International Journal of Research Studies in Biosciences (IJRSB)
Volume 3, Issue 9, September 2015, PP 1-5
ISSN 2349-0357 (Print) & ISSN 2349-0365 (Online)
www.arcjournals.org

Optimization of Reaction Time for the Assay of Protease Activity in a Local Strain of *Aspergillus niger*

***Chidi-Onuorah, L.C., Onunkwo, A.U., Agu, K.C., Ogbue, M., Kyrian-Ogbonna, E.A., Awah, N.S., Okeke, C.B. and Nweke, G.U.**

Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University P M B 5025 Awka, Anambra State, Nigeria
lc.ezeanya@unizik.edu.ng

Abstract: *As the essence of science is to discourage the use of chemically synthesized enzymes, the need for locally produced enzymes of biological (microbial) origin has been increased and will also maximize the reserve of huge foreign exchange spent on the importation of enzyme. Fermentation periods for enzyme (protease) production have been optimized by some researchers but none has optimized the reaction time for enzyme assay, hence the need for this study. Protease enzyme was produced by an Aspergillus isolate obtained from samples of spoilt beans and meat and its activity assayed at different reaction times of 15, 30, 45 and 60 minutes. The samples were cultured on skimmed milk agar (SMA) and fungal organisms isolated were screened for proteolytic activity. The species that showed zones of clearance were identified macroscopically as well as microscopically as Aspergillus niger, Aspergillus fumigatus and Aspergillus flavus with Aspergillus niger having the highest zone of clearance of 40mm followed by Aspergillus fumigatus with 36mm and Aspergillus flavus, 16mm. Using shake submerged fermentation, crude enzyme was obtained from Aspergillus niger, which had the highest zone of clearance, assayed for protease activity and their results extrapolated from the optical density of the different concentration of tyrosine standard. Protease activities of 38.67U, 25.00U, 21.78U and 19.33U were obtained for 15, 30, 45 and 60 minutes reaction times respectively. A reaction time of 15 minutes was found to be optimum for assay of protease activity with the Aspergillus niger strain isolated.There was no significant difference ($p>0.05$) between the reaction time of enzyme assay and protease activity. Thus, protease activity decreased with increased reaction time.*

Keywords: *Aspergillus, protease, optimized, assay, fermentation*

1. INTRODUCTION

Industrial enzymes have seen a spectacular rise in their production in the last three decades. The growth of industrial enzyme market has expanded to nearly eighty five (85) enzymes, which are currently in commercial production. With the discovery of a variety of new and more active enzymes and rising need for more environmental friendly and sustainable solution in every aspect of life, the enzyme market has been forecasted to go up to US$3.74 Billion by 2015 according to a new report by Global Industry Analyst (2013). The major enzymes used in industrial enzymes market are amylase, lipase, protease, ligase, cellulose, xylanase etc.

Enzyme-producing microorganisms include bacteria, molds and Yeasts (Fodiloglu and Erkman, 1999; Corzo and Revah, 1999; Kulkani and Gadre, 2002; Agu *et al*., 2013). In addition, to their role in synthetic organic chemistry, microbial enzymes also find extensive applications in chemical, pharmaceutical, food and leather industries (Gulati *et al*., 2005; Gunstone, 1999; Agu *et al.*, 2013). Proteolytic enzymes are included in a sub-class of the enzymes hydrolases. These enzymes cause breakdown of proteins into smaller peptides and amino acids by catalyzing the breakdown of peptide bonds. Proteases refer not to a single enzyme but to a mixture of enzymes which include proteinases, peptidases and amidases. The proteinases hydrolyze intact protein molecules to proteoses, peptones and some amino acids. Peptidases hydrolyze peptones to amino acids while amidases hydrolyze amino acids and release ammonia. Proteases are the most important class of industrial enzyme and constitute the largest product segment in the global industrial enzymes market as it account for about 60% of total enzyme market (Woods *et al.*, 2001). They are high temperature resistant with high specific activities and superior physical and chemical characteristics, that is why they have wide applications in a large number of industrial processes (Rao *et al*., 1998 and Temiz *et al*., 2008) for

example in food industry for cheese ripening, meat tendering, the production of protein hydrolysate and bread making (Poldermans, 1990), and with the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields, such as clinical, medicinal and analytical chemistry. To meet the current largely increased demand, studies on the cost-effective production of industrially important enzymes have become the need of today.

Plant, animal and microbial sources are employed in enzyme production. Microbial proteases are preferred to plant and animal sources to various advantages: rapid growth, the limited spa**ce** required for their cultivation, their broad biochemical diversity and the ease with which they can be genetically manipulated to generate new enzymes with altered properties (Rao *et al*., 1998).

A variety of microorganisms such as bacteria, fungi, yeast and *Actinomycetes* are known to produce these enzymes (Madan *et al.*, 2002). Molds of the genera *Aspergillus, Penicillium and Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Sandhya *et al.*, 2005).

The present study describes the production of protease from locally isolated *A. niger* by submerged fermentation. Reaction time was optimized for the assay of protease by *A. niger* in order to determine the time that gives the highest activity which will help maximize the reserve of huge foreign exchange spent on the importation of protease enzyme and discourage the use of chemically synthesized enzymes.

2. MATERIALS AND METHODS

2.1. Collection of Samples

Fresh meat was bought from a local market and spoilt beans were collected from a domestic waste in the local habitat both in Awka, Anambra State, Nigeria. The meat was mashed in a mortar, kept covered in a plastic bowl and allowed to undergo spoilage for three (3) days. The samples were transported to the microbiology laboratory of Nnamdi Azikiwe University Awka, Anambra State, Nigeria.

2.2. Isolation and Screening of Proteolytic Fungi

The fungal organism used in this study was isolated from spoilt beans and meat through serial dilution method. 1gram each of both samples was suspended in 9ml of sterile distilled water and shaked vigorously to release fungal spores responsible for the spoilage of the samples. 1ml each of the samples suspension was pipetted and transferred into another 9ml of sterile distilled water. This was further diluted in nine more 9ml of sterile distilled water blanks.

About 0.2 ml of the diluted samples from the 10^{-5} and 10^{-6} dilution was plated in duplicates on skimmed milk agar (SMA) for protease production by pour plate method. The plates were incubated at room temperature for 4-5 days. Fungal isolates with zone of clearance were further purified by repeated subcultures on SMA and identified as *Aspergillus niger, A. fumigatus and A. flavus* based on their morphological and microscopic characteristics according to Larone, (2011). The isolate with the highest zone of clearance was transferred to Sabouraud dextrose agar (SDA) slants and maintained at 4^0C.

2.3. Inoculum Preparation

Homogenous spore suspension of the fungal isolate with the highest zone of clearance was obtained by adding 10ml of sterile distilled water to the slants and shaked vigorously for 1minute.

2.4. Shake Submerged Fermentation

Fermentation conditions: Formulation used was a Modified Reese Medium (Ph 9.0) which contained KH_2PO_4, 2.0g; $(NH_4)_2SO_4$, 1.4g; $MgSO_4.7H_2O$, 0.3g; $CaCl_2$, 0.3g; Urea 0.3g; trace element solution 1.0ml; Tween 80, 0.5ml and supplemented with 0.30% glucose, 0.5% casein and 0.05% yeast extract. Trace element solution contained $MnSO_4.H_2O$, 1.56g; $FeSO_4.7H_2O$, 5.00g; $ZnCl_2$, 1.67g; $CoCl_2$, 2.00g per litre.

Fermentation process: Fermentation was carried out in 250ml Erlenmeyer flask containing 100ml of Reese medium (modified) pH 9.0 which was supplemented with glucose (0.25%), casein (0.5%) and yeast extract (0.5%). The medium was inoculated with 2ml of fungal spore suspension. The flask after inoculation was placed in the orbital shaker rotating at 200 rpm and at 30^0C for 72 hours.

2.5. Extraction of the Crude Enzyme Solution

At the end of the three days fermentation period, the culture medium was centrifuged at 5000rpm to remove fungal mycelia and medium debris, the supernatant was used as the crude enzyme solution.

2.6. Determination of the Optical Density for the Concentration of Tyrosine Standard

To six labeled test tubes, 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml of tyrosine was added. 1.0ml (0.5% casein) and 1.9ml 0.1M Tris-HCl buffer pH 9.0 were also dispensed and the tubes incubated at 37^{o}C for 30minutes.

At the end of the incubation period, the content of the test tubes were centrifuged and 1.0ml of Folin-Ciocalteu was added to 0.5ml of the supernatants and read spectrophotometrically to determine the optical density at 280nm for the different concentration of tyrosine (table 1).

2.7. Assay of Enzyme Activity for Isolate

The activity of the enzyme was measured in terms of its action on casein, and this was determined spectrophotometrically by Lowry *et al.*, method (1951).

0.1ml of the enzyme was added to four test tubes containing 1.0ml (0.5% casein) and 1.9ml 0.1M Tris-HCl buffer pH 9.0. These tubes were incubated at 37^{o}C for 15, 30, 45 and 60 minutes respectively in a water bath. The reaction was arrested by the addition of 2.0ml of 5% Trichloroacetic acid. The mixtures in the tubes were centrifuged and the supernatants obtained. 1.0ml of Folin-Ciocalteu reagent was added to 0.5ml of the supernatants and allowed to stand for 30minutes for colour development. The absorbance of the tubes were read at wavelength 280nm and their optical density obtained and extrapolated from that of tyrosine standard to determine the concentration of the tyrosine liberated by the protease enzyme produced. The activity of the enzyme assay was calculated for the different concentrations of tyrosine determined and recorded.

3. RESULTS

Of the three (3) fungal strains isolated from spoilt beans and meat which tested positive for protease, *Aspergillus niger* showed maximum hydrolysis zone (40mm) followed by *A. fumigatus* (32mm) and finally *A. flavus* (16mm) on skimmed milk agar and hence was selected for substrate screening.

The assay of protease activity at different reaction times showed that 15minutes reaction time had the highest activity (38.67U) with 60minutes showing the least activity as shown in table 2.

Table1. *Optical densities of the different tyrosine concentrations used*

Tube	Tyrosine Concentration (mg/ml)	Optical density(280nm)
1	0.0	0.0
2	0.2	0.032
3	0.4	0.069
4	0.6	0.104
5	0.8	0.138
6	1.0	0.176

Table2. *Activities of protease assay at different reaction times*

Tube density(280nm)	Activity(unit)	Time(mins)	Optical	Conc.(mg/min/ml)	Activity x 10^{-2}(mg/min/ml)
1	15	0.10	0.58	3.867	38.67
2	30	0.13	0.75	2.500	25.00
3	45	0.17	0.98	2.178	21.78
4	60	0.20	1.16	1.933	19.33

4. DISCUSSION

The result of this work has shown that *Aspergillus* species are good producers of protease enzyme which is in accordance with the works of Oyeleke *et al.*, 2010, Radha *et* al., 2011 and Srividya, 2012.

Work has not been reported on the optimization of reaction time for enzyme assay though some researchers have optimized the fermentation periods for enzyme production. Most of the works where fermentation time was optimized showed that protease production/yield decreased with increased incubation time except for a few one of which is the result of Oyeleke *et al.*, 2010 which showed that

protease production increased with passage of time as high yields of proteases by *A. flavus* and *A. fumigatus* were noticed after 144 h with protease activities of 0.96 µg/ml for *A. flavus* and 0.84 µg/ml for *A. fumigatus.* Regardless of the time optimized, the result of Oyeleke *et al.,* 2010 is in contrast with the result of this work which showed highest protease activity of 38.67U at reaction time of 15minutes with a sudden decrease to 25.00U at 30minutes. In relation to the result of this study, Malathi and Chakraborty (1991) reported that there was a steep increase in enzyme activity produced by *Aspergillus flavus* from 36 hour on, with a maximum being reached after 48 hour. After 70 hour, less than 50% of the maximum enzyme activity was retained.

This is also similar to Hamid and Ikram-Ul-Haq (2009) who reported that maximum acid protease activity of 7.2U/g by *Aspergillus niger* was achieved after 72 hours of incubation. After 72 hours, the enzyme activity was slightly declined but after 96 hrs, there was a sharp decline in the production of protease.

The result of Srividya, 2012 indicated that protease yield increased gradually and attained high titer of enzyme activity (280 U/g) at 120 hours of incubation. Further incubation reduced the yield. This also agrees with the result of this work which has shown that increased time decreased enzyme activity.

The variations in the enzyme activities of the aforementioned works could be attributed to species differences, fermentation methods and fermentation/assay periods among many others.

It is therefore concluded from this study that *A. niger* is a good producer of protease enzyme and increased reaction time of enzyme assay decreased enzyme activity.

5. APPENDIX

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CORRELATIONS
/VARIABLES=Time Activity
/PRINT=TWOTAIL NOSIG
/MISSING=PAIRWISE.
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Correlations

		Different reaction times in minutes	Activities of protease assay in unit
Different reaction times in minutes	Pearson Correlation	1	-.916
	Sig. (2-tailed)		.084
	N	4	4
Activities of protease assay in unit	Pearson Correlation	-.916	1
	Sig. (2-tailed)	.084	
	N	4	4

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