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Enzyme synthesis from *Aspergillus niger* using solid-state fermentation of Agro - waste Substrates

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Abstract:- Cellulose is considered to be the most abundant renewable lignocellulosic material on earth. The technique employed for the conversion of these agricultural residues to simple sugars by cellulases thus has a great industrial and commercial potential and can open new application fields in the areas of industrial processes. The process of this conversion of cellulosic materials by cellulase which is a hydrolytic enzyme was studied using solid-state fermentation (SSF) and agricultural wastes as substrates and microorganisms (*Aspergillus niger*)in a controlled environment while giving the organism free access to water molecules. The fungal organism used was isolated from the area where the bi-products were obtained. The microbe (*A.niger*) was subjugated for the production of an enzyme carboxymethyl cellulase in a CMC solution by the DNSA method ata varying range of temperatures and other varying parameters were studied as well such as the pH, Carbon and nitrogen sources and effects of metals ion concentration on the enzyme. It was analyzed using the Lineweaver-Burk plot which offered a practical graphical method for analyses of the Michaelis-Menten equation to evaluate the imperative terms in enzyme kinetics such as K_m and V_{max}. The yield was also compared using the aforementioned equation and kinetics and it was observed that the enzymatic yield was higher on rice bran than on corn cob. The enzyme was tolerant and stable at a varying range of temperatures, as such is a viable tool in industrial processes and hence can provide veritable potential in developing andspurring the economy of the country if well harnessed.

Keywords:- *cellulose, Aspergillus niger, fermentation, substrates, synthesis*

I. INTRODUCTION

Cellulase is one of the several enzymes produced chiefly by microorganisms (bacteria, fungi, and protozoans) that catalyze cellulolysis, cellulose decomposition, and some related polysaccharides. Complete cellulose hydrolysis to glucose demands the action of exoglucanases, endoglucanases and β-glycosidases. These enzymes could act serially or synergistically to decompose cellulosic materials.The production of cellulase has been reported in a wide variety of bacteria (Bhat & Bhat,1997) and fungi (Anita, Namita, Narsi, & Bishnoi,2009). However filamentous fungi are preferred for commercial enzyme production because the quantity/level of enzymes produced by these cultures are higher than those obtained from yeast and bacteria (Bakri, Jacques & Thonart, 2003).Almost all genera of *Aspergillus* synthesize cellulase, the latter has the potential to dominate the enzyme industry. However, cellulase may reach the largest volume of industrial enzyme; if the ethanol from lignocellulosic biomass through the enzymatic route becomes a major transportation fuel (Singhania, Sukumaran, Patel, Larroche & Pandey, 2010). Filamentous fungi are the most commonly used microorganisms in solid state fermentation (SSF) because they can grow on solid materials with low water contents (Pandey, Selvakumar, Soccol, & Nigam, 1992). Interestingly, there are several reports describing the use of agro-industrial residues for the production of cellulases such as wheat straw, corn cob, wheat bran, and rice bran as substrates (Alegre, Polizeli, Terenzi, Jorge, & Guimaraes, 2009). The emphasis on the use of microbial cellulase in the market has potentially led to a dramatic and extensive interest in bio-based fuels and chemicals. To create a viable and sustainable economy, cellulases need to be produced cost-effectively and t, a pospressive or high level of biocatalyst properties. Solid state fermentation (SSF) offers a low-cost alternative for the production of cellulases using natural agro-industrial wastes. Conservatively, solid state fermentation is an alternative phase where microorganisms grow on the surface of moist (water) particles as well as in between them. This process providesa viable design as the spaces in between the particles are occupied by a continuous gas phase which is a key factor in the production of enzymes. Moreover, the gas phase is greatly influenced by the size, and shape of the network of gas-filled pores. The availability of spaces in between the particles ensures the availability of oxygen that improves enzyme production in aerobic fungal culture. Experiments have that showed that substantial improvements made during enzyme production may have resulted in the bed porosity of the substrates. In Industrial scaleprocesses, where the production of enzymes iscommercialized, the bed porosity is essential but not all-sufficient in the process. Other parameters such as the physiology of the microbial cell, the structures or composition of the solid substrate and substrate-microbial reactivity could influence the production process.Importantly, substrate reactivity in cellulosic substrates is greatly influenced by the physicochemical characteristics of the substrate at varying levels of degrees, as regards the microfibril level, it is the crystallinity of cellulose, whereas at the fibre level, the specific area. The optimum reaction in cellulase reactivity due to the increase in specific surface area is credited to the increase in or creation of surface openings or spaces by the removal of cell wall components that enhance the direct contact between the enzyme (cellulase) and the substrates. During the growth phase of the cellulosic substrates, the propagation of the fungal mycelium (*A. niger*) occurs through the production of the enzymes that drive the hydrolytic reactions. The reaction is responsible for the production of soluble sugars that in turn facilitate/promote fungal growth. It is interesting to note

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that hydrolysis occurs effectively when the process or spaces are large enough to accommodate both largeand small enzymes tomaintain the synergistic reaction/action of the enzyme system, hence the mechanical preparation of the substrates. Whereas on the other hand, reduced surface area inhibits this synergy.(Rahardjo,& Jolink, 2005)

The growth of fungal natural substrate is usually slow and drawbacks could beovercome by mechanical and chemical pre-treatment. Consequently, pre-treatments are known to introduce structural changes, especially in cellulosic substrates which in turn alter the physicochemical properties of the substrates. The present study is aimed at determining the microorganism (*A niger*), through solid-state fermentation, Enzymes assay and protein determination, and optimization of the physical and chemical parameters (pH, temp, Carbon and Nitrogen sources) and comprising of enzyme yield between substrates. This enzyme has enormous potential applications in the production of food, animal feeds, textile, fuel, chemicals, pharmaceutical industries, paper and pulp industries and waste management (Benguin, 1990 & Tarek, 2007).

II. MATERIALS AND METHOD

A. Identification of the Organism

The fungus (Aspergillus niger) was isolated from corn cob and rice bran retting ground area at market areas of Onitsha and Asaba respectively. The isolate was grown on a potato dextrose agar (PDA). After 7 days of culture the isolate was identified using colony morphology and microscopic examination and, molecular analysis of the organism, Aspergillus niger was identified using polymerase chain reaction. The isolate was further cultured on a CMC agar medium and screened for its ability to synthesize cellulose according to Wood and Bhat (1998). The isolated fungal colony was further sub-cultured until solid state fermentation.

The procured cellulosic substrates (corn cob and Rice bran) were ground mechanically using a mortar and pestle. The substrates were roughly grounded to provide a large surface area for enzyme activity. The substrates were treated with (1% 9w/v) of NaOH solution in the ratio of 1.10 (substrate solution) for 1 hr and were brought to a neutral PH (7) by washing thoroughly with distilled water and dried at room temperature. The treated substrates were autoclaved at 1210 C for I hr (Gomes, et al 2006).

The fermentation was carried out using (20) 250ml of Erlenmeyer flash with 100ml of Czapek-dox broth and 15ml of distilled water (moistening agent) and 10g of the substrates. The experiment was replicated for each subtract (corn cob and Rice brain). The flasks (20), (10 for corn cob and 10 for Rice bran) were sterilized at 1210C for 15mins and cooled at room temperature 1ml of the inoculum (was added, mixed thoroughly and incubated at various temperature ranges in a humidified incubator for 96hrs. (The flasks contained the inoculum (microbes and salts, sugars and Nitrogen sources). They were gently mixed periodically by shaking. The enzyme was extracted from the substrates (corn cob and Rice brain) by mixing the Erlenmeyer flasks gently and homogeneously in a rotatory shaker (120rpm) at 300 C with a contact time of 1 hr. A dampened muslin cloth was used to filter the extract and the pooled extracts were centrifuged at 6000rpm for 15mins and the clear supernatant was used as a source of extracellular enzyme. The Cellulase (carboxymethyl cellulase) was assayed according to the method of Cehose (1987) where one unit of the enzyme is the amount of enzyme which releases per mol of reducing sugars per minute with glucose as standard. The protein content of the supernatant secreted by the fungus (A. niger) was estimated by lowry's method (lowry et al, 1951). The optical density obtained from the spectrophotometer was compared with BSA standard curve to calculate the amount of protein (mg/ml) in the supernatant used in the cellulose assay. Glucose Stock; 100mg of glucose was dissolved in 10ml of distilled water. Citrate buffer: 210 of citric acid monohydrate (C6 H80 7H2 0) was dissolved in 750ml distilled water. Na0H was added until the pH was equal to 4.3 and the final make-up was 100ml. that is M citrate buffer had a pH of 4.5 when it was diluted to 0.05 pH was maintained at 4.8. Dintrosaticylic acid (DNSA). 30G K-Na tartrate, dissolved in 50ml of distilled water, was further added by 1g DNSA and 20ml of 2N N2OH; the final make-up was 100ml. The data was collected and the glucose standard curve was used as a standard according to Kondo et al.(1994).However, the kinetics of the enzyme cellulase was assayed with increasing concentrations of CMC (carboxymethylcellulose) (0.25-0.45mg\0.5ml) at 25 and 37^{o}C at varying pH values.

III. RESULT

organism	Organism grp	strain	sample	assembly	Size(Mb)	GC%	scaffold
Aspergillus niger	Eukaryota, fungi Ascomycetes	B20044	Sam34006482	GCA-023625455.1	35.02	49.30	822

Table 1: Molecular results of the fungi

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Temperature (^{0}C)	**Enzyme Activity (O.D)**	
	Corn cob	Rice bran
25	0.89	1.67
30	0.82	0.8
35	0.75	0.84
37	0.98	0.98
40	0.75	0.85

Table 2: Effects of temperature on the Enzyme Activity at 540nm

pH	**Enzymatic Activity O.D**	
	Corn cob	**Rice bran**
3.0	0.92	1.63
4.0	0.97	1.45
4.5	1.62	1.36
5.0	1.46	1.33
6.5	1.36	1.08
7.0	1.21	0.96
8.5	1.17	0.73

Table 3: Effects Of pH On Enzymatic Activity At 540nm

(g/mol)	**Sucrose Enzymatic Activity**		**Galactose Enzyme Activity (O.D)**		**lactose Enzyme Activity (O.D)**	
	Corn Cob	**Rice Bran**	**Corn Cob**	**Rice Bran**	**Corn Cob**	**Rice Bran**
0.25	0.86	0.96	0.74	0.89	0.62	1.74
0.36	0.36	0.97	0.84	0.84	1.22	1.32
0.40	0.86	0.93	0.80	0.82	1.22	1.27
0.45	0.75	0.89	0.79	0.78	0.92	0.94

Table 4: Effects of Sugar on Enzymatic Activity at 540nm

(g/l)	**KNO_3;/Enzyme Activities (OD)**		**Peptone Enzyme Activities (OD)**		**Urea Enzyme Activities (OD)**		**NH_4N0_3Enzyme Activities (OD)**	
	Corn Cob	Rice Bran	Corn Cob	Rice Bran	Corn Cob	Rice Bran	Corn Cob	Rice Bran
2.0	**0.65**	**0.93**	**0.65**	**0.86**	**0.68**	**1.73**	**0.90**	**1.36**
4.0	**0.82**	**0.98**	**0.45**	**0.89**	**0.63**	**1.84**	**0.93**	**1.39**

Table 5: Effect of Nitrogen sources on Enzyme Activity at 540mm

Temperature ^{o}C	pH	Carbon sources(g/mol)	Nitrogen sources(g/l)	E.A (O.D)
37^{o}C	4.5	Lactose 1.46	H_4NO_3	1.99nm

Table 6: Effect of the Optimized parameters for *A. niger* on the corn cobatoptimized Temperature, pH, Carbon and Nitrogen at 540nm.

Temperature ^{o}C	pH	Carbon sources(g/mol)	Nitrogen sources(g/l)	E.A (O.D)
25^{o}C	3.0	Lactose 0.25	1.84	1.96

Table 7: Effect of the Optimized parameters for A. niger on the Rice bran at optimized Temperature, pH, Carbon and Nitrogen at 540nm.

Protein estimation content of *A. niger* on corn cob and Rice branat 620nm.

Temp ^{o}C	Enzyme Activities (OD)	
	Corn cob	Rice bran
25	1.75	1.63
30	1.73	1.96
35	1.71	1.54
37	1.62	1.38
40	1.58	1.34

Table 8: Effect of Temperature on Enzymatic Activity at 620nm

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pH	Enzyme Activities (OD)	
	Corn cob	Rice bran
3.0	1.06	1.37
4.0	1.44	1.39
4.5	1.41	1.46
5.0	1.49	1.24
6.5	1.46	1.23
7.0	1.05	1.18
8.0	0.98	1.04

Table 9: Effect of pH on Enzymatic Activity at 620mm.

(g/mol)	**Sucrose Enzymatic Activity**		**Galactose Enzyme Activity (O.D)**		**lactose Enzyme Activity (O.D)**	
	Corn Cob	Rice Bran	Corn Cob	Rice Bran	Corn Cob	Rice Bran
0.25	1.02	0.97	1.07	0.87	0.53	0.97
0.36	0.93	0.92	1.04	0.84	0.86	0.92
0.40	1.36	**0.93**	0.96	0.79	1.37	0.93
0.45	1.56	0.58	1.32	0.69	1.46	1.04

Table 10: Effect of Carbon Source on Enzymatic Activity at 620nm.

(g/l)	**KNO_3/Enzyme Activities (OD)**		**Peptone/Enzyme Activities (OD)**		**Urea/Enzyme Activities (OD)**		**NH_4N0_3/Enzyme Activities (OD)**	
	Corn Cob	Rice Bran	Corn Cob	Rice Bran	Corn Cob	Rice Bran	Corn Cob	Rice Bran
2.0	1.13	1.35	1.26	1.14	1.21	1.25	1.63	1.43
4.0	1.94	1.46	1.34	1.53	1.53	1.99	1.96	1.64

Table 11: Effect of Nitrogen Source on Enzymatic Activity at 620nm

metals(mg)	**Zn/Enzyme Activities (OD)**		**Mg/Enzyme Activities (OD)**		**Fe/Enzyme Activities (OD)**		**Ca/Enzyme Activities (OD)**		**Cu/Enzyme Activities (OD)**		**Na/Enzyme Activities (OD)**	
	Corn Cob	Rice Bran	Corn Cob	Corn Cob	Rice Bran	Corn Cob	Rice Bran	Corn Cob	Rice Bran	Rice Bran	Corn cob	Rice Bran
10	0.42	0.48	0,42	0.38	0.32	0.35	0.64	0.28	0.36	0.29	0.49	0.76
20	0.38	0.25	0.47	0.46	0.29	0.43	0.68	0.24	0.27	0.98	0.52	0.39
30	0.32	0.92	0.49	0.58	0.87	0.57	0.74	0.29	0.47	0.45	0.42	0.64
40	0.47	0.52	0.36	0.74	0.46	0.46	0.97	0.44	1.25	0.57	0.89	0.58
50	0.34	0.32	0.35	0.86	0.25	0.35	0.43	0.32	0.35	0.83	0.78	0.98

Table 10: Effects of Metal Ions Concentration On Cellulase Activity at 520nm (OD)

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- **Glucose Curve**

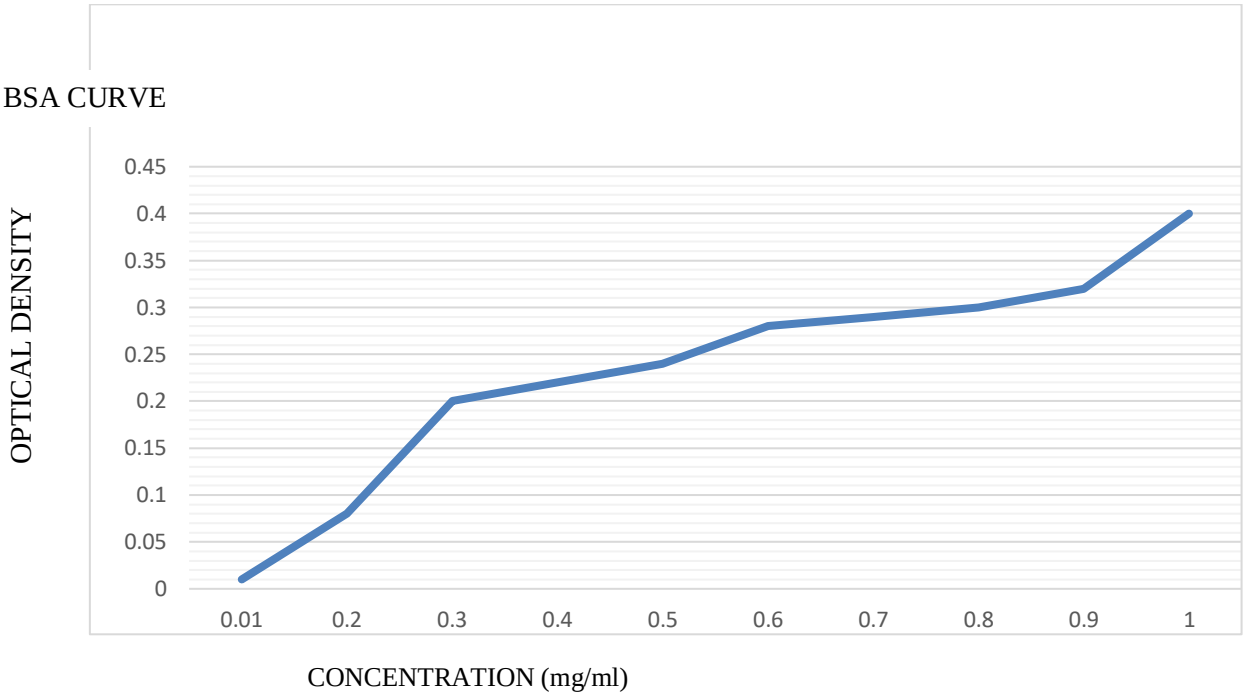

Fig. 1

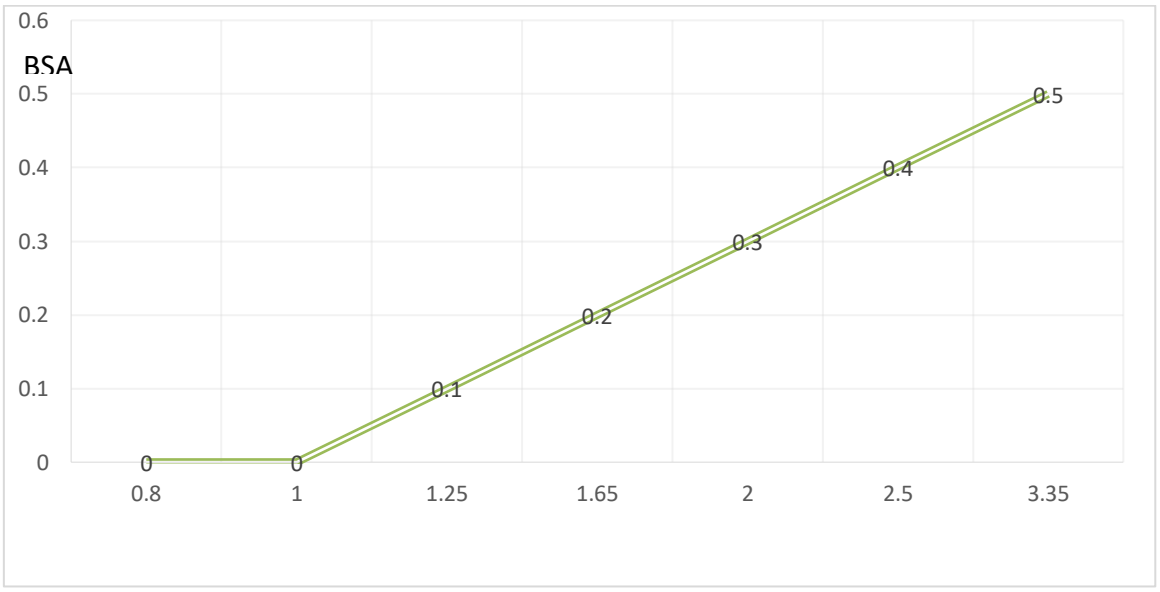

Fig. 2: CONCENTRATION OF GLUCOSE (Mg/Ml) At Various Optical Density

- **Glucose Curve:**

The enzymatic reactions often exhibit a form of kinetics known as Michaelis Menten kinetics, which is known for a hyperbolic relationship between velocity (V) and substrate concentration (S). Normal enzyme kinetic reactions are measured under steady-state conditions and such conditions usually occur in the cell. The expression is given as V=V max S/K_m+S. The curve was obtained using the line weaver –Burk plot for important parameters of cellulase enzyme such as km and Vmax. The obtained curve is gotten by imputing the values 1/S on the x-axis and 1/vmax on the y-axis. The y-intercept denotes the 1/Vmax whereas the x-intercept denotes the 1/km.The turnover number which depicts the enzyme yield is characterized by the number of substrates that are converted to products per unit time per molecule of the enzyme was obtained when the values of Vmax are divided by the amount of enzyme used. Typically, the values range from 10^2-10^3 S-1.

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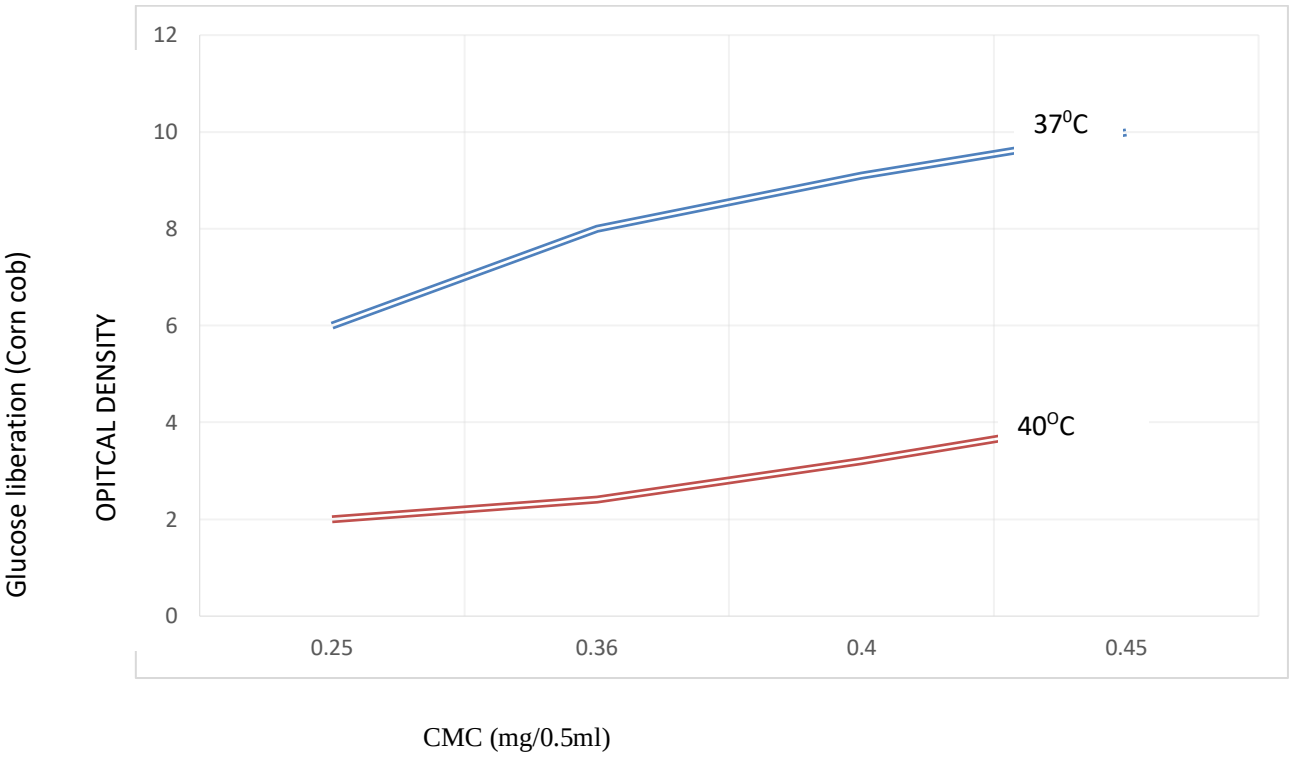

Fig. 3: Glucose liberation At Various Temperature Range (Corn cob)

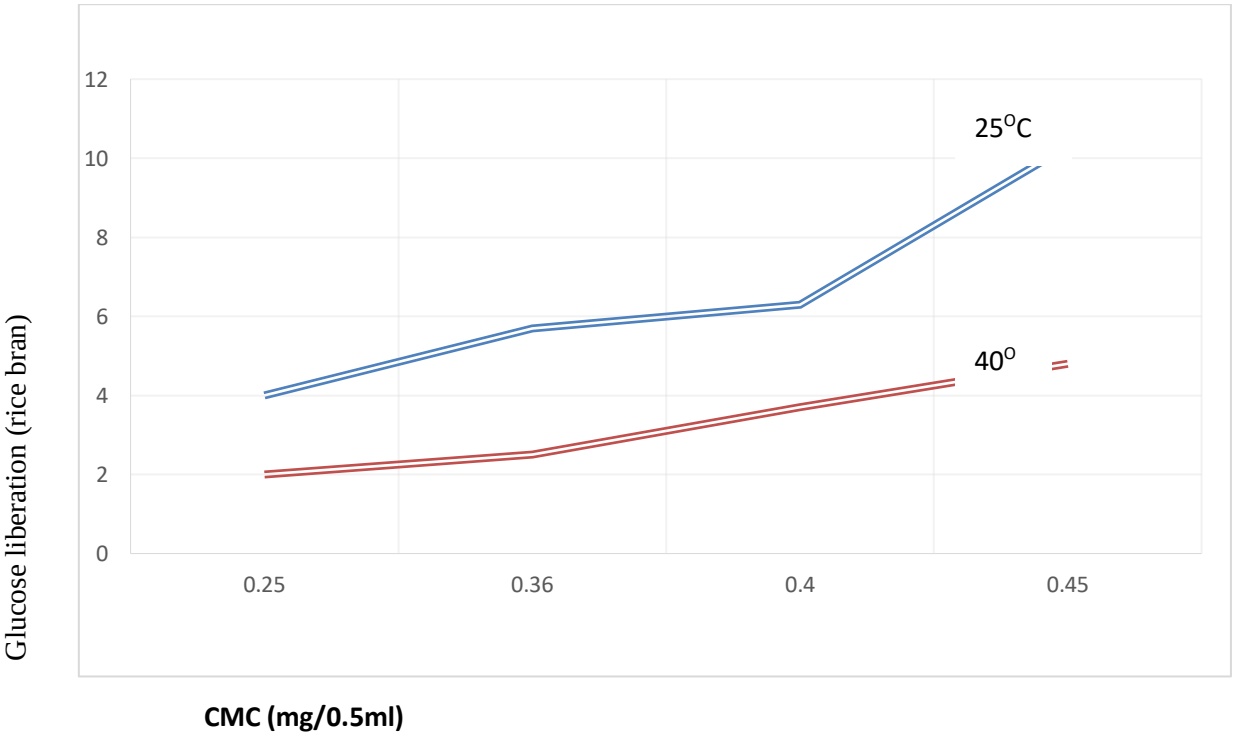

Fig. 4: Glucose liberation At Various Temperature Ranges (rice bran)

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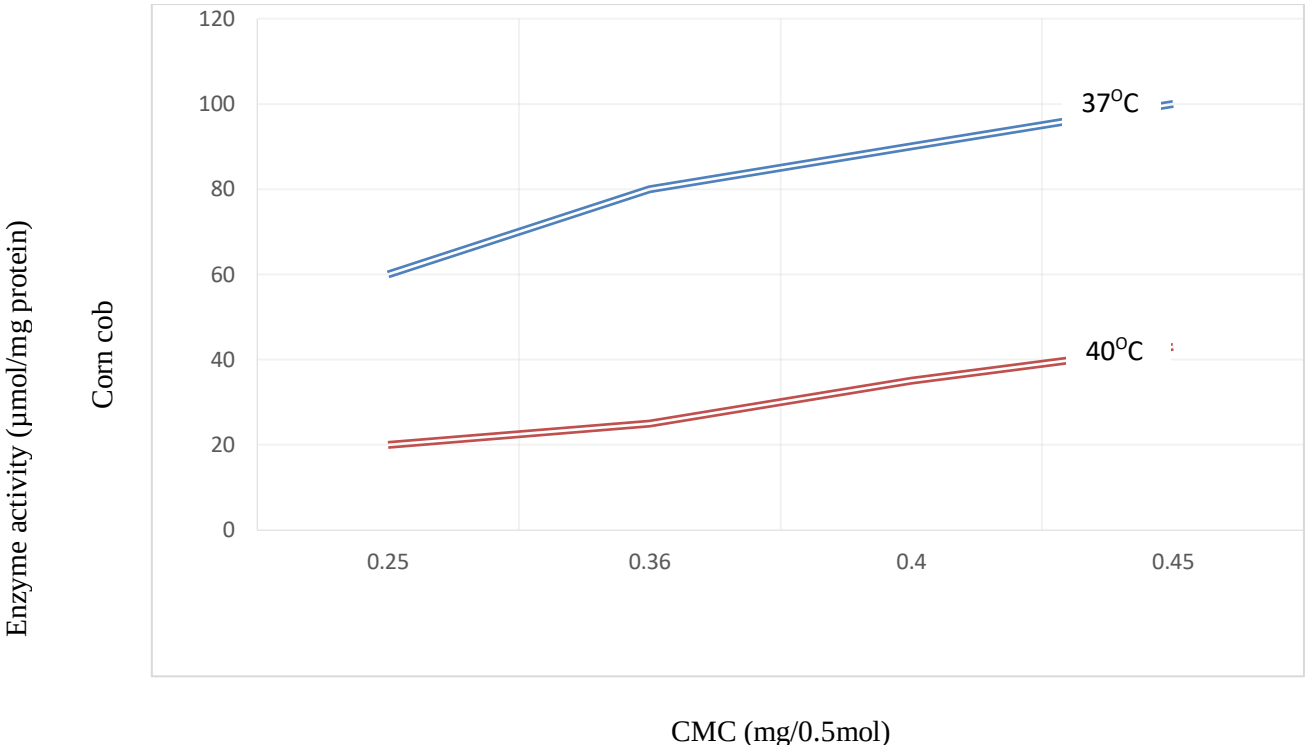

Fig. 5: Enzyme activity (µmol/mg protein) Released At Different Temperature Range Using Corn Cob

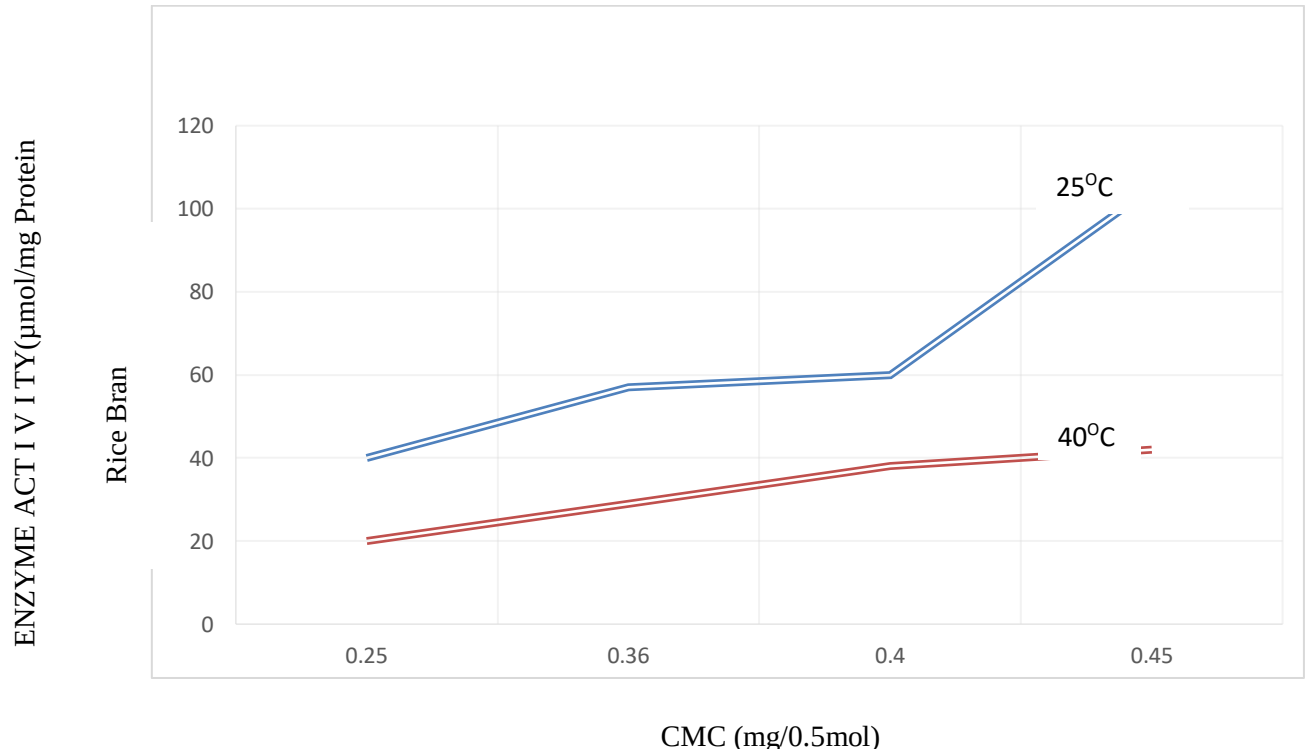

Fig. 6: Enzyme activity (µmol/mg protein) Released At Different Temperature Range Using Rice Bran

zyme/30mins

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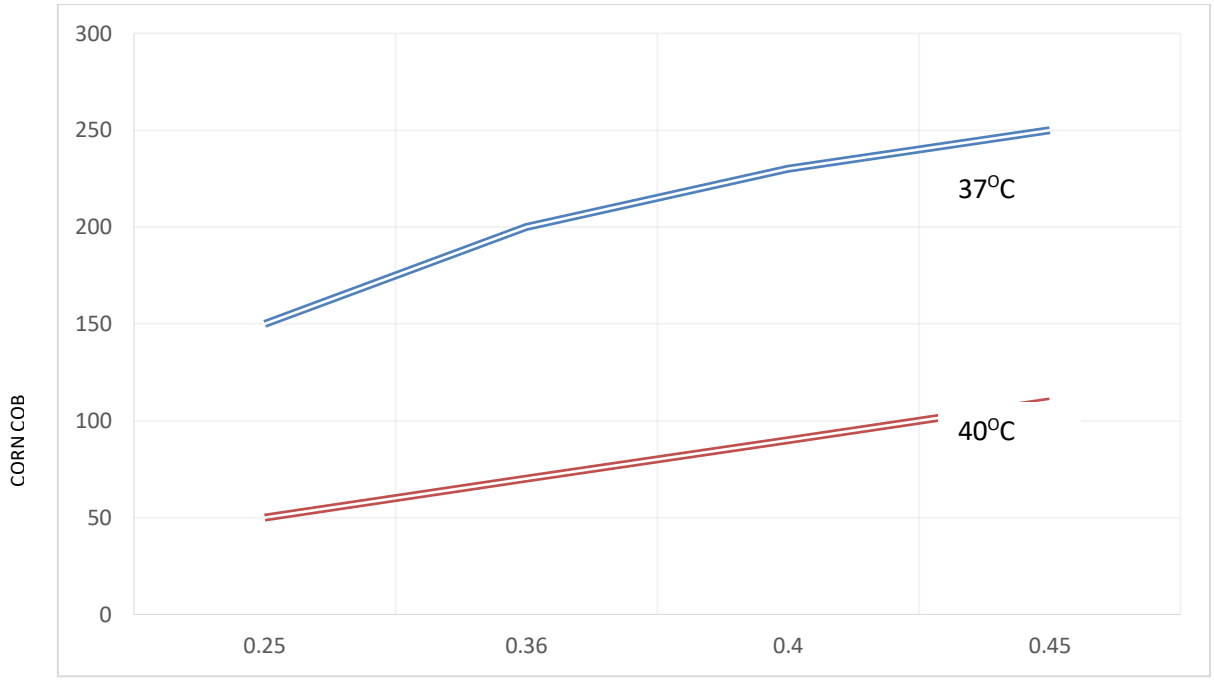

Fig. 7

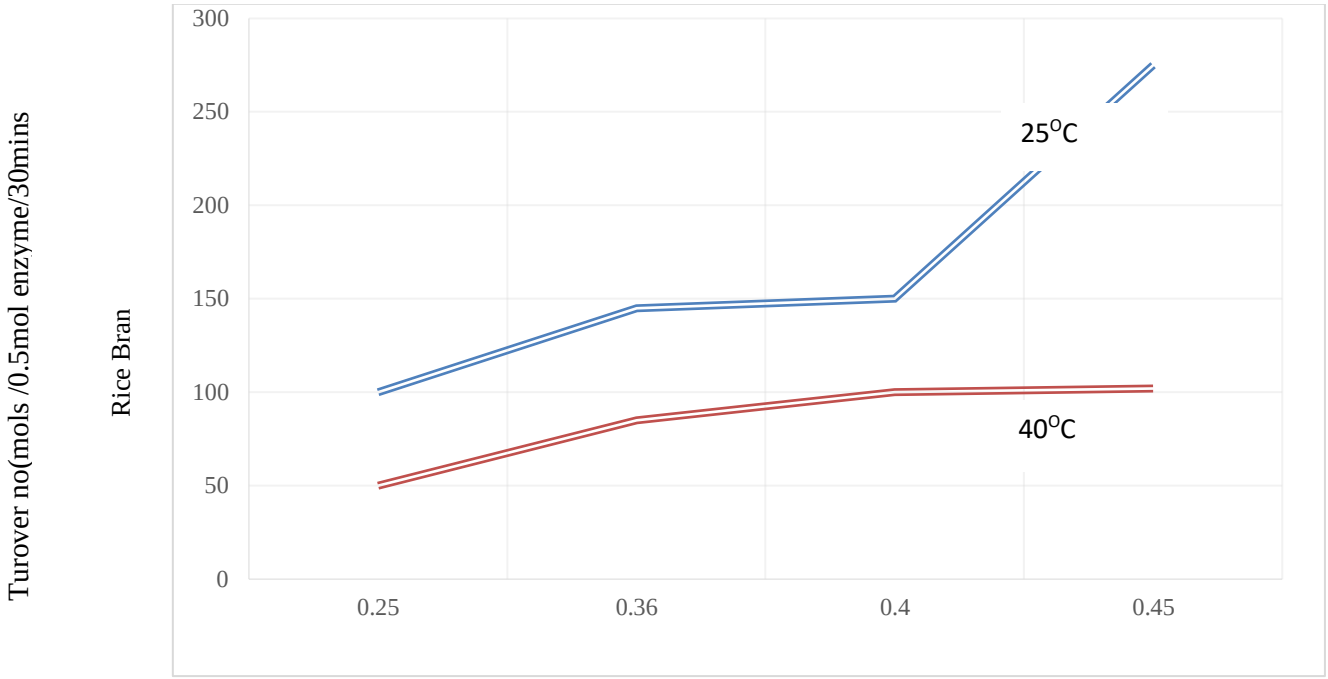

Fig. 8

Fig. 7 AND FIG 8 Turnover OF Mg /0.5mol Enzyme/ At Various Temperature Range in 30mins for Corn Cob And Rice Bran

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IV. DISCUSSION

Aspergillus niger was screened (GCA-023625455.1) for the production and activity of the carboxymethyl Cellulase enzyme. All enzymes that acted upon the cellulose substrate were presumed to move about the surface in a random walk (Ninlos et al; 2007) resulting in steps of the dimensions of one glucose per line step.The activity of the enzyme was assayed at optimum and end temperatures (25^0C and 40^0C) for Rice bran substrate and(37^0C and 40^0C)for corn cob substrates. The glucose production by the enzymeparticipation at (25^0C and 40^0C) and (20^0C and 40^0C)by DNSA method thereby generating a reddish-brown colour of amino acid compounds. The glucose liberationin enzymatic reaction withthechromogenic agents occurs chromogenically in the reaction. The absorbance was measured by the spectrophotometer method at a wavelength of 540nm.(Coleman et al,2007). Enzyme activity with 0.45mg/0.5ml CMC concentration was found to be 42µmol/mg protein and 100µmol/mg protein at(40^0C and 37^0C) for corn cob substrate and 43.5µmol/mg protein at 110µmol/mg protein at (40^0C and 25^0C) for rice bran substrate. It was observed that the enzyme remained very active at both optimized temperatures and high temperatures. The maximum turnover at 37^0C for corn cob substrate was 250mol/ 0.5ml enzyme/30mins and 252mol/0.5ml enzyme/30mins at 25^0C for corn cob and rice bran substrates respectively when acted upon by the enzyme at 0.45mg/0.5ml. It was also noted that the characteristic of the enzyme from *Aspergillus niger* was highly affected by the concentration of the substrates. Itcould have arisen, owing to the varying character or parameters or the intrinsic properties such as polymerization or the surface area of the substrates. A key finding is that enzymes maintained a high-level tolerance to varying temperatures which makes them an enzyme of choice for scientists (Fazem, 2012). It was equally noted that the K_m of the enzyme is considered to be the most criterion for evaluating the enzyme for various t**ools** or uses, L+S values indicate the efficiency of the enzyme, lesser K_m value, and higher efficiency. The K_m value of the enzyme cellulase determined at 25^0C and 40^0C for rice bran is 0.219 and O.5911 respectively and at 37^0Cand 40^0 C for corn cob, it is valued at 0.328 and 0.65 respectively. It was noted that the enzyme remained more active at 37^0 C for corn cab and 25^0 for rice bran at the CMC concentration of 0.45mg/0.5mol. Given that industrial enzymes operating on different temperatures allow industrial technologists to develop an effective approach and efficient processes in nature. The optimal pH for cellulase activity on Rice bran and corn cob substrates are 3.0 and 4.5 respectively. Kinetic parameters for CMC hydrolysis provide an interesting insight into kinetic optimization at different temperatures (Tong el; 1980). Because of the extreme molecular stability of cellulases at a wide array of temperatures, *Aspergillus niger* forms an interesting source of enzyme for industrial applications which can be greatly commercialized. The enzymatic activity of cellulase at low and high temperatures provides an in-depth understanding of its physiological adoption to cold and high temperatures at the enzyme level, as it compensates for the reduction of chemical reactions induced by low and high temperatures(Feller and Gerday; 2003). Also, it was observed that the increase in the concentration of Nitrogen sources greatly affects the enzyme activity as it produces more yield. The metal ions acted as a co-factor during enzymatic hydrolytic reactions. It was also observed that iron (Fe) ion was the best cofactor in rice bran substrate while magnesium favoured corn cob substrate. For purpose of comparisonof yield between the two substrates, it was observed that enzymatic activity on rice bran was greater than on corn cob given their respective values of k_m (0.219mM and 0.328.mM). For comparison of yield between the two substrates, it was observed that enzymatic activity on Rice bran was greater than on corn cob given their respective values of km (0.219mM and 0.328mM). In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has spurred great interest in the exploration of extracellular enzymatic activity in several microorganisms while using Agro-industrial wastes as a source of the substrate (Abu et al; 2005).

V. CONCLUSION

This study confirmed that the enzyme cellulase from *Aspergillus niger* can tolerate verging degrees of temperatures and other physical parameters under a controlled environment and still survive and remain active. It also goes to prove that agro-industrial waste could provide a sustainable source for exploring various biotechnological assays of industrial enzymes and in turn spur an economic upturn in the country.

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