THE INTERNATIONAL JOURNAL OF SCIENCE & TECHNOLEDGE

Protease Production by Aspergillus Niger in Solid State Fermentation Using Bambara Beans (*Vigna Subterranean*)

Sole-Adeoye O. D.
Postgraduate Student, Department of Chemical Engineering, Obafemi Awolowo University, Ile-ife, Nigeria
Dr. E. F. Aransiola
Senior Lecturer, Department of Chemical Engineering, Obafemi Awolowo University, Ile-ife, Nigeria

Abstract:
Bambara beans, a readily available and under-utilized legume, constitute a valuable source for enzyme production. An attempt was made to study protease production by Bambara beans using Aspergillus niger on under solid-state fermentation. Bambara beans has proved to be efficient substrate for protease enzyme production. To study the maximum enzyme production, the process parameters of fermentation were optimized. Maximum protease production i.e. (42U/ml) was obtained with an initial moisture content of 50% (w/w) on incubation period of 72h, the crude protease was most active at pH 12, 55 ^{o}C, and was stimulated by Cu^{2+} but inhibited in the presence of Zn^{2+}. Starch and maltose were the best organic substrates while corn steep liquor was the best nitrogen source and pure sugars such as fructose, glucose, and sucrose could not enhance its production. The above data revealed that Bambara beans which is a cheap raw material under optimized condition of solid-state fermentation can be successfully employed for protease production by Aspergillus niger.

Keywords: *Protease, Aspergillus niger, Bambara beans, Solid state fermentation*

1. Introduction

The high costs of importation and production of enzymes is a major concern to manufacturing industries. The world is now searching for alternative, cheap, underutilized and readily available substrate that can help in lowering of cost of production of these enzymes [i] as the high cost of enzymes is attributed to about 40% substrate's cost; hence the need to use cost-effective substrate in the production process [ii]. Proteases are primarily obtained from plant, animal and microbes but microbial proteases are preferred to those from plant and animal sources because they are cheaper to produce and their enzyme contents are more predictable, controllable and reliable [iii]. It is one of the most important commercial enzymes constituting two third of the enzyme used in various industries [iv], detergents, pharmaceuticals, leather, food and agriculture industries [v], the need to bring down the cost of production is eminent by using inexpensive substrates. Some of the substrates that have been used included tomato pomace, an expensive substrate [vi], fish flour mixed with polyurethane foam [vii], wheat flour, corn flour, steamed rice, steam pre-treated willow [viii] aspen pulp, sugar beet pulp, sweet sorghum pulp, steamed rice, etc., which are all competing with the food market. Hence, the need for an alternative, cheaper raw material, Bambara beans.

The botanical name for Bambara beans is *Vigna subterranean.* It is an indigenous, under-utilized legume grown across the sahara and sub-sahara Africa [ix]. Although it thrives well in sandy soil, it still grows readily in all soil types even under conditions of drought and low soil fertility. It has been described as an indigent crop [x] that is extremely hardy and not commonly eaten due to its requirement of high cooking time thereby increasing energy demand. It is also under-utilized by farmers as they rarely feed animals with it owing to its very strong nature thereby not aiding digestibility in animals. It is not commonly eaten due to its requirement of high cooking time thereby increasing energy demand. Its nutritional proximate composition is 16, 9.7, 5.9, 2.9 and 64.9% for crude protein, moisture, crude fat, ash and carbohydrate compositions respectively and contains appreciable amount of lysine with minimum amount of trypsin and chymotrypsin; with a carbohydrate to protein ratio of 1:4[xi] Despite its nutritional value, it is still considered as one of the prioritized neglected and underutilized specie [xii].

Figure 1: Bambara beans

Proteases of bacteria and fungi are increasingly studied due to its importance and subsequent applications in industry and biotechnology, but fungal proteases have increased in their industrial demand since they can be easily grown on inexpensive substrates [xii] [xiv]; are considered to be safe in producing enzymes, GRAS (generally recognized as safe), and they are produced extracellularly which makes its recovery from the fermentation broth easy.

2. Methodology

2.1. Sample Collection

Bambara beans obtained from Ore market, Ondo state were sorted, rinsed, air-dried and milled into powdery form and then stored for further use. Ten gram of soil sample was collected from a dumpsite at Obafemi Awolowo University environs and taken to the laboratory for microbial analysis.

2.2. Screening for Proteinase from Aspergillus Isolates

The isolated fungus was grown on Reese's medium [xiv] containing 0.5% casein as protein substrate and the plates were incubated at 28 ^{o}C to allow the growth of test fungi for 4 days. These were then examined for the formation of zone of clearance around the colony. The zone was made clearer, by flooding the plates with a solution of 5% Trichloroacetic acid (TCA) and kept for 30 min to allow the precipitation of residual proteins in the medium.

2.3. Preparation of Inoculum

The inoculum was prepared by dispersing the spores from a week-old fungal slant culture containing profuse growth of the microorganism and production of spores in 0.1% Tween -80 solution with the help of a sterile inoculation needle under aseptic condition and the test tube was shaken to get homogenized spores suspension. One milliliter of the spore's suspension was then transferred to the cotton plugged Erlenmeyer flasks containing fermentation substrate and then incubated at 28°C for 5 days.

2.4. Preparation of Fermentation Media used for Protease Production

Two hundred grams of milled Bambara beans was moistened with 100 ml of protease specific fermentation salt solution containing KH_2PO_4 (7 g), $MgSO_4.7H_2O$ (0.5 g), Na_2CO_3 (10 g), sucrose (1 g) and yeast extract (5.5 g) with pH adjusted to 4.8 in 500mL Erlenmeyer flasks. The flasks were plugged with cotton wool, sterilized in an autoclave at 121 ^{o}C for 15 minutes after which they were removed and allowed to cool before inoculating.

2.5. Fermentation Process

After cooling to room temperature, the fermentation media was inoculated with 1 ml of spore suspension from the inoculum broth and mixed carefully under aseptic conditions to achieve uniform distribution of inoculum throughout the medium. This was carried in an incubator shaker at 28°C for 5 days. At 24 h intervals samples were taken for the determination of protease activity and carbon utilization. The fermentation was run in duplicates and flasks shaken twice daily.

2.6. Extraction of Crude Enzyme

In extracting the crude enzyme produced after fermentation, 10 ml of distilled water was added to the fermented broth and the content was homogenized on a rotary shaker at 180 rpm (revolution per minute) for 1 hour and centrifuged at 3000 rpm for 20 mins to obtain a clear supernatant in extracting the crude enzyme. The cultural filtrate which is the crude protease enzyme were collected by filtering using Whatman's number 1 filter paper and used for enzyme assay.

2.7. Protease Enzyme Assay

The activity of protease in the crude enzyme was assayed using casein as substrate [xv]. Casein solution of 0.01% (4ml) was incubated with 1ml of enzyme solution and 1ml of sodium carbonate buffer (pH 10) for 5 minutes at 37℃. The reaction was stopped using 5ml Tricholoroacetic acid solution. After 30 minutes, the mixture was centrifuged at 5,000rpm for 5minutes. After centrifugation, the supernatant was developed with Bradford reagent and read at 580nM. Four milliliters of 0.01% casein solution was put into test and blank tubes and were incubated at 37 ^{o}C for 5 minutes. One milliliters of crude enzyme extract were added to T-test tube, mixed properly and incubated at 37 ˚C in a water bath for 30 min to allow enzymatic reaction to occur. The reaction was terminated by addition of 5 ml of trichloroacetic acid (TCA) solution in both test and blank tubes and allowed to settle for 30 minutes prior to centrifugation at 5000 rpm for 5 minutes, after which solution from both test tubes were filtered off using Whattman No 1 filter paper. Tyrosine liberated during casein hydrolysis was measured in the supernatant at 540nm using the method of [] Lowry *et al.* One unit of protease activity is defined as the amount of enzyme required to produce an increase or the amount of protease that liberates 1 µmol of tyrosine under the applied condition.

2.8. Effects of Operating Parameters on Protease Activity

a) Temperature: The inoculated substrate was incubated at different temperatures ranging from 30 to 70 ^{o}C at 10 ^{o}C interval to observe the effect of temperature by keeping all other conditions at their optimum and measuring the activity with standard proteolytic activity assay. Prior to addition of enzymes, the casein was pre-incubated for 10 minutes.

b) pH: The optimum pH was determined by incubating enzyme solution The effect of pH on the protease production was carried out using the following pH levels 7 – 12 at 1.0 unit interval, Erlenmeyer flasks containing 50 ml quantities of modified standard growth broth containing 1% casein prepared in buffers of different pH and carbon sources were taken with pH of the broth was adjusted to 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 in different flask using pH meter with 1 N HCl or 1 N KOH and sterilized, after which standard assay production procedure reported by Joo *et al.* (2002).

c) Incubation Period: The effect of incubation period on protease was determined by incubating fermentation medium for different incubation periods in hrs. i.e. 24, 48, 72, 96 and 120h at 35°C with initial substrate pH 7.0.

d) Metal ions: The effect of various metal ions on the enzyme activity was studied. The enzyme was incubated with various metal ion (5mM); manganese, magnesium, zinc, calcium and copper for 30min under the standard assay conditions.

e) Nitrogen: Seven different nitrogen sources consisting of peptone, whey protein, urea, beef extract, corn steep liquor, yeast extract and tryptone were employed to determine effect of nitrogen source on protease activity. The nitrogen sources were homogenized separately into a medium comprising of $(NH_4)_2SO_4$, $CaCl_2$ $(7H_2O)$, KH_2PO_4, Na_2HPO_4, $MgSO_4$ $(7H_2O)$, $ZnSO_4$, NaCl at 10 g/l concentration and autoclaved at 120 ^{o}C for 20 minutes. Cultures were inoculated with inoculum and incubated in a rotatory shaker (120 rpm) for 72 hours and centrifuged at 8,000 rpm for 15 minutes to remove fungi mycelia and the supernatant was used for estimation of proteolytic activities.

f) Carbon: Seven different carbon sources namely glucose, maltose, lactose, fructose, sucrose, galactose and starch of equal concentration (10 g/l) were examined for maximum protease production. The carbon sources were homogenized separately into a medium comprising of $(NH_4)_2SO_4$, $CaCl_2$ $(7H_2O)$, KH_2PO_4, Na_2HPO_4, $MgSO_4$ $(7H_2O)$, $ZnSO_4$, NaCl at 10 g/l concentration and autoclaved at 120 ^{o}C for 20 minutes. Cultures were inoculated with inoculum and incubated in a rotatory shaker (120 rpm) for 72 hours and centrifuged at 8,000 rpm for 15 minutes to remove fungi mycelia and supernatant used for estimation of proteolytic activities.

3. Results and Discussion

Accurate selection of substrate is necessary to reach higher quantities of enzyme desired [xvi]. By Proximate analysis, the moisture content, fat, ash, crude fibre and carbohydrate composition were found to be: 8.54, 6.32, 3.90, 18.14; 4.55 and 58.55% respectively. With high carbohydrate (58.55%) and protein composition (18.14%), Bambara is suitable to serve as good carbon and nitrogen sources for protease enzyme production.

3.1. Fungal Strain

A total number of five different (5) *Aspergillus* strains were isolated from the soil samples. And when grown on PDA, only three (3) were identified as *Aspergillus niger* strains. Assaying using Reese medium, the highest zone of clearance was 35mm

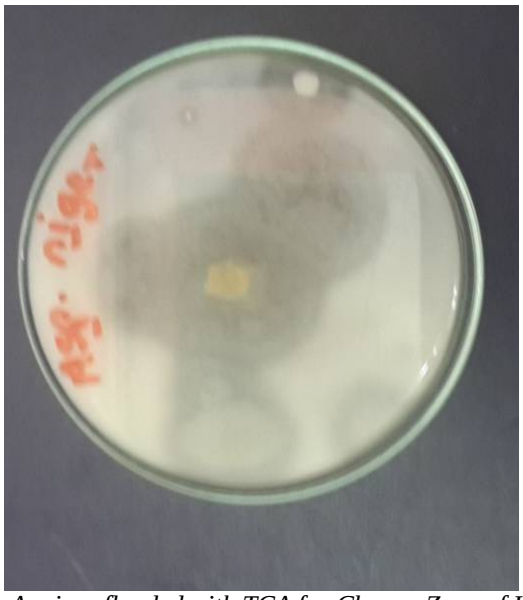

Figure 2: A. niger flooded with TCA for Clearer Zone of Inhibition

3.2. Fermentation

After five days of fermentation (Figure 4.3, extraction was done. Centrifugation was done to obtain a clear brownish colour. The supernatant obtained with proteolytic activity of 11.84 U/ml and specific activity of 1.11 U/mg was used as crude enzyme solution. Use of low cost substrates like sunflower meal, wheat bran, soybean meal, cottonseed meal, and rapeseed meal gave 5.2, 3.2, 4.8, 4.0 and 3.3 U/ml respectively [xvii] for the biosynthesis of protease by *Aspergillus niger* where sunflower meal gave the maximum enzyme activity of protease enzyme. Evaluation of different protein substrates for the production of proteolytic enzyme by *Aspergillus niger* and found soybean meal as a best substrate [xviii].

3.3. Effects of Operating Parameters on Enzyme's Activity

3.3.1. Effect of Temperature

The effect of temperature on the activity of the enzyme is shown in Fig.3. The optimum temperature for caseinolysis was recorded was at 55 °C, which gave maximum production of 55.9 U/ml. As the temperature was increased above 55 ^{o}C, the enzyme production was decreased. The observation in this study falls in line with reports [xviii] given that protease activity increases up to 60 ^{o}C and a reduction in activity was observed above 60 ^{o}C [xix]. The rate of the process was increased by increasing temperature but beyond certain limits it started decreasing because enzymes are sensitive to temperature [xx]. Rise of temperature within certain limits has the effect of increasing the activity of enzymes but very high temperatures are destructive [xxi].

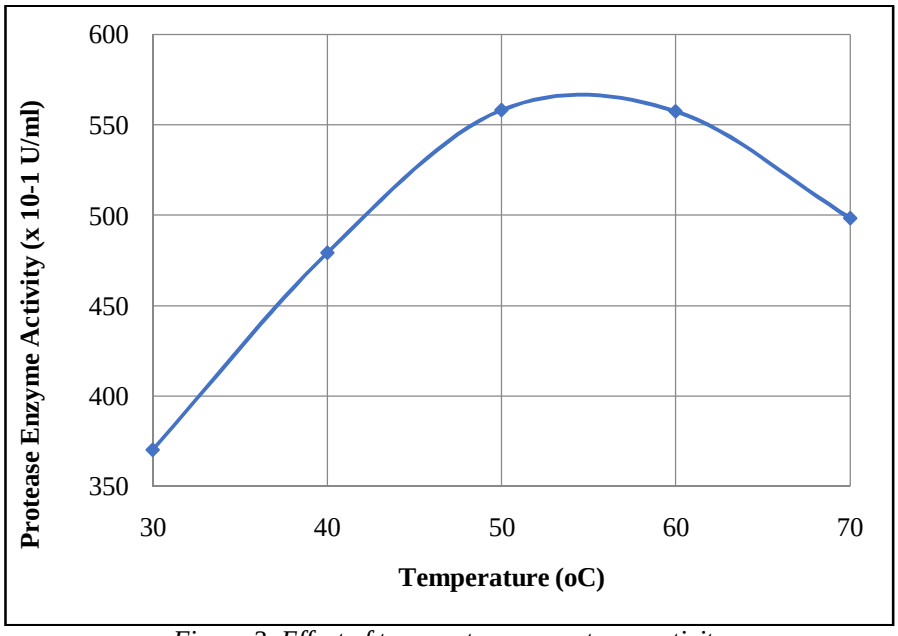

Figure 3: Effect of temperature on protease activity

3.3.2. Effect of pH

Fig 4 shows optimum pH for the protease activity of the fungi, range of pH (7-12) were used and it was observed that activities between pH 9.0 and 10.0 were relatively high, but was seen to be optimum at pH of 12 with an activity of 12.9 U/ml. From the result obtained, it is well seen that the organism produced alkaline protease demonstrating the alkalinophilic nature of the fungus. It has been reported widely that protease production from microbial source can be acidic or alkaline proteases as reported by many researchers depending on the organisms and source of the isolation. An optimum pH for protease activity at 8 was recorded for extracellular protease from *Aspergillus niger* and *Bacillus subtilis* high pH between 3.0 and 9.0 was also noticed [xxii].

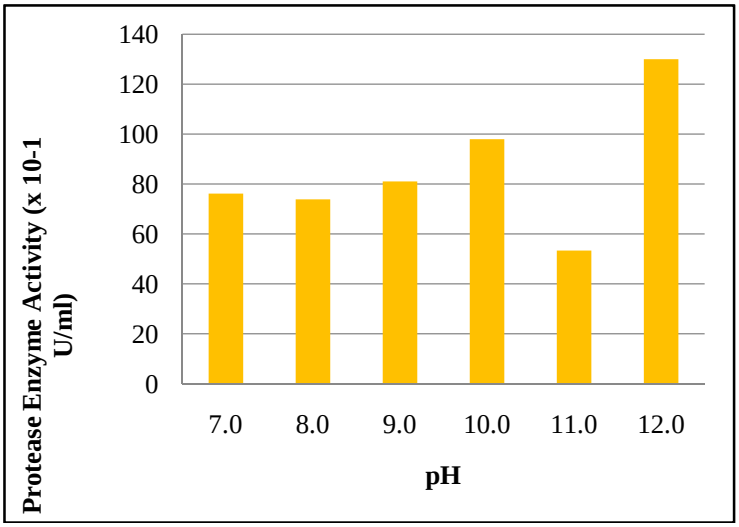

Figure 4: Effect of pH

3.3.3. Effect of Incubation Time

For the optimum incubation time, according to the results obtained at different time intervals (Figure 5), it was clearly indicated that the production of protease by *Aspergillus niger* grown on bambara beans gradually increased with time and that the optimum incubation time was 72 hours (42 U/ml) as also reported by Paranthaman who used rice mill as substrate in protease production [xxiii]. The exponential phase is observed at 24 h; the rate of growth was between 24 h to 72 h reaching peak growth at 72 h of incubation. The growth started declining after 72h before entering the death phase. A gradual decrease in enzyme units was observed with increasing incubation period, clearly suggesting the enzyme's role as a primary metabolite, being produced in log phase of the growth of the fungi for utilization of nutrients i.e. protein present in the solid substrate [xxiv].

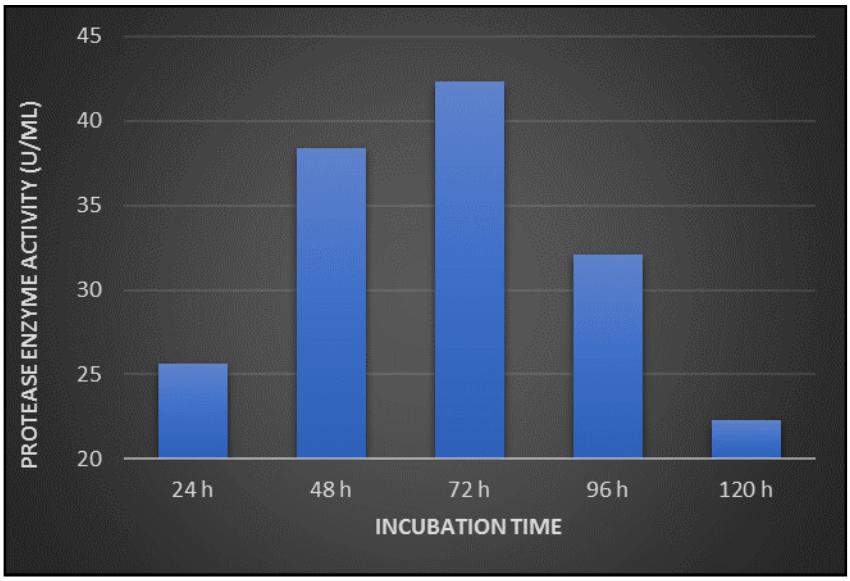

Figure 5: Effect of incubation time

3.3.4. Effect of Metallic Ions

Presence of specific metallic ions can inhibit or enhance protease activity. Figure 6, shows that the activity of the protease enzyme was promoted by cu^{2+} as it showed maximum activity at 1068.78 U/m and zn^{2+} inhibited the activity with the lowest at 311.64 U/ml. This is in contrast to the report given [xxv] that zn^{2+} caused acceleration of alkaline protease activity by *Aspergillus flavus* and the activity was inhibited by mg^{2+} and ca^{2+}. Cu^{2+} and Zn^{2+} was reported to have strongly decreased protease enzyme activity [xxvi]. The protease from Bambara beans is a metallo- protease as metal ions affects its function which in line with the report [xxvii]. The demonstration of cu^{2+} as a good stimulant to the study enzyme activity is in line with the report from [xxviii]. This result therefore suggests that cu^{2+} apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of the enzyme at high temperature.

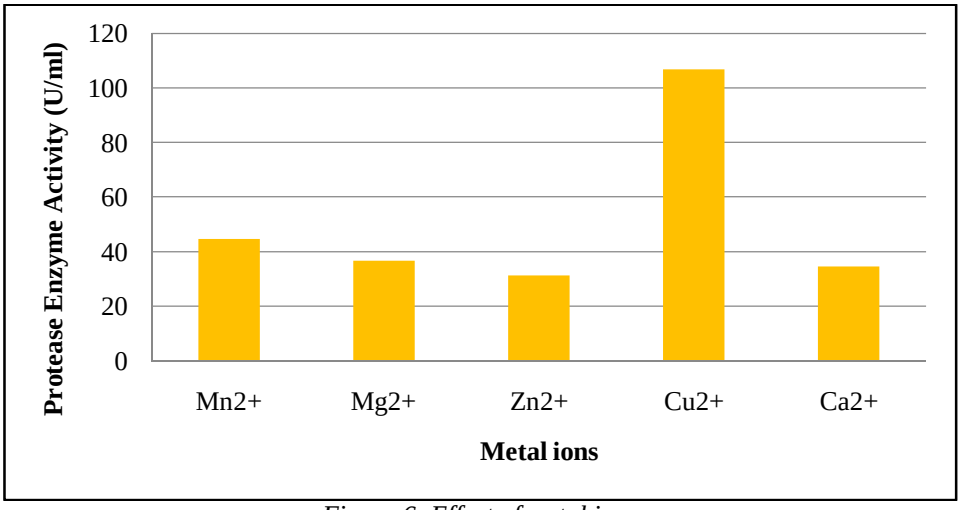

Figure 6: Effect of metal ions

3.3.5. Effect of Nitrogen Sources

Nitrogen is very important in metabolism of microorganisms especially in the synthesis of enzymes and other proteins. It has always been one of the important components of fermentation substrate or media. Certain nitrogenous salts tend to decrease the pH of the culture medium and had the adverse effect on enzyme production although they supported the growth of the organism [xxix] (Chinnasamy *et al*. 2011).

Maximum production of the enzyme (324 U/ml) was observed when corn steep liquor was used as an organic nitrogen source which indicates its affinity for complex sugars; all other nitrogen sources such as yeast extract, beef extract, and whey protein gave good amount of enzyme production, but they were less effective than corn steep liquor. In a similar study, [xxx] Singh *et al*. (2009) used cheap nitrogen sources such as corn steep liquor for the production of a thermostable acid protease by a strain of *Aspergillus niger*.

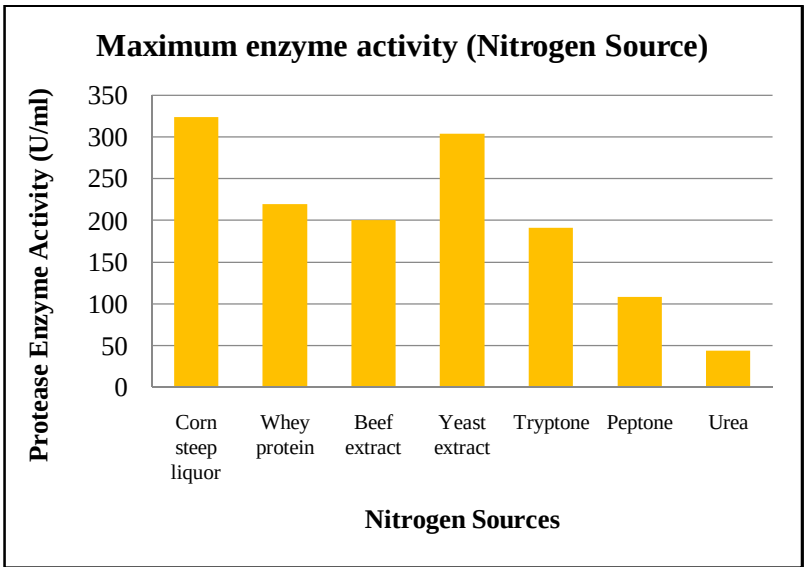

Figure 7: Effect of Nitrogen sources

3.4. Effect of Different Carbon Sources

Different carbon sources such as sucrose, starch, maltose, glucose, fructose and sucrose were added to the fermentation substrate to study their effect on the production of proteases by *Aspergillus niger* (Figure 4). The synthesis of alkaline protease was found maximum (337.0 U/ml) with starch as a carbon source in the medium. It was decreased with maltose, (330.0 U/ml), galactose (287 U/ml) and lactose (219.0 U/g). Minimum production of alkaline protease was obtained when the substrate was supplemented with glucose (52.0 U/ml), fructose (73.0 U/ml) and sucrose (49.0 U/ml).

The reason for obtaining highest protease activity by using starch as a carbon source may be due to the breaking down of starch into simple forms which can then be easily used by the microorganism for growth and enzyme production. From the results obtained, it was also observed that sugars like fructose, glucose had little effect on its activity, thereby showing the affinity for complex substrates making it show diauxic growth. This is in agreement with previous report by [xxxi] Da-Silva *et al.* (2005) and [xxxii] Murthu and Christudhas. (2012), which showed that starch caused high level of enzyme expression in *Bacillus* species and concluded that starch is the best carbon source for both growth and protease production.

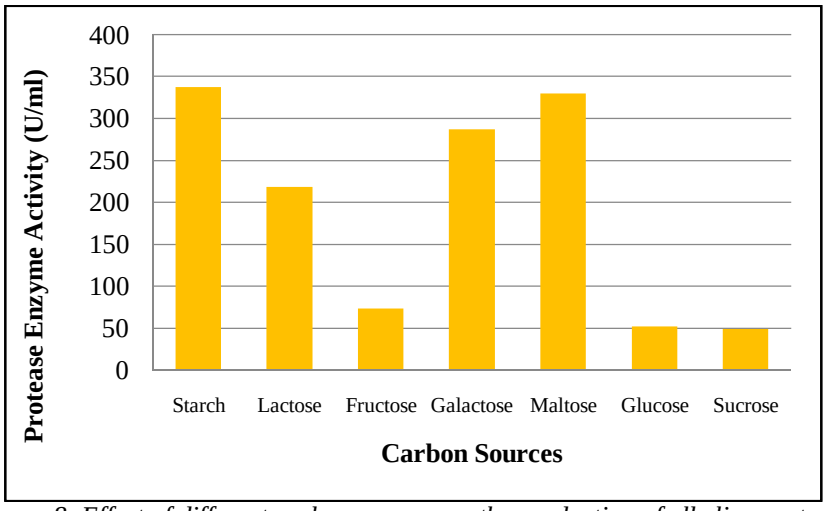

Figure 8: Effect of different carbon sources on the production of alkaline protease

4. Conclusion

It is concluded from the present study that Bambara beans, a viable carbon source with low cost, negligible applications and high protein composition can be exploited as substrate in fermentation process for protease production. Culture conditions such as incubation temperature, incubation period, diluent pH, etc. had a profound effect on the production of enzyme. It was also found that starch and corn steep liquor were cheap carbon and nitrogen sources that resulted in significant rise in the protease production. The optimum pH and temperature for enzyme activity was determined as 12.0 and 55 °C; which implies that the strain was mesophilic, also displaying its activity for casein predominantly in the alkaline region of pH 10 and 12.

5. References

i. O.D. Sole-Adeoye, "Production of protease enzyme from Bambara beans (Vigna subbterranean) using Aspergillus niger''. M.Sc. Thesis. Department of Chemical Engineering, Obafemi Awolowo University, Ile Ife, Nigeria. March, 2016.

ii. El –Enshasy, H., Abuoul-Enein, A., Helmy, S.and El Azaly, Y. (2008). Optimization of the industrial production of alkaline protease by Bacillus licheniformis in different production scales. Australian Journal of Basic and Applied Sciences, 2(3): 583-593.

iii. Burhan, A., Nisa, U., Gokhan, C., Ashabil, A. and Osmair, G. (2003). Enzymatic Properties of a novel thermostable, thermophilic alkaline and chelator resistant amylase from an alkaphilic Bacillus sp Isolate ANT-6. Journal of Process Biochemistry. 38: 1397–1403.

iv. Gupta, R., Beg, Q.K. and Lorenz, P. (2002b). Bacterial alkaline proteases: molecular approaches and industrial applications. Journal of Applied Microbiology and Biotechnology. 59:15.

v. Sawi, S.A., Labdane, H.M., Abiet, P. (2008). An editerpene from the fruits of Juniperus phoenicea L. grown in Egypt and their activities against human liver carcinoma, Australian Journal of Medicine and Herbal. 2: 115–122.

vi. Belmessikh, A., Boukhalfa, H., Mechakra-Maza, A., Gheribi-Aoulmi, Z. and Amrane, A. (2013). Statistical optimization of culture medium for neutral protease production by Aspergillus oryzae: Comparative study between solid and submerged fermentation on tomato pomace. Journal of Taiwan Institute of Chemical Engineering. 44(3): 377-385.

vii. García-Gómez, M.J., Huerta-Ochoa, S., Loera-Corral, O. and Prado-Barragán, L.A. (2009). Advantages of a proteolytic extract by (Aspergillus oryzae) from fish flour over a

viii. Oyeleke, S.B and Oduwole, A.A. (2009). Production of amylase by bacteria isolated from a cassava dumpsite in Minna, Niger state, Nigeria. African Journal of Microbiology Research, 3: 143-146.

ix. Atiku, N., Aviara, N.A. and Haque, M. (2004). Performance evaluation of bambara groundnut sheller, Agricultural Engineering international. Journal of Scientific Research and Development. 4: 2.

x. Barimalaa, I.S., Achinewhu, S.C., Yibatama, I. and Amadi, E. N. (1994). Studies of the solid substrate fermentation of bambara groundnut (Vigna subterranean (L) verdc). Journal of food science and Agric. 66: 443-446.

xi. Olanipekun, B.F., Otunola, E.T., Adejuyitan, J.A. and Adeyanju, J.A. (2012). Proximate and fatty acid composition of bambara groundnut (voandzeia subterranean l. thouars) as influenced by fermentation with a combination of rhizopus oligosporus and rhizopus nigricans. Transnational Journal of Science and Technology. 2(9):77- 84.

xii. Dansi, A.; R. Vodouhe; P. Azokpota; et al. (19 April 2012). "Diversity of the Neglected and Underutilized Crop Species of Importance in Benin". The Scientific World Journal. **2012**: 932947. doi:10.1100/2012/932947. PMC 3349165 . PMID 22593712

xiii. Murthy, P.S. and Naidu, M.M. (2010). Production and application of xylanase from penicillum sp. utilizing coffee by-products. Journal of Food and Bioprocess Technology. 5: 657-664.

xiv. Benazir, J. F., Suganthi, R., AnjanaHari., Ramesh Kumar, V., Aswathi, M. P., Niraimathi, G., Mala, M. K., Sukanya, S. and Santhi, R. (2011). Bio utilization of agro industrial waste in solis state fermentation by Aspergillus niger for production of protease. Asiatic Journal of Biotechnology resources. 2: 422-43.

xv. Vaishali Choudhary. (2013). Recovery of Silver from used X-ray films by Aspergillus versicolor protease. Journal of Academia and Industrial Research. 2: 39- 41.

xvi. Joo, H S., Kumar, C.G., Park, G.C., Paik, S.R. and Chang, C.S. (2003). Oxidant and SDS-stable alkaline protease from Bacillus clausii - production and some properties. Journal of Applied Microbiology, 95: 267.

xvii. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951). Protein measurement with folin phenol reagent. The Journal of Biological Chemistry. 193: 265-275.

xviii. Mukhtar, H. and Hoq, I.U. (2008). Production of alkaline protease by Bacillus subtilis and its application as a depilating agent in leather processing. Journal of Botany. 40(4): 1673-1679.

xix. Heneri, P., Chamtal, B., Odile, T. and Aimee, P., 1988. Semi alkaline proteolytic enzyme by Aspergillus niger by using various protein substrates. Journal of Fermentation and Technology. 66: 380-382.

xx. Pagare, R.S., Ramdasi, A.M., Khandelwal, S R., Lokhande, M.O. and Aglave, B. (2009). Production and Enzyme Activity of An Extracellular Protease from Aspergillus niger and Bacillus subtilis. International Journal of Biotechnology and Biochemistry. 44(2): 211-220.

xxi. Daniel, R.M., Peterson, M.E. and Danson, M.J. (2010). The molecular basis of the effect of temperature on enzyme activity. Journal of Biochemistry. 425(2): 353-360.

xxii. Haq, I., Mukhtar, H., Daudi, S., Ali, S. and Qadeer, M.A. (2003). Production of proteases by a locally isolated mould culture under lab conditions. Biotechnology. 2: 30-36.

xxiii. Pagare, R.S., Ramdasi, A.M., Khandelwal, S R., Lokhande, M.O. and Aglave, B. (2009). Production and Enzyme Activity of An Extracellular Protease from Aspergillus niger and Bacillus subtilis. International Journal of Biotechnology and Biochemistry. 44(2): 211-220.

xxiv. Paranthaman, R., Alagusundaram, K. and Indhumathi, J. (2009). Production of protease from rice mill wastes by Aspergillus niger in solid state fermentation. World Journal of Agric Science. 5: 308-312.

xxv. Alagarsamy, S., Christian, L. and Ashok, P. (2006). Microbiology and Industrial Biotechnology of Food-Grade Proteases: A Perspective. Journal of Food Technology and Biotechnology. 44(2): 211-220.

xxvi. Chinnasamy, M., Duraisamy, G., Dugganaboyana, G., Kumar, G., Ravikumar, M. and Chandrasekar, U. (2011). Production, Purification and Characterization of Protease by Aspergillus flavus under Solid State Fermentation. Jordan Journal of Biological Sciences. 4(3): 137-148.
xxvii. Temiz, H., Okumus, E., Aykut, U., Dervisoglu, M. and Yazici, F. (2008). Partial purification of pepsin from turkey proventriculus. World Journal of Microbiology and Biotechnology. 24: 1851–1855.
xxviii. Khan, H., Subhan, M., Mehmood, S., Durrani, M.F., Abbas, S. and Khan, S. **(**2008). Thermo-alkaliphilic halotolerant detergent compatible protease(s). Journal of Biotechnology. 7(1): 94-99.
xxix. Ogbonna, A.C., Obi, S.K. and Okolo, B.N. (2003). Studies on malting parameters, purification, and characterization of proteolytic enzymes from sorghum varieties. Journal of Instant Brewery. 109: 51- 56.
xxx. Da-silva, R., Lago, E. S., Merheb, C. W., Macchione, M.M., Park, Y.K. and Gomes, E. (2005). Production of xylanase and CMCase on solid state fermentation in different residues by Thermoascus aurantiacus miehe. Brazilian Journal of Microbiology. 36(3): 235-241.
xxxi. Chinnasamy, M., Duraisamy, G., Dugganaboyana, G., Kumar, G., Ravikumar, M. and Chandrasekar, U. (2011). Production, Purification and Characterization of Protease by Aspergillus flavus under Solid State Fermentation. Jordan Journal of Biological Sciences. 4(3): 137-148.
xxxii. Singh, A., Singh, N. and Bishnoi, N.R. (2009). Production of cellulases by Aspergillus heteromorphus from wheat straw under submerged fermentation. International Journal of Civil and Environmental Engineering. 1: 23-26.
xxxiii. Mukhtar, H. and Hoq, I.U. (2008). Production of alkaline protease by Bacillus subtilis and its application as a depilating agent in leather processing. Journal of Botany. 40(4): 1673-1679.
xxxiv. Muthu, P. and Christudhas, W. (2012). Purification and characterization of neutral protease