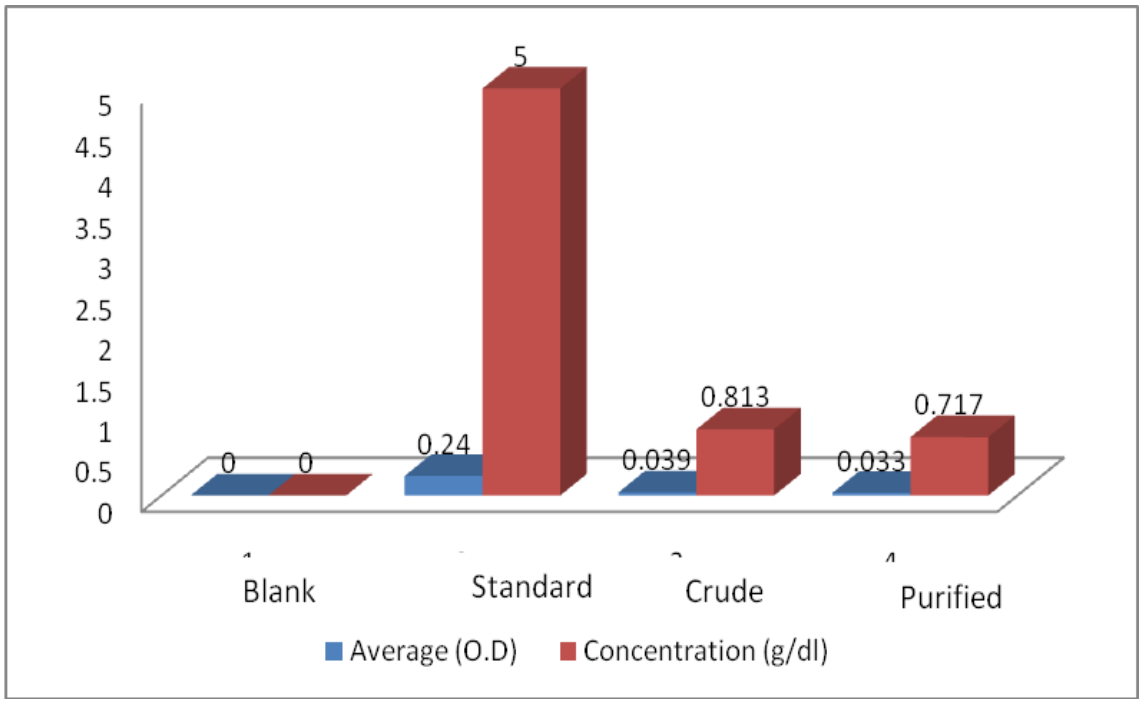

Figure 1: Total protein of the native amylase using Biuret method

The chart shows that the protein content of the crude enzyme is higher than the purified enzyme even as the purified records higher enzyme activity. This is in line with that of [18], which showed that the amount of protein content of α amylase decreases, during purification process hence, crude enzyme has a higher than the purified. Similarly, the purification process improved the specific activity of the amylase during the study. This finding is in agreement with that of [6] who reported that activity increases with amylase purification.

The protein content of the crude amylase for the cloned amylase is higher than the purified enzyme though with a reduced enzyme activity as seen in figure 2 below.

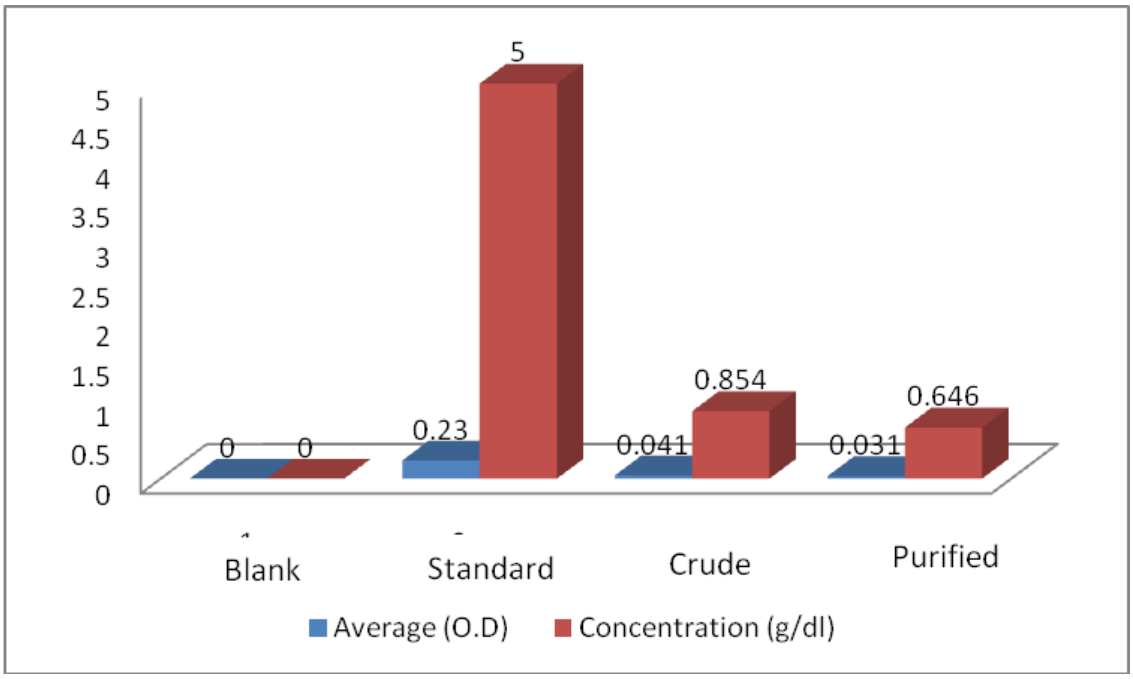

Figure 2: Total protein of the cloned amylase using Biuret method

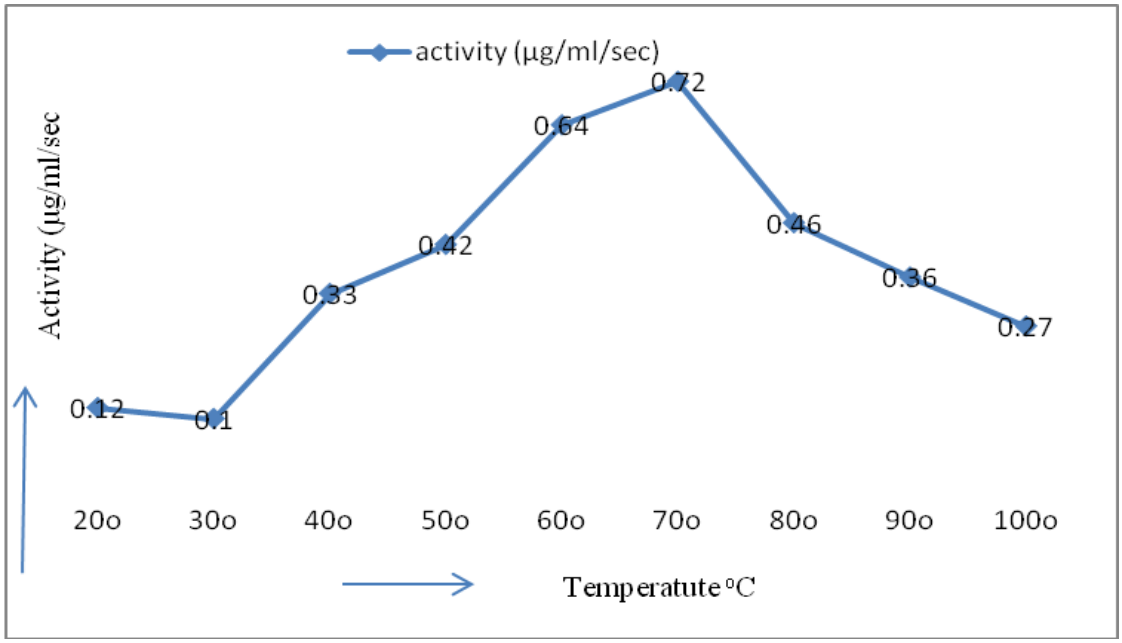

Figure 3: Effect of temperature on the activity of the native amylase

The result of the temperature increase on the native amylase is as portrayed in table Figure 3. The native enzyme demonstrated a wide range of temperature activity from 50-80°C with an optimum of 70°C and 0.72µg/ml/sec activity. [7], had earlier made the same submission of optimum temperature at 70°C. Again, the optimum temperature of 70°C agrees with the findings of [7] though at a different range of 50-70°C. Furthermore, record has shown that *Bacillus* sp. has a range of 50-90°C [3] though with activity is extensively reduced at temperatures lower than 50°C [4, 5]. This collaborates with the result of this research work for the native enzyme sourced directly from *Bacillus subtilis.* [6], reported 60°C optimum temperature for α-amylase in sprouting maize and rice.

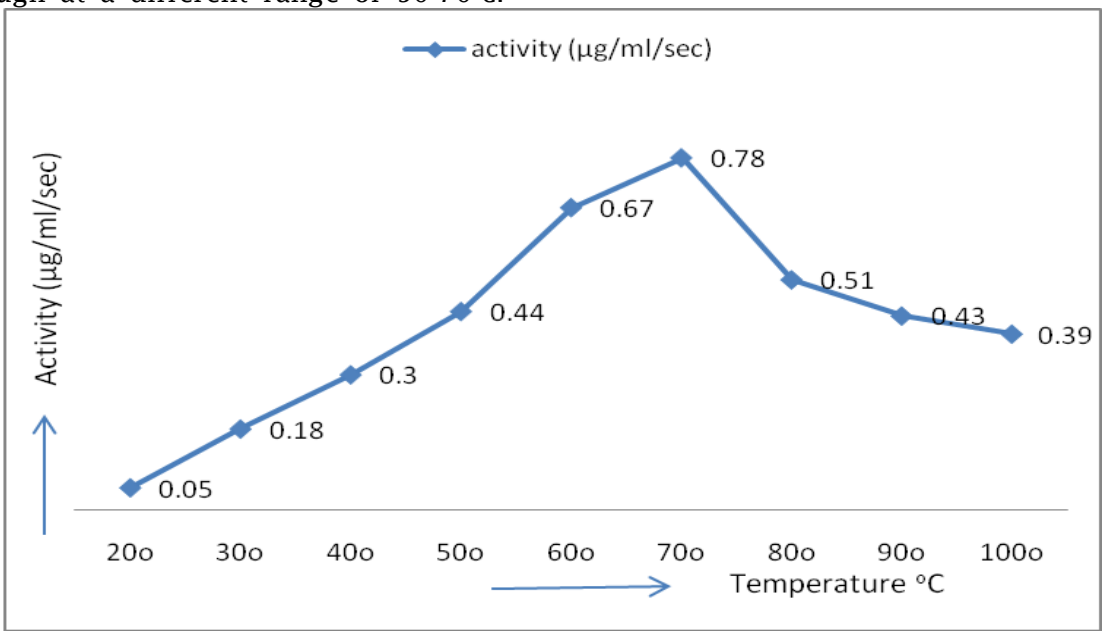

Figure 4: Effect of temperature on the activity of the cloned amylase

The result of increased temperature change on the activity of the cloned amylase had the same trend as the native. The cloned amylase had a range of 50-90°C with an optimum temperature of 70°C and 0.77µg/ml/sec activity. The pattern of the activity showed a decrease at temperatures lower than 50°C. These properties are important in starch processing industries which require an extensive range of temperature. Also, this attribute including the measure of activity puts the enzyme in the primary need of basic research, laundry or dishwashing. This is because the elimination of starch from porcelain and cloth is difficult at lower temperatures. So new detergents fortified with α-amylases, capable of surmounting this problem at low or moderate temperatures is an advantage [7]. This favours the enzyme relevance in inactivation processes like the baking industry [9]. It is important to note that the optimum temperature refers to the temperature at which the maximum number of active sites is available for reactivity.

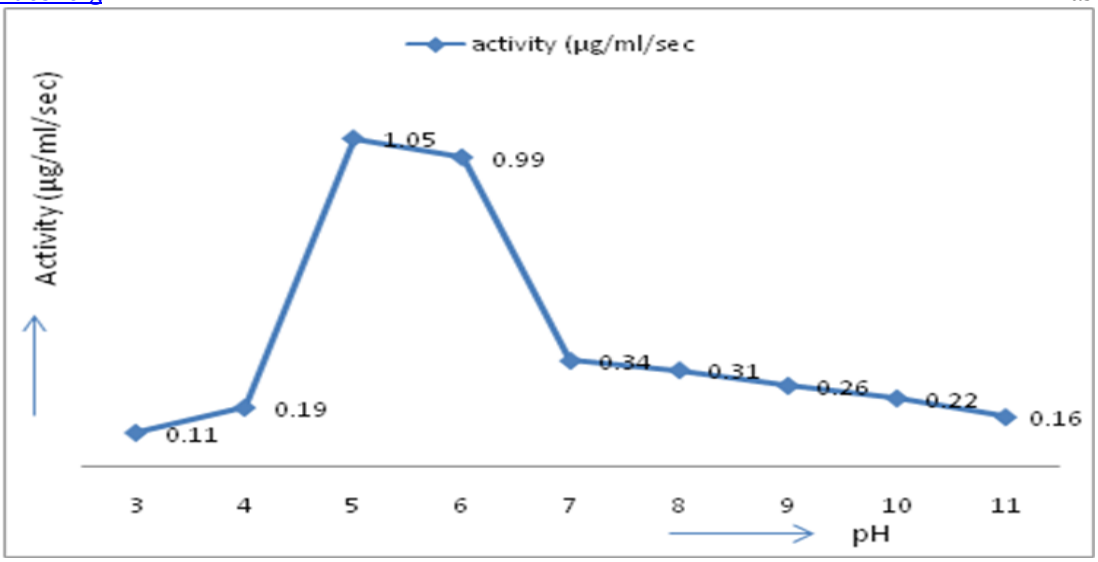

Figure 5: Effect of pH on the activity of the native amylase

The result of the pH rise on the activity of the native enzyme is as monitored in table 4.6a, Appendix K. The optimum pH for the native amylase was 5.0 with an activity of 1.06µg/ml/sec. It has been revealed that α-amylases from different *Bacillus* sources operates at a range of pH: 6.0-7.0 or 5.0-7.0 [9, 14, 15] though [15] posted a pH range of 5.0-7.0. In this study, the native enzyme had activity spread across the pH though decreased rapidly after pH 6 and sharp increase at pH 5.0. This observation indicates that the enzyme has a wide range of action though performs better in acidic medium. This view was collaborated by [12] who reported that the enormous activity of amylase at an assorted pH implies its extensive relevance in nature. This deduction agrees with the report of [4].

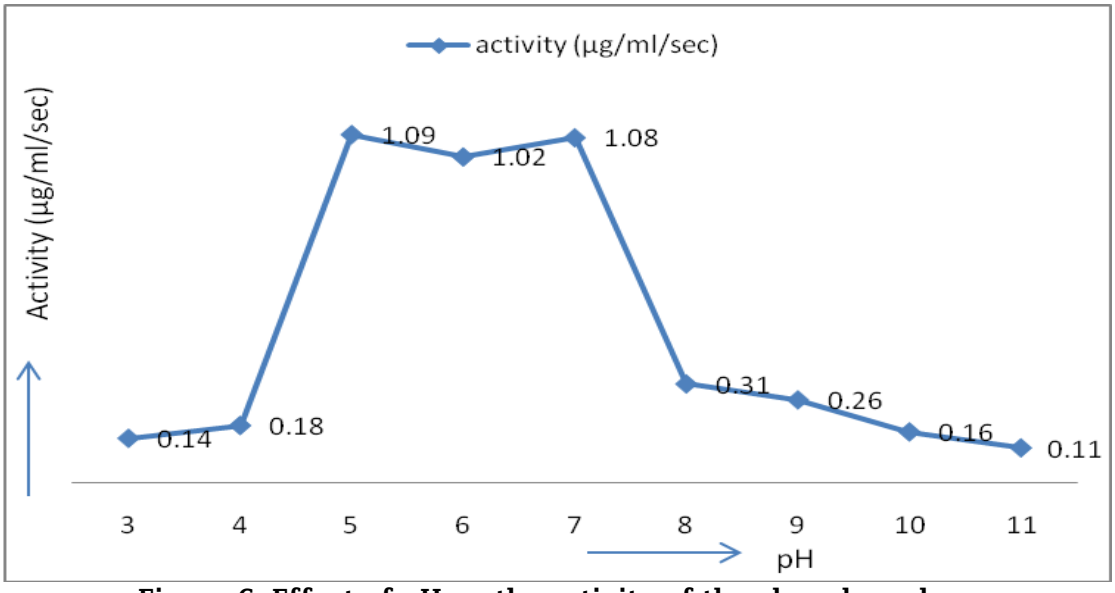

Figure 6: Effect of pH on the activity of the cloned amylase

However, the cloned enzyme showed two peaks of pH 5 and 7 with activities of 1.09µg/ml/sec and 1.08µg/ml/sec respectively. The different pH values revealed that the amylase can do well both in acidic and neutral media. This illustrates the importance of the enzyme in processes requiring acidity and

alkalinity since it retained activity across the board. The optimum pH is that at which the maximum numbers of active sites are available for reactivity.

CONCLUSION

This study of amylase gene cloning from the *Bacillus subtilis* isolated from a cassava waste dumpsite showed that the cloned enzyme compared favourably with the native enzyme following the usual catalysed reactions and in fact showing more returns. This is because the enzyme and specific activities of the cloned amylase were found to be higher than that of the native amylase. In addition, the cloned amylase exhibited sufficient thermostability with more glucose yield at various temperature ranges. A combination of these properties makes the cloned amylase very competitive for industrial and economic growth. Considering the foregoing, the rDNA method may be a better means of amylase production in industries for commercial purposes.

REFERENCES

1. Adejumo, A. L., Agboola, F. K., & Layokun, S.K (2012). Production and partial characterization of a thermostable extracellular α-amylase by a bacterium isolated from Cow dung microflora. *Fermentation Technology and Bioengineering, 2*(3), 14-22.
2. Adrianne, M. & Helen, K. (2001). Recombinant DNA and biotechnology: A Guide for Students. Washington, D.C: ASM Press.
3. Aiyer, D. P. V. (2004). Effect of C: N ratio on α-amylase production by *Bacillus licheniformis* SPT 27. *African Journal of Biotechnology, 3, 519-522.*
4. Ajayi, A. O. & Fagade, O. E. (2006). Growth pattern and structural nature of amylases produced by some *Bacillus* species in starchy substrates. *African Journal Biotechnology*, *5* (5), 440-444.
5. Alberts, B., Alexander, J., Julian, L., Martin, C., Keith, R. & Peter, W. (2002). Molecular biology of the cell. New York: Garland Science. Pp. G: 35.
6. Ali, S., Mahmood, S., Alan, R. & Hossain, Z. (1998). Culture conditions for production of glucoamylase from Rice bran by *Aspergillus terreus. Mircen Journal of Applied Microbiology and Biotechnology, 5*, 525 – 532.
7. Ara, K., Ozaki, K., Nakamura, K., Yamane, K., Sekiguchi, J. & Ogasawara, N. (2007). *Bacillus* minimum genome factory: Effective utilization of microbial genome information. *Biotechnology and Applied Biochemistry, 46* (3), 169-78.
8. Atiyeh, M., Reza, H. S., Mehdi, R. & Vahab, J. (2010). Characterization of an α-amylase with broad temperature activity from an acid-neutralizing *Bacillus cereus* strain. *Iranian Journal of Biotechnology, 8* (2), 103-111.
9. Ayogu, T. E. & Amadi, E. S. (2010). Amylase production by *Rhizopus nigricans* using mashed Maize. *The Internet Journal of Microbiology, 8*, 1. DOI: 10.5580/f95.
10. Bandow, J. E., Brutz, H. & Hecker, M. (2002). *Bacillus subtilis* Tolerance of moderate concentrations of Rifampin Involves the B-dependent general and multiple stress response. *Journal of Bacteriology*, *184* (2), 459-467.
11. Barnett, M. J., Fisher, R. F., Jones, T., Komp, C., Abola, A. P., Barloy-Hubler, F., ... Long, S. R. (2001). Nucleotide sequence and predicted functions of the entire Sinorhizobium meliloti pSymA megaplasmid. *Proceedings of the National Academy of Sciences*, *98* (17), 9883-9888.
12. Bennett, B.M., Brien, J.F., Nakatsu, K., & Marks, G.S. (1985) Role of hemoglobin in the differential biotransformation of glyceryl trinitrate and isosorbide dinitrate by human erythrocytes. *Journal of Pharmacology and Experimental Therapeutics 234*, 228-232.
13. Bertrand, T. F., Frederic, T. & Robert, N. (2004). Production and

partial characterisation of a thermostable amylase from *Ascomycetes yeast* strain isolated from starchy soil (pp. 53-55). New York: McrGrawHill Inc.

14. Burdett, I. D. J., Kirkwood, T. B. L. & Whalley, J. B. (1986). Growth kinetics of individual *Bacillus subtilis* cells and correlation with nucleoid extension. *Journal of Bacteriology, 167* (1), 219-230.
15. Burhan, A., Nisa, U., Gokhan, C., Ashabil, A. & Osmair, G. (2003). Enzymatic properties of a novel thermostable thermophilic alkaline and chelator resistant amylase from an alkaphilic *Bacillus* sp isolate ANT-6. *Process Biochemistry, 38*, 1397–1403.
16. Castellani, A. & Chalmers, A. J. (1919). Manual of tropical medicine (3rd ed.). New York: Williams Wood and Co.,
17. Cazorla, F. M., Romero, D., Pérez-García, A., Lugtenberg, B. J. J., de Vicente, A. & Bloemberg, G. (2007). Isolation and characterization of antagonistic *Bacillus subtilis* strains from the Avocado rhizoplane displaying biocontrol activity. *Journal of Applied Microbiology, 103*, 1950–1959.
18. Cheesbrough, M. (2003). Medical laboratory manual: Tropical health technology, low priced edition (pp. 20-35). United Kingdom: Doddington, Cambridgeshire.