

Production and Optimization of Protease from *Aspergillus niger* and *Bacillus subtilis* using Response Surface Methodology

M. A. Milala, I. A. Jatau and A. A. Abdulrahman

Department of Biochemistry, Faculty of Science, University of Maiduguri, Borno state, Maiduguri, Nigeria

Abstract: *Proteases are enzymes that perform proteolysis. In this study we investigated the production and optimisation of protease from Bacillus subtilis and Aspergillus niger using response surface methodology. This was achieved by isolating the Fungus and Bacterium from contaminated soil and bovine intestinal sample and screening for protease activity was carried out using casein as substrate. Optimization of different conditions for maximum protease activity (pH, Temperature, Carbon source and Mineral Salts) and Response of microorganisms to mineral salts in the culture medium was observed. The time course for the production of protease by both isolates was found to be maximum at 48 hours. The optimum pH for protease activity was found to be at pH 8.0 for Bacillus subtilis, while Aspergillus niger has optimum activity at pH 4.0 and pH 8.0. The optimum temperature for Aspergillus niger was at 60°C while for Bacillus subtilis was at 40°C. This study had also shown the effect of decrease in carbon source and mineral salt (NaNO₃ and MgSO₄) concentration on protease activity. When Carbon source was decreased, a significant decrease in protease activity was observed by both isolates of Aspergillus niger and Bacillus subtilis as compared with the control which has activity of 0.0686U/ml and 0.0827U/ml respectively. Mineral salts (MgSO₄) were decreased and protease activity decreased for Aspergillus niger and Bacillus subtilis which has activity of 0.0473U/ml and 0.0495U/ml as compared with control which has activity of 0.0686U/ml and 0.0827U/ml respectively, NaNO₃ when decreased shows decrease in protease activity decreased for Aspergillus niger and Bacillus subtilis which has activity of 0.0392U/ml and 0.0571U/ml as compared with control which has activity of 0.0686U/ml and 0.0827U/ml respectively. The findings of this study indicate the possibility that the isolates produce protease which has wide range of applications for Industrial and Domestic purposes.*

Keywords: *Microbial protease; Aspergillus niger; Bacillus subtilis; Response surface methodology.*

I. Introduction

Enzymes are macromolecular biological catalyst. Enzymes accelerate or catalyse chemical reactions. The molecules at the beginning of the process are called substrates and the enzymes convert these into different molecules, called products. Almost all metabolic processes in the cell need enzymes in order to occur at rates fast enough to sustain life (Stryer *et al.*, 2002).

Biologically active enzymes may be extracted from any living organism. Of the hundred or so enzymes being used industrially, over halves are from fungi and yeast and over a third are from bacteria with the remainder divided between animal (8%) and plant (4%) sources. A very much larger number of enzymes find use in chemical analysis and clinical diagnosis. Microbes are preferred to plants and animals as sources of enzymes because; they are generally cheaper to produce, their

enzymes contents are more predictable and controllable, unlike the plant and animal tissues, contain less potentially harmful materials (Godfrey and West, 1996).

Proteases are enzymes that catalyse hydrolytic reactions in which proteins molecules are degraded to peptide and amino acids. Proteases (Serine protease (EC.3.4.21), Cysteine(thiol) protease (EC.3.4.22), Aspartic protease (EC.3.4.23) and metalloprotease (EC.3.4.24) constitute one of the most important groups of industrial enzyme market (Nunes *et al.*, 2001; Singh *et al.*, 1998).

Microbial proteases play an important role in biotechnological processes and they account for approximately 59% of the total enzymes used (Gupta *et al.*, 2002). Proteases are produced by a wide range of microorganism including bacteria, fungal, moulds, and yeasts. Fungal protease is one of the major reasons for the wide popularity of fungi in fermentation industry. They find application in modern and biochemical industries; food environment and pharmaceutical processing.

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variable). An experiment is series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response.

Fungal protease routinely used in bakery industry, are indispensable to start the primary proteolysis of gluten (Rizello *et al.*, 2007). Among fungi, proteases of *Aspergillus niger* used for bakery application have been found to degrade gluten polypeptides. Bacterial proteases have also been used in industries. Among these *Bacillus* genus has gained importance at industrial scale. Despite this, only a few studies have been done on proteolytic enzymes from *Bacillus spp.* Furthermore studies have showed that nutritional factors including sources of carbon and nitrogen can influence protease production (Yossan *et al.*, 2006). Presently, both fungal and bacterial proteases are of great demand because of potential industrial applications. In the present report we investigated the production and optimisation of protease from *Bacillus subtilis* and *Aspergillus niger* using response surface methodology.

II. Materials and Methods

All chemicals and reagents used in this study were of analytical grade.

2.1 Protease Producing Isolates

The bacterium and Fungus were isolated respectively from Soil samples collected at Sardauna Hall (University of Maiduguri) sewage and at Maiduguri Abattoir in tightly closed containers. Bacterial and fungal species were identified in Crop Protection Pathology laboratory, Faculty of Agriculture, University of Maiduguri. The isolates were identified as *Aspergillus niger* and *Bacillus subtilis*.

Screening for protease production from bacterium was done by the method of Maharana and Ray (2013). Screening for protease from fungus was done by method of Lovrien *et al.*, (1985). Forty eight hour old culture in nutrient broth medium, incubated at 30°C was taken for both fungus and bacterium inocula for production of protease.

2.2 Protease production by submerged fermentation

Protease production was done using 250ml Erlenmeyer flasks. Production consisting mineral salt medium in g/L

- Bacteria/Fungi

Bacteriological peptone (2.0)/200.0 peeled Irish potatoes and 20.0 dextrose, Na₂NO₃ (3.0), NaCl (0.5), MgSO₄ (0.5), KCl (0.02), CaCl₂ (0.5), FeSO₄ (0.2), NaH₂PO₄ (0.5), and Casein (0.5). Production was done at 40 degrees for 96 hours and protease activity was determined from

supernatant collected by samples with intervals of 24 hours after centrifugation at 2000 rpm for 5minutes.

2.3 Protease activity assay

Protease activity was assayed by the method of Lovrien *et al.*, 1985. Three millilitres (3ml) of reaction mixture containing 0.5% casein in 2.95ml of 0.05M acetate buffer, pH 8.0 and 0.1ml of crude enzyme was incubated at 40°C. After 30 minutes, the reaction was stopped by adding 3ml trichloroacetic acid (TCA). After one hour each of the culture filtrate was centrifuged at 2,000rpm for 5minutes to remove precipitate and absorbance of the supernatant was read spectrophotometrically at 540nm. The amount of amino acid released was calculated from a standard curve plotted against a range of known concentration of tyrosine.

2.4 Optimisation of protease production

2.4.1 Effect of pH on protease activity

The optimum pH for protease activity was determined with casein 0.5% (w/v) as substrate dissolved in 0.5M Sodium acetate buffer pH 7.5 and the pH of the buffer was adjusted to pH range from 2.0, 4.0, 5.0, 6.0, 8.0, 9.0 and 10.0 using Lactic acid and sodium chloride.

2.4.2 Effect of temperature on protease activity

The effect of temperature on protease activity was carried out by performing the standard assay procedure at pH8.0 with temperatures of 30°C, 40°C, 50°C, 60°C, 70°C and 80°C.

2.4.3 Effect of substrate concentration on protease activity

The effect of substrate concentration on protease activity was carried out by performing the standard assay procedure at pH8.0 and temperature 40°C with varying concentrations of Casein (substrate) of 0.25%, 0.5%, 1%, 1.5% and 2% respectively.

2.4.4 Effect of decrease in carbon source and mineral salt medium on protease activity

The effect of decrease in carbon source and mineral salt medium on protease activity was determined by performing the standard assay procedure at pH 8.0 and temperature 40°C while decreasing the quantity of the carbon source and minerals salts in the medium(MgSO₄ and NaNO₃).

III. Results and Discussion

3.1 Evaluation of Optimum incubation period for protease production

The isolates of *Aspergillus niger* and *Bacillus subtilis* was investigated for the better production of protease in various incubation period from 6-96 h which showed that maximum activity was at 48 h of incubation (0.0686U/ml and 0.0827U/ml)at 40°C(the early stage of stationary phase) using casein as the substrate. After that there was a slight fall in protease activity which is due to exhaustion of nutrients (Fig1). This finding is in line with the works of Abbas and Leila, 2011; Oyeleke *et al.*, 2010 in which they reported 48 hours as the optimum incubation period for maximum protease activity from the genus *Aspergillus niger* and *Bacillus subtilis*.

3.2 Influence of pH on protease activity

The activity of protease increased as the pH of the buffer was increased for isolates of *Bacillus subtilis*. The protease was optimally active at pH 8.0 (0.0827U/ml)(Fig 2.0). This is also in line with the work of Abbas and Leila, 2011. Further increase in pH above 8.0 resulted to a decrease in protease activity and thus loss in the activity of the enzyme. Usually, maximum protease activity for *Bacillus subtilis* is observed at pH 8.0 as reported by Sangeetha (2011).

The activity of protease increased as the pH of the buffer was increased for *Aspergillus niger*. The protease was optimally active at pH 4.0 (0.1223U/ml)(Fig 2.0)This finding was in line with the

works of Oyeleke *et al.*, 2010 on protease activity who reported optimum pH of 4.0. Further increase in pH above 4.0 resulted in decrease in protease activity until pH 8.0(0.06886U/ml) which shows an increase in protease then decreases in protease activity above 8.0 and lost in the activity of the enzyme. Usually, maximum protease activity for *Aspergillus niger* is observed at pH 4.0(acidic) and pH 8.0(alkaline) as reported by Oyeleke *et al.*, 2010. The enzyme lost activity at higher pH because changes in pH can make and break inter and intra molecular bonds and changing the structure of the enzyme and its effectiveness.

3.3 Influence of temperature on protease activity

The activity of protease also increases as temperature of the medium was raised from 30°C for *Bacillus subtilis*, the activity increased until an optimum of 60°C (0.1U/ml) was obtained (Fig 3.0). Beg and Gupta, 2003 reported an optimum temperature of 60°C. A further increase in temperature resulted in a decrease in enzymatic activity and thus the denaturation of the enzyme at higher temperature.

The activity of protease increases as temperature of the medium was raised from 30°C for *Aspergillus niger*, the activity increased until an optimum of 40°C (0.0686U/ml) was obtained (Fig 3.0).Shumi *et al.*, 2003 reported an optimum temperature of 40°C and 50°C respectively. A further increase in temperature resulted in decrease in enzymatic activity and thus the denaturation of the enzyme at higher temperature. Thermo alkaline proteases are the most commonly used of the alkaline proteases because they function at a pH range of 7.0 – 12.0 and a temperature of 40°C– 60°C (Beg and Gupta, 2003). Fungal proteases are usually thermo labile and show reduced activities at high temperatures (Shumi *et al.*, 2003). Higher temperature is found to have adverse effects on metabolic activities of microorganisms (Sharma *et al.*, 1980).

3.4 Influence of different carbon sources and mineral salts

On decrease in the quantity of mineral salts, protease activity was lower than the control. When carbon source (glucose) is decreased, the enzyme activity in both isolates showed decrease in protease activity. Imandi *et al.*, (2010) reported that glucose is important in protease production. For decrease in MgSO₄ and NaNO₃ (0.1g respectively) there is significant decrease in protease activity for both *Bacillus subtilis* and *Aspergillus niger*.

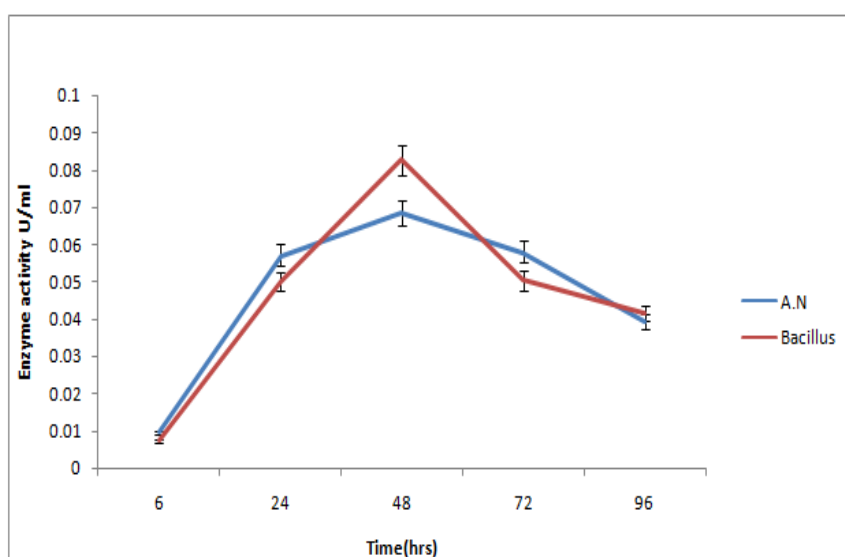


Figure.1 Time course for the production of protease from *Aspergillus niger* (A.N) and *Bacillus subtilis* from 6-96hours.

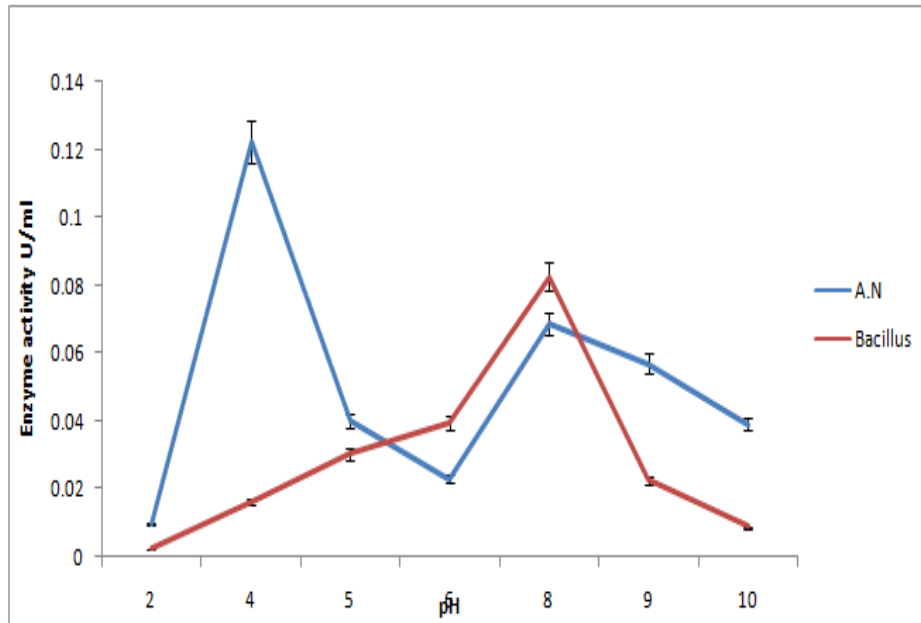


Figure.2 Effect of pH on protease activity by *Aspergillus niger* (A.N) and *Bacillus subtilis* with pH ranging from 2-10

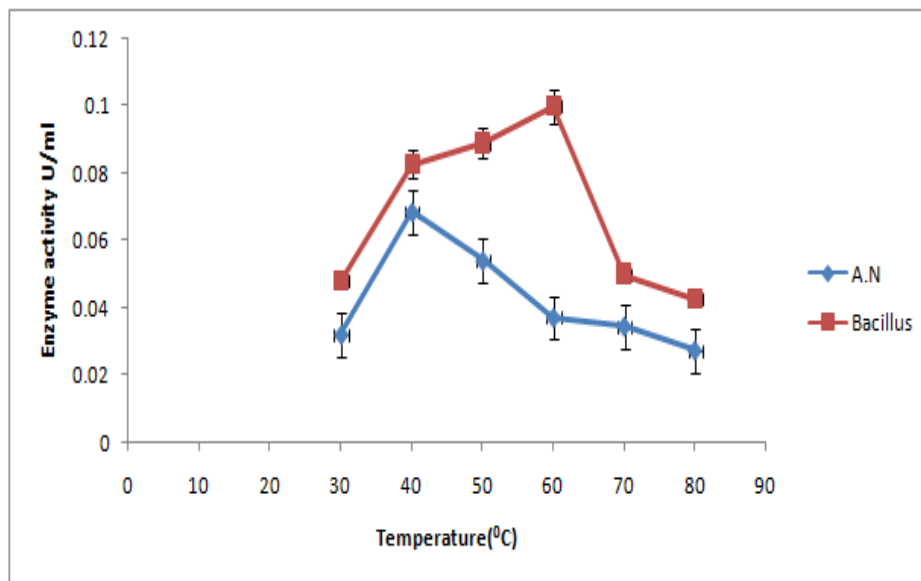


Figure.3 Effect of temperature on protease activity of *Aspergillus niger* and *Bacillus subtilis* with temperature ranging from 30°C to 80°C

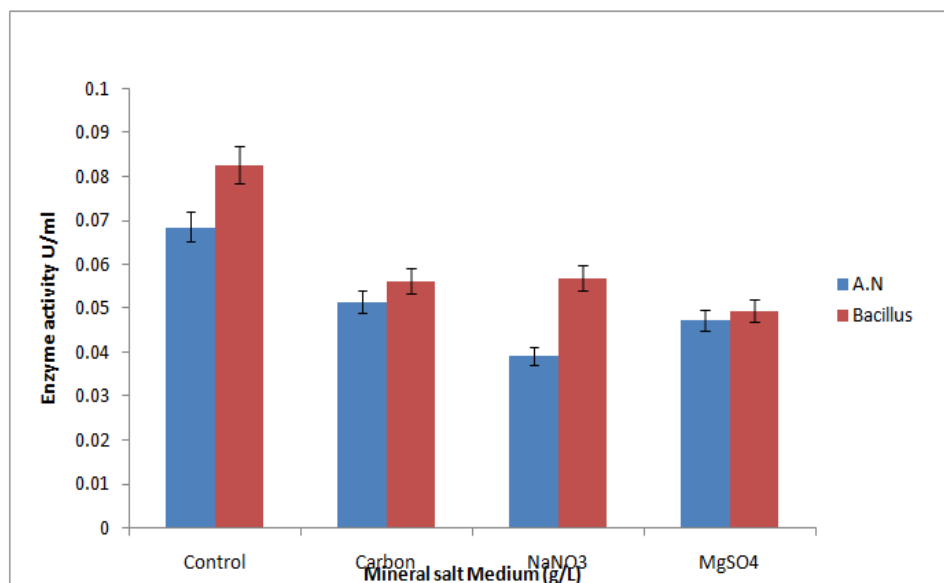


Figure.4 Effect of carbon source and mineral salts on protease activity of *Aspergillus niger*(A.N) and *Bacillus subtilis* using Response Surface Methodology (RSM)

IV. Conclusion

This study investigated the production and optimisation of protease from *Bacillus subtilis* and *Aspergillus niger* using response surface methodology. The findings of the study indicate the possibility that the isolates produce protease which has wide range of applications for Industrial and Domestic purposes.

References

- [1]. Abbas, A.S and Leila J (2011) Effect of the culture conditions on the production of an extracellular protease by *Bacillus* sp isolated from soil sample of Lavizan Jungle Park. *Journal of Applied Microbiology*.
- [2]. Gupta, R., Beg, Q K., Khan, S and Chauhan, B. (2002). An overview on fermentation, downstream processing and properties of microbial proteases. *ApplMicrobiolBiotechnol*: **60**, 381-395.
- [3]. Nunes, A.S, and M. L. L. Martins (2001) "Isolation, properties and kinetics of growth of a thermophilic *Bacillus*," *Brazilian Journal of Microbiology*, vol. 32, no. 4, pp. 271–275.
- [4]. Nunes, A.S, M.L.L. Martins and Chellapandi, P. (2010). Production and Preliminary Characterization of Alkaline Protease from *Aspergillus flavus* and *Aspergillus terreus*. *EJ. Chem.*, **7(2)**: 479-482
- [5]. Lovrien R.E, Gusek T, Hart B (1985). Cellulase and protease specific activities of commercially available cellulase preparations. *Journal of Applied Biochemistry*. **7**: 258 -272.
- [6]. Rizzello, C.G, M De Angelis, R Di Cagno, A Camarca, I Losito, M De Vincenzi, M De Bari, F Palmisano, F Maurano, C Gianfrani and Subathra Devi, C. (2012). Isolation, Screening and Identification of Protease producing Fungi from Rhizosphere Soil and Optimisation of pH, Incubation Time and Inducer Concentration For Enhanced Protease Production. *International Journal of Pharma and Bio Sciences*: Vol. 3, 2.
- [7]. Sangeetha R, Geetha A and Singh, R. (2002). Production of alkaline protease by a UV mutant of *Bacillus polymyxa*. *Indian J. Microbiol.*, **42**: 155-159
- [8]. Sharma OP., KD. Sharma and K. Nath, (1980). Production of proteolytic enzyme by fungi. *Rev. Roum. Biochemistry*, **17**:209-15.

- [9]. Shumi, W., M.T. Hossain and M.N. Anwar, (2003). Protease from *Fusariumtumidum* Sherbakoff. The chittgong Uni. *Journal of Science.*, 27:79-84
- [10]. Yossan, Q, Y. Ping, X. LI, H. Wang, Randir, S. (2002). Production of alkaline protease by *Aspergillus* ssp. Under submerged and solid substrate fermentation. *Indian J Microbiol* **42**:43–47
- [11]. Stryer L, Berg JM, Martin, M L L. (2004). Production and properties of extracellular protease from thermophilic *Bacillus* sp. *Brazilian J. Microbiology.*, **35**: 91-96.