

BRC 2002039/15108

## Cellulase and amylase production by *Aspergillus niger* Sl.1 in ammonia treated agrowastes and evaluation of some kinetic parameters of the enzymes

E. A. Abu<sup>1</sup>; D. A. Ameh<sup>1</sup>; S. I. Ibrahim<sup>1</sup>; A. S. Agbaji<sup>2</sup> and J. B. Ameh<sup>3</sup>

<sup>1</sup>Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria.

<sup>2</sup>National Research Institute for Chemical Technology, Zaria, Nigeria.

<sup>3</sup>Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria.

(Received June 18, 2002)

**ABSTRACT:** production of cellulase and amylase by *Aspergillus niger* Sl.1 in carboxy methyl cellulose (CMC) and in four ammonia – treated agrowastes, maize cob (MC), rice bran (RB), sorghum bran (SB) and wheat bran (WB) were investigated. Some kinetic parameters of the enzymes were also investigated. Among the agrowastes, maize cob recorded the highest cellulase activity ( $6.02 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ) while wheat bran recorded the lowest cellulase activity ( $5.02 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ). The highest amylase activity ( $4.64 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ) was recorded in wheat bran medium. Optimum pH for cellulase and amylase activities range from 5.5 – 6.0 and 6.0 – 7.0 respectively in all the media studied. Optimum temperature for cellulase and amylase activities conducted on the crude enzyme extract indicated that cellulase in carboxymethylcellulose medium was the least stable both at room and refrigerated temperatures. The least stable amylase enzyme was recorded in rice bran and wheat bran media at room and refrigerated temperatures respectively after 21 days.

The implications of these results on the industrial production cellulase and amylase using the agrowastes are discussed.

**Key Words:**

### Introduction

Cellulase and amylase are among the industrially useful enzymes produced by micro-organisms especially members of the genus *Aspergillus* (Sanyal et al., 1988; Shambe, 1999; Okolo et al., 1995).

Production of cellulase and amylase using agrowastes has been extensively studied and various pre-treatments such as ball milling, steaming, alkali, combined alkali and steaming have been employed to enhance cellulase production with combined alkali and steaming standing out as the most acceptable method of pre-treatment (Raji et al., 1998; Udotong, 1997; Ali et al., 1991). results from these studies could not be compared because cellulase production was carried out under different conditions using different organisms. In our effort to assess the effect of five pre-treatment methods on cellulase production by *Aspergillus niger* SL.1 in four agrowastes media under similar conditions, ammonia steeping was found to be generally studied (Abu et al., 2000; Abu et al., 2001).

Further study was therefore carried out to investigate the cellulase and amylase production by *Aspergillus niger* SL.1 in ammonia treated agrowastes and evaluate some parameters related to enzyme activities and stabilities in the crude extract.

## Materials and Methods

**Agrowastes:** Four agrowastes, maize cob; rice bran; sorghum bran and wheat bran; obtained from harvesting dump in Guinea Savannah Zone of Nigeria were used.

**Organism:** *Aspergillus niger* SL.1 isolated from compost soil at Ahmadu Bello University, Zaria, characterized and maintained on potato dextrose agar (PDA) at 4°C was used.

### *Substrate Pre-treatment*

The agrowastes were pre-treated by steeping 20g of the milled agrowastes in 100ml of 2.9M ammonia solution for 24 hours (Cao et al., 1996). The mixture was filtered and the residue was washed with distilled water and dried to a constant weight at 80°C in an oven.

### *Enzyme production*

The organism was cultured in a mineral salt-cellulosic media (g/l)  $\text{KH}_2\text{PO}_4$ , 10.0;  $(\text{NH}_4)_2\text{SO}_4$  10.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.33;  $\text{CaCl}_2$ , 0.5;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.013;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.004;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.004;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.0067; yeast extract, 0.5 and 40g of CMC or maize cob, or rice bran or sorghum bran or wheat bran in their respective media. The initial pH was adjusted to 5.0 following autoclaving. A spore suspension ( $2.0 \times 10^5$ ) of *A. niger* SL.1 was used as inoculum for each of the culture medium. The media were incubated at 28°C in an orbital shaker set at 100 rpm for 72 hours.

### *Assay for Cellulase and Amylase Activities*

Cellulase activity was determined by measuring the amount of reducing sugar released as glucose in 3.0ml reaction mixture containing 0.1M acetate buffer (pH 5.0), 2% CMC solution and culture supernatant at 30°C during 30 min. The reducing sugar was determined by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme, which released  $1\mu\text{mole glucose min}^{-1}$ . Amylase activity was assayed by incubating 2% starch in 0.1M acetate buffer (pH 5.0) with the culture supernatant at 30°C for 30 min (Wood and Bhat, 1988). One unit of enzyme activity was defined as the amount, which released  $1\mu\text{mole glucose min}^{-1}$ . Protein was determined by the methods of Lowry et al (1951).

## Result and Discussion

The extracellular cellulase and amylase production by *Aspergillus niger* SL.1 in some locally available and abundant agrowastes are summarized in Table 1. It is apparent from the results that all the agrowastes tested supported and amylase production comparable to carboxymethylcellulose. Among the agrowastes maize cob recorded the highest cellulase activity ( $6.02 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ), which represents 77.8% of carboxymethylcellulose medium ( $7.73 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ). Wheat bran however recorded the lowest cellulase activity ( $5.02 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ). Amylase production was highest in wheat bran medium ( $4.64 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ) and lowest in rice bran medium ( $1.60 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ). Production of amylase in maize cob and wheat bran media represents 132 – 161% of carboxymethylcellulose medium ( $2.87 \text{ unitsmg}^{-1}\text{ml}^{-1}$ ). Difference in cellulase and amylase production in the agrowastes could be due to differences in the structural features of the cellulosic wastes and the degree of susceptibility of the substrates to chemical modifications (Weinmer et al., 1995; Karunananda et al., 1992).

Specific advantage of ammonia treatment is the associated residual nitrogen, which enhances biomass growth and activities (Macdonald et al., 1994). Combination of substrate susceptibility to chemical modification and specific advantage of ammonia treatment could be responsible for higher amylase production in wheat bran and maize cob than carboxymethylcellulose medium. These as well may be responsible for comparable cellulase activities in some of the agrowastes studied (Sanhya and Losane, 1994).

A study of the effect of pH on the cellulase activities of crude extract showed that pH range of 5.5 – 6.0 supported the highest cellulase activities in the five media (Figure 1). The optimum pH values for cellulase activities from fungal species has been reported to vary from species to species ranging from 3.0 to 6.0 (Ali et al., 1991; Shambe, 1999).

Similar study of the effect of pH on amylase activities revealed that all the media had optimal pH of 6.0 – 7.0 (Figure II). The optimal values recorded in this study is similar to those reported for *bacillus subtilis* amylase (Krishma and Chandrasekaran, 1996) and Fungal species; *Cephalosporium Nodulisporium* and *Aspergillus* (Baldensperger et al., 1985; Shambe, 1999).

Figure III showed the effect of changes in temperature on cellulase activities. Optimum temperature range between 35 – 40°C in the five media. The optimum temperature values observed for this organism is similar to those reported by other workers on fungal sources of cellulase (Gbekeloluwa and Moo-Young, 1991).

Effect of changes in temperature on amylase activities shown in Figure IV indicated an optimum range of 40 – 50°C in all the media. Shambe (1999) reported an optimum temperature of 50°C for some fungal species of amylase. An optimal temperature of 35°C for bacterial sources of amylase has been reported (Krishma and Chandrasekaran, 1996; Ramesh and Losane, 1989).

Tables II and III showed storagability studies of crude enzyme extracts of both cellulase and amylase. Stability of the enzyme decrease with increase in the number of days both at refrigerated and room temperature. However room temperature storage showed a higher rate of decrease in enzyme activities. Cellulase in carboxymethylcellulose medium was the least stable both at room and refrigerated temperatures. The least stable amylase enzyme was recorded in rice bran and wheat bran media at room and refrigerated temperatures respectively after 21 days.

On the basis of the results of the present study, it is concluded that the utilization of the four agrowastes as substrates when subjected to ammonia treatment, could lead to large scale production of industrial enzymes and also contribute to safe and economics waste management in the environment. The present study also clearly indicates the variations in pH and temperature influence the efficiency of the enzyme irrespective of the type of substrate used and the crude enzyme extracts was fairly stable over 21 days, which could reconstitutes significant economic benefit.

Table 1: Cellulase and amylase activities in ammonia treated agrowastes.

Sample	Cellulase (Units/ml)	Amylase (Units/ml)	Protein (mg/ml)	Specific activities (Unitsmg <sup>-1</sup> ml <sup>-1</sup> )	
				Cellulase	Amylase
CMC	6.03	2.24	0.78	7.73	2.87
Maize cob	5.48	3.46	0.91	6.02	3.80
Rice Bran	3.28	1.32	0.58	5.66	2.28
Sorghum Bran	5.57	1.50	0.94	5.93	1.60
Wheat Bran	4.37	2.83	0.87	5.02	4.64

Values are means of triplicate determinations.

FIGURE I: EFFECT OF pH ON CELLULASE ACTIVITIES

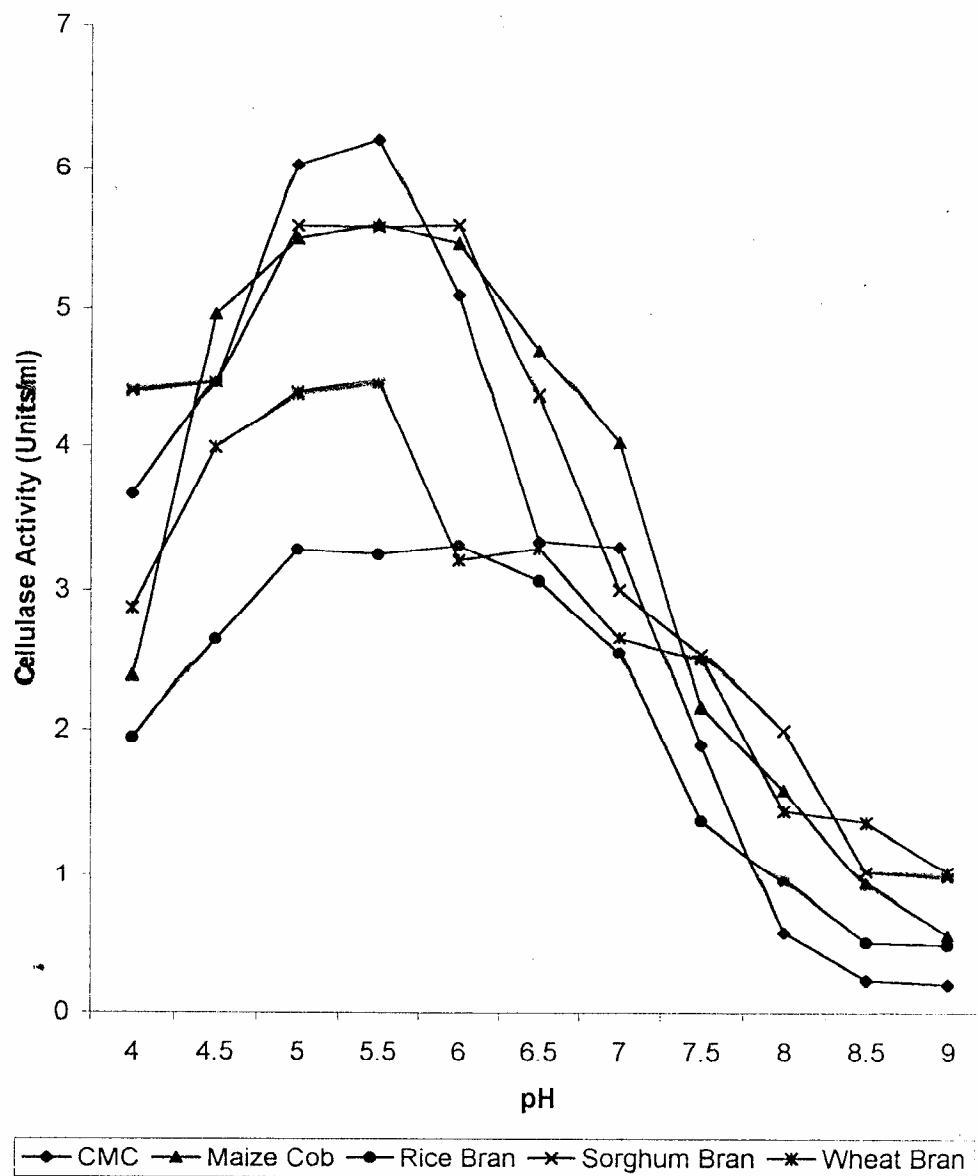


FIGURE II: EFFECT OF pH ON AMYLASE ACTIVITIES

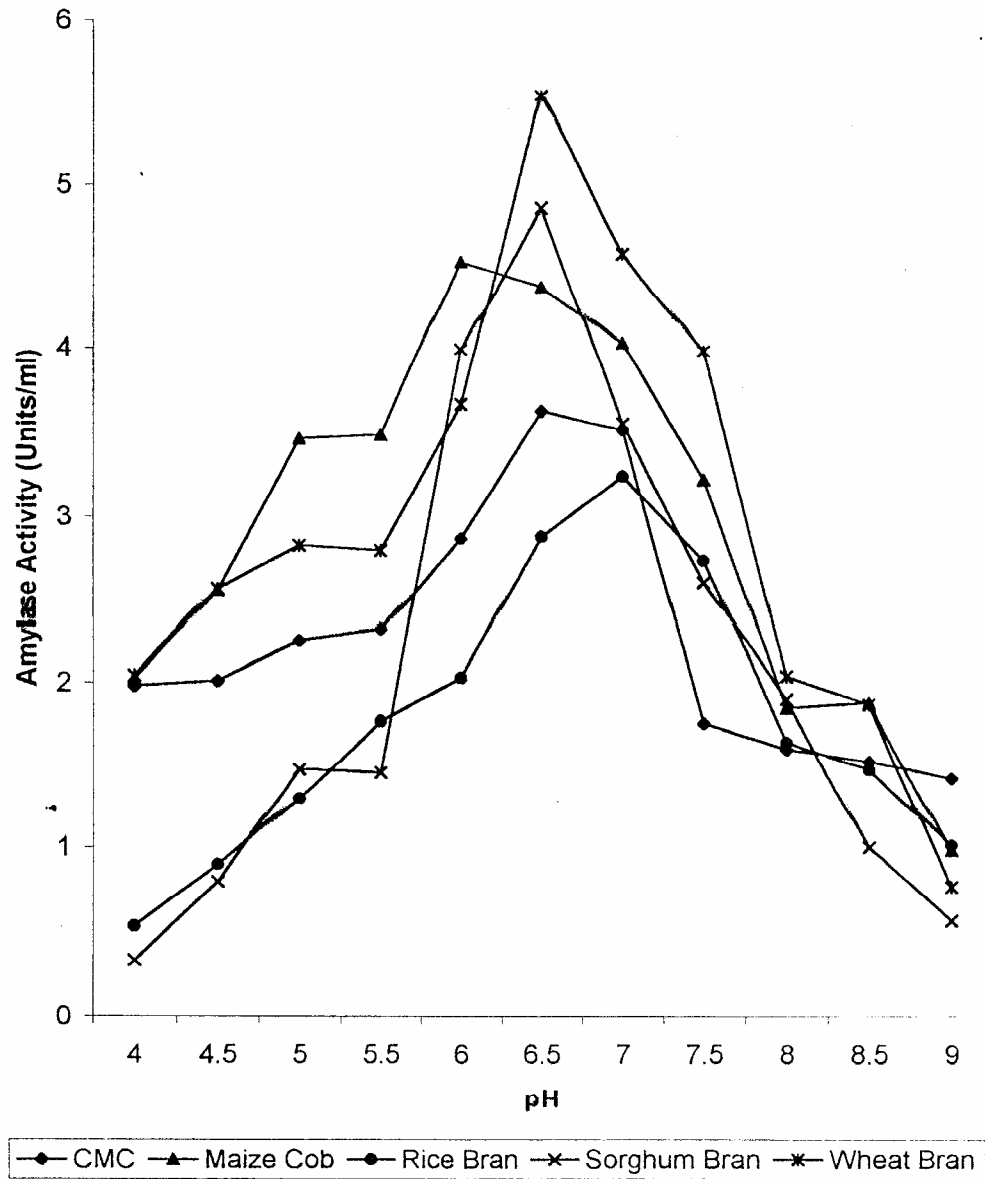


FIGURE III: EFFECT OF TEMPERATURE ON CELLULASE ACTIVITIES

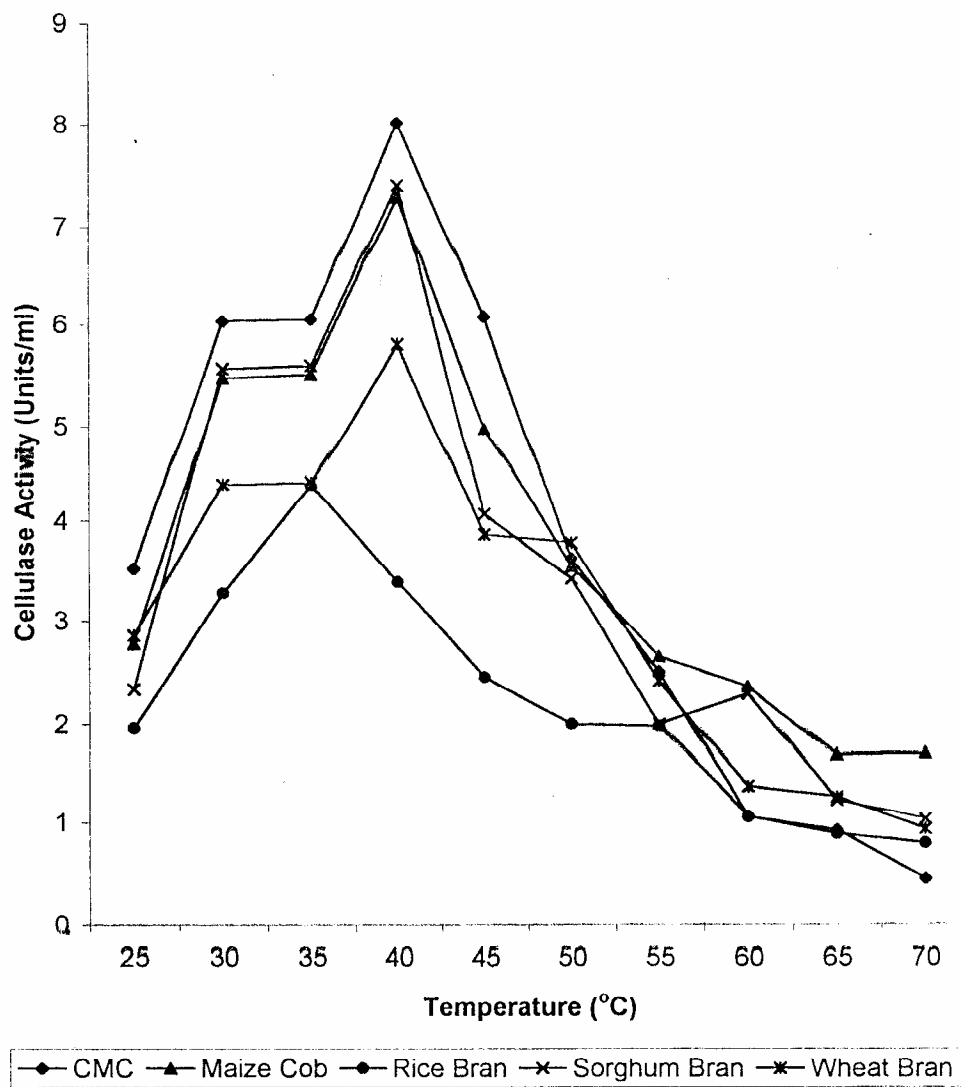


Table 2: Effect of storage time on cellulase activities.

DAY	CMC		Maize Cob		Rice Bran		Sorghum Bran		Wheat Bran	
	RMT	RFT	RMT	RFT	RMT	RFT	RMT	RFT	RMT	RFT
0	6.03	6.03	5.48	5.48	3.28	3.28	5.57	5.57	4.37	4.37
3	6.01	6.02	5.39	5.46	3.25	3.28	5.54	5.56	4.33	4.36
7	5.49	5.89	5.04	5.40	2.99	3.21	5.49	5.46	4.04	4.30
14	3.32	5.50	4.10	4.96	2.01	3.00	4.02	5.31	3.86	3.99
21	1.36	4.80	2.09	4.62	1.65	2.65	2.32	4.64	2.47	3.68

values are means of triplicate determinations.

Key: RMT = Room Temperature; RFT = Refrigerated Temperature.

Table 3: Effect of storage time on amylase activities (Units/ml).

DAY	CMC		Maize Cob		Rice Bran		Sorghum Bran		Wheat Bran	
	RMT	RFT	RMT	RFT	RMT	RFT	RMT	RFT	RMT	RFT
0	2.24	2.24	3.46	3.46	1.32	1.32	1.50	1.50	2.83	2.83
3	2.25	2.23	3.43	3.45	1.28	1.32	1.49	1.49	2.80	2.81
7	2.20	2.21	3.36	3.40	1.20	1.30	1.46	1.47	2.76	2.79
14	1.88	1.99	2.75	3.31	0.86	1.26	1.01	1.40	2.05	2.56
21	1.04	1.69	1.84	2.76	0.41	1.10	0.86	1.32	1.66	2.01

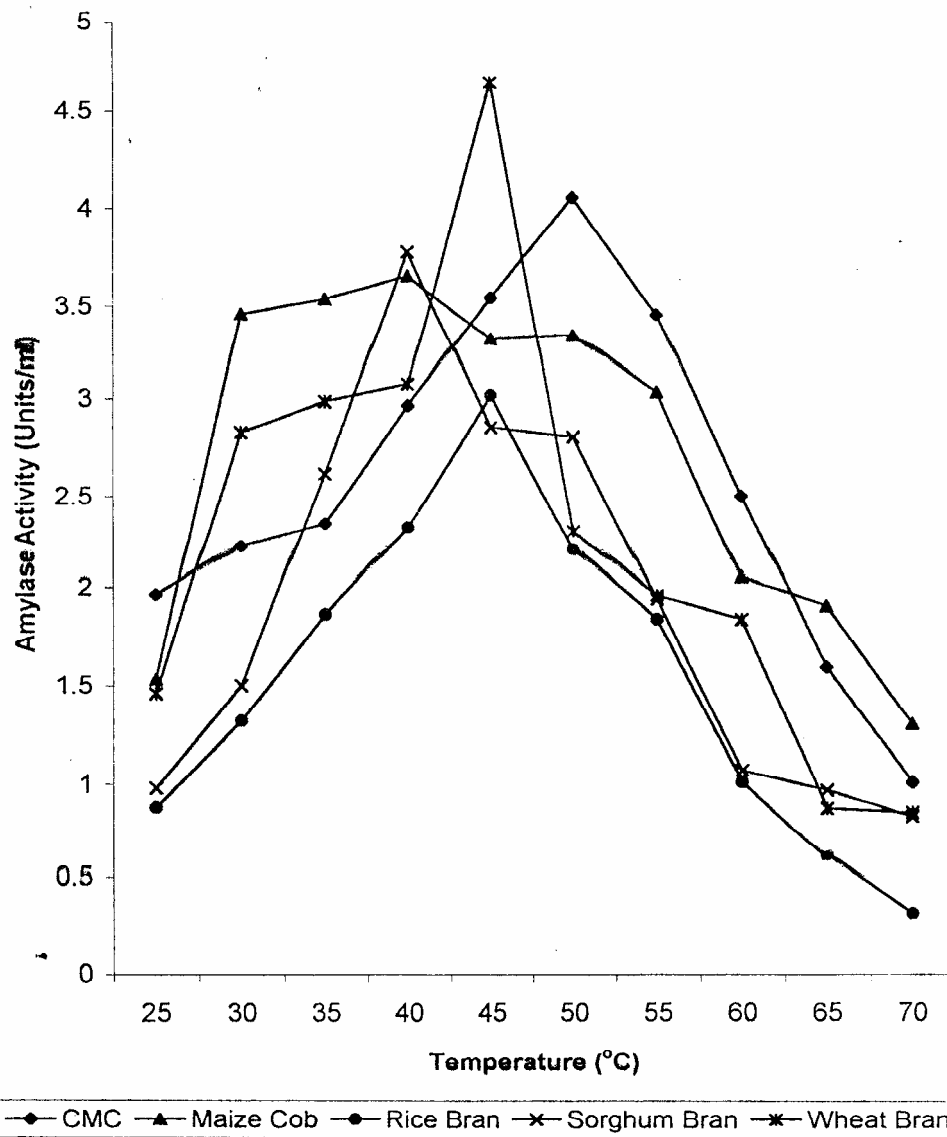
Values are means of triplicate determinations.

Key: RMT = Room temperature; RFT = Refrigerated Temperature.

## References

- Abu, A.E.; Ameh, D.A.; Agbaji, A.S.; Onyenekwe, P.C.; Ado, S.A. and Nwaeze, A.R. (2001). Effect of Pre-treatment of maize cob and wheat bran and cellulase production by *Aspergillus niger* SL.1. Nig. J. Biotechnol. 12(1): 55 – 63.
- Abue, A.E.; Onyenekwe, P.C.; Ameh, D.A.; Agbaji, A.S. and Ado, S.A. (2000). Cellulase production from sorghum bran by *Aspergillus niger* SL.1: An assessment of pre-treatment methods. A paper presented at the International Symposium on Biotechnology; Commercialization and Food Security, 25<sup>th</sup> – 27<sup>th</sup> October, 2000, Abuja.
- Ali, S.; Sayed, A.; Sarker, R.T.; Akin, R. (1991). Factors affecting cellulase production by *Aspergillus terreus* using Water Hyacinth. W.J. Microbiol. and Biotech., 7: 62 – 66.
- Baldensperger, J.; Lemer, J.; Hannabal, L.; Quinto, R.J. (1985). Solid-state fermentation of banana wastes. Biotech. Lett., 7: 743 – 748.

FIGURE IV: EFFECT OF TEMPERATURE ON AMYLASE ACTIVITIES





- Cao, J.; Krishan, M.S.; Du, J.X.; Ho, W.Y.; Chen, Z.D.; Tsao, G.T. (1996). Ethanol production from corn cob pre-treated by ammonia steeping process using generically engineered yeast. *Biotech. Letters*, 18(9), 1013 – 1016.
- Gbekeloluwa, B.D. and Moo-Young, M. (1991). Production and properties of  $\beta$ -glucosidases by *Neurospora sitophila*. *W.J. Microbiol. and Biotech.*, 7: 4 – 11.
- Krishna, C. and Chandrasekaran, M. (1996). Banana waste as substrate for  $\alpha$ -amylase production by *Bacillus subtilis* (CBTK 106) under solid – state fermentation. *Appl. Microbiol. Biotechnol.*, 46: 106 – 111.
- Karunananda, K.; Fales, S.L.; Varga, G.A.; Royse, D.J. (1992). Chemical composition and bio-degradability of crop residues colonized by white rot fungi. *J. Sci. Food Agric.* 60: 105 – 115.
- Lowry, O.H.; Rosebrough, N.S.; Farr, A.L.; Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265 – 275.
- McDonald, P.; Edwards, R.A.; Greenhalch, J.F.D. (1994). Effect of ammonia treatment on the nitrogen content of straws. In: *Animal Nutrition*, Longman Publ., pp. 426 – 428.
- Miller, G.C. (1959). Use of the dinitrosalicylic acid reagent for the determination of reducing sugar. *Anal. Chem.* 31: 426 – 428.
- Okolo, B.N.; Ezeogu, L.I. and Mba, C.N. (1995). Production of raw starch digesting amylase by *Aspergillus niger* grown on native starch sources. *J. Sci. Food Agric.* 69: 109 – 115.
- Raji, A.I.; Ameh, J.B. and Ndukwe, M. (1998). Production of cellulase enzyme by *A. niger* CS14 from delignified wheat straw and rice husk substrates. *Nig. Journ. Educ.* 15(1): 57 – 63.
- Ramesh, M.V. and Losane, B.K. (1989). Solid state fermentation for production of higher titres of thermostable  $\alpha$ -amylase with two peaks of pH optima by *Bacillus licheniformis* M27. *Biotech. Lett.* 11: 49 – 52.
- Sanhya, X. and Losane, B.K. (1994). factors influencing fungal degradation of total soluble carbohydrates in sugarcane-press mud and under solid state fermentation process. *Biochem.* 29: 295 – 301.
- Sanyal, A.; Kudu, R.K. and Duber, D.K. (1988). Extra cellular cellulolytic enzyme system of *Aspergillus japonicum*. Effect of different carbon sources. *Enzyme and Microbiol. Technol.* 10: 85 – 90.
- Shambe, T. (1999). Production of amylases and cellulases: degradation of starch and carboxymethyl cellulase by extra cellular enzymes from four fungal species. The effect of cation and sulphhydryl group on the activity of the enzymes. *Nig. Journ. Biotechnol.* 6: 131 – 137.
- Udotong, I.R. (1997). Comparative energy yields from three lignocellulosic wastes. *Nig. Journ. Ren. Ener.* 15: 143 – 147.
- Weimer, P.J.; Hackney, J.M.; Frenceh, A.D. (1995). Effects of chemical treatments and heating on crystallinity on cellulase biodegradation. *Biotech. and Bioeng.* 48: 69 – 78.
- Wood, T.M. and Bhat, K.M. (1988). Methods for measuring cellulase activities in: Aselson, J.N.; Simon, M. (Series eds.) *Methods enzymology series. Biomass Part A. Cellulose and Hemicellulose*. Academic press, N/Y, pp. 87 – 112.