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Production and Partial Characterization of Cellulases from *Apergillus fumigatus* Using Two Distinct Parts of Corn Cob as Carbon Sources

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Abstract

Corn cobs were sun-dried for three days and separated into the hard outer part (Corn Cob Outer, CCO) and the soft inner part (the pulp) (Corn Cob Inner, CCI). Each part was milled separately. *Aspergillus fumigatus* isolated from sewage water was grown on and adapted to each part of corn cob. Using CCI and CCO, as the sole carbon sources for submerged fermentation, the organism produced cellulase. Activity was highest on the 3rd and 4th days using CCI and CCO, respectively. The crude cellulases were partially purified by 50% ammonium sulphate precipitation followed by dialysis. The partially purified cellulases were then characterized with respect to pH, Temperature and Themostability. While the optimum pH of the CCI cellulose was 6.0, that of CCO cellulase was pH 7.0. The optimum temperature of CCI was 55°C whereas that of CCO was 50°C. The stability of enzymes from 35°C to 70°C was studied. At 70°C, CCO cellulase has lost 45.88% of its original activity while CCI cellulase lost 58.14%. The results show that corn cob could serve as a cheap carbon source for the production of fungi cellulase. The study indicates that waste could be converted to wealth.

Key words: Corn cob, cellulose, *Aspergillus fumigates*, submerged fermentation, partial purification

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Introduction

Corn cob is a waste material obtained after removing the seeds from corn. Corn cob as a lignocellulosic biomass has been estimated to contain 45% of cellulose and 15% lignin (Betts *et al.* 1991; Sun and Cheng 2002). Lignocellulose is one of the earth's most abundant, renewable resources and its degradation and utilization by microorganisms is considered very important (Kato *et al*., 2006). It has become of considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Balasaravanan *et al.,* 2013). Cellulase is an enzyme system that acts in synergy to cleave β -1, 4-glycosidic bonds in cellulose. In general, bacterial cellulases are constitutively produced, whereas fungal cellulases are produced only in the presence of cellulose (Suto and Tomita, 2001). Filamentous fungi, particularly *Aspergillus and Trichoderma spp.* are well known efficient producers of cellulase (Peig *et al*., 1998). These enzymes have wide range of applications in industries such as textile, laundry, pulp and paper and fruit juice extraction. Cellulase also has great potential in animal feed and saccharification of lignocellulosic biomass to fermentable sugars useable in the production of bioethanol. The aim of this study was to produce and characterize cellulases from fungi using the hard outer part of corn cob as well as the pulp as distinct carbon sources.

Materials and Methods

Plant Material: Dry corn cobs (mixed varieties) were collected from the National Cereals Research Institute, Yandev, Benue State. These were supplemented with local varieties obtained from the University of Nigeria Agric. Farms and Ogige market, all in Nsukka, Enugu State.

Processing of Corn Cob

The corn cobs were sun-dried for three days to remove the remaining moisture. The hard outer part was then carefully separated from the soft inner part (pulp). Each was milled to powder and the outer part was labeled CC-Outer while the pulp was labeled CC-Inner.

Isolation of Cellulolytic Microorganisms: A total of sixteen microoganisms were isolated and screened for cellulolysis and cellulase production. Five samples were collected of which three were soil samples collected from different sites: soil from saw dust dump-site, decomposing litter and garden soil. The last two were sewage sediment and sewage water from a Sewage Treatment Plant. All the five samples were used in the microbial isolation according to conventional soil microbiology methods (Alef and Nannipieri, 1995). Mineral broth (400ml) containing 0.1% NH_4Cl, K_2HPO_4. $3H_2O$ and $MgSO_4$. $7H_2O$ was prepared and 90ml was dispensed into each of the four clean dry Erlynmeyer flasks. 20g garden soil was mixed with 20ml of water and stirred and filtered through Whatman No. 1 filter paper. Then 5ml of the filtrate was used to enrich the mineral broth in the four flasks.

Three filter paper strips (1x3cm) were shredded and added to each flask to serve as carbon source giving a nutrient broth. Each soil sample (20g) was mixed with 20ml of water and 5ml of the extract used to inoculate sterile nutrient broth. The sewage samples were used to inoculate the remaining two flasks. All four flasks were wrapped in black sheet and placed in dark cupboard to prevent algal growth. Sabouraud Dextrose Agar (SDA) was aseptically inoculated by streaking with sterile wire loop dipped in the fourteen-day old culture. Sixteen organisms were isolated after repeated sub culturing on SDA media.

Screening for Cellulolysis: The isolates were subcultured on a media containing 1% pure crystalline cellulose and the organism that thrived on the media was selected and used for cellulase production. This organism identified to be *Aspergillus fumigatus* based on morphological characteristics was adapted on media in which corn cob (CC-Outer and CC-Inner) were the carbon source.

Cellulase Production and partial purification: Corn cob broths each containing mineral broth of 0.1% NH_4Cl, K_2HPO_4. $3H_2O$ and $MgSO_4$. $7H_2O$ and then 1%(w/v) of either CC-Outer or CC-Inner as sole carbon source, were prepared an used for cellulase production. A sterile 6.0mm^2 cork borer was used to inoculate 50ml sterile corn cob broth. Replicate cultures were left to stand at room temperature for several days. Cell free supernatants were harvested each day by filtration and subjected to cellulase assay to determine the day of peak cellulase secretion for each of the two substrates (CC-Outer and CC-Inner). For large enzyme production, the whole process was repeated but fermentation was ended on the day of highest activity. The harvested cell free supernatant was used as crude enzyme. The crude enzyme was subjected to 50% ammonium sulfate precipitation and dialyzed for 12hr in 0.2M acetate buffer pH 5.5. The dialyzed enzyme was used for further studies and subsequently now referred to as the enzyme.

Cellulase Assay: 0.1ml each of the enzyme was incubated with a strip of filter paper (1x3cm) for 6h at 50°C in 2.4ml of 0.2M sodium acetate buffer pH 5.5. The activity of the enzyme was determined from the amount of glucose released using DNS (Miller, 1959). One unit of enzyme activity, U, is defined as the amount of enzyme that releases one µmole of glucose per minute. Specific activity is given as U/mg protein. The protein content was determined by the method of Lowry *et al*. (1951).

Effect of pH on Cellulase Activity: Activity of the enzyme was determined in buffers of pH ranging from 0.2M sodium acetate buffer (pH 3.5 – 5.5), 0.1M sodium phosphate buffer (pH 6 – 7.5) and 0.1M Tris – HCl buffer (pH 8.0 – 10.0) at 0.5 intervals. The pH optima was evaluated from a plot of activity against pH.

Effect of Temperature on Cellulase Activity: Activity of the enzyme was determined by incubating the enzyme with pectin solution at 25-70°C interval of 5°C for 1hour and at pH 5.5 (0.2M sodium acetate buffer). The optimum temperature was evaluated from a plot of activity against temperature.

Heat- stability of Cellulase: The enzyme incubated for 1h at each of the temperatures ranging from 35-70°C. After cooling for 30 min activity of the enzyme was determined in 0.2M sodium acetate buffer of pH 5.5 at 50°C.

Results and Discussion

Peak cellulase activity was recorded on the 4th day (3.82U) using CC-Outer as the sole carbon source while for CC-Inner the peak activity was recorded on the 3rd day (4.083U). The protein assay conducted on the crude culture filtrate after mass production showed that *Aspergillus fumigatus* secreted higher amount of extracellular protein when grown on CC-Inner (0.164 mg/ml) than when grown on CC-Outer (0.112 mg/ml), shown in Fig.1. This shows that the substrate, CC-Inner supported higher protein secretion than did CC-Outer The specific activity for dialysed and undialyzed cellulase (CC-INNER) were 1.20U/mg and 6.24U/mg, respectively as shown in Table 2. While the specific activity for dialysed and undialyzed cellulase (CC-outer) were 3.154U/mg and 7.685U/mg, respectively as shown in Table 1. The fact that the organism secreted higher amount of cellulase when grown on CC-Inner than when grown on CC-Outer suggests that the corn cob pulp is a better substrate for cellulase production than the outer part.

For dialysed CC-INNER, approximately two -fold purification was achieved while 29-fold purification was achieved in dialysed CC-OUTER (Tables 1 and 2). When *A. fumigatus* was grown on different carbon sources including alfa alfa, corn cob, saw dust, wheat straw, rice straw and wheat bran, it was shown that corn cob and sawdust proved to be the poorest substrates in supporting cellulase secretion (Sherief *et al.,* 2010). It is possible that the poor cellulase secretion could be because the corn cob was used whole. Most of the previous studies on cellulase production from *Aspergillus fumigatus* using corn cob have been on corn cob as a whole, and not on the distinct parts of the cob. Several factors could affect the extracellular secretion of cellulase during fermentation with either increase or decrease in extracellular enzyme activity. The depletion of micro- and macro-nutrients in the medium as fermentation progresses could limit the potentiality of the organism. Some toxic metabolites secreted into the medium by the same organism could also result in a change in the pH of the medium. This in turn could have a critical effect on cellulase production. Glucose, the end product of cellulase action, is a potent catabolite repressor of cellulase biosynthesis (Ilmen *et al.,* 1997; Zhang and Lynd, 2005). Cellobiose inhibits both endoglucanase and β-glucosidase (Ojumu *et al.,* 2003).

The cellulase activity and total protein of CC-OUTER were always lower than those of CC-INNER. This would suggest that CC-Outer did not support cellulase production as much as did CC-Inner. The outer part of the corn cob is very hard compared to the inner part (the pulp) which is very soft and light. CC-Outer is probably rich in lignin since hardness of plant materials is associated with lignifications associated with age of the plant material. Lignin shields the cellulose and makes it inaccessible to hydrolytic action of enzymes.

The pH profile of the CCI enzyme showed a sharp rise in cellulase activity between the pH 4.0 (17.778 Units) and pH 5.0 (79.583 Units). The optimum activity of the enzyme was pH 6.0 (88.819 Units) (Fig. 3). The pH profile of the CCO enzyme also showed a sudden rise in cellulase activity between pH 5.0 (9.306 Units) and pH 6.0 (46.181 Units). The enzyme from CC-OUTER proved to be a neutral cellulase with optimum activity (57.153 Units) at pH 7.0 (Fig. 4). This finding agrees with that of Immanuel *et al* (2006). Ray *et al* (2007) also reported neutral cellulase. More so, Abdelnasser and El-diwany (2007) were able to isolate from a thermophilic bacterium, cellulases with peak activity at pH 7.0. These reports, however, disagree with that of Abo-State *et al* (2010) on cellulase production using *Aspergillus terreus* Mam-F23 and *Aspergillus flavus* Mam-F35 and reported optimum pH of 4.5 and 4.0 for CMCase and Fpase activities, respectively. Lowe *et al.* (1987) reported optimal pH of 6.0 for both CMCase and FPase. Ponpium *et al.* (2000) also reported a cellulolytic multi-enzyme complex with pH optima of 6.0. However, Gilna and Khaleel (2011) reported pH optima of 6.5 for a strain of *A. fumigatus*. This disagrees with the current findings and those of Ahmed *et al.* (2009) who produced cellulases from the fungus *Trichoderma harzianum* and reported optimum pH of 5.5 for exoglucanase, endoglucanase and β-glucosidase. Coral *et al*. (2001) reported cellulase from *Aspergillus niger*, with CMCase activity over a broad range of pH but having peak activities at pH 4.5 and 7.5. These multiple peaks and hence variation from our finding could

be as a result of isoforms of the same enzyme being present but acting maximally at different pH. It may also be traced to differences in the species of organisms used. The fairly low activity observed in CC-OUTER over the pH range (3.5 - 5.0), and in CC-INNER over the pH range (3.5-4.0) could probably be due to deprotonation of the catalytic amino acid residues in the active and binding sites of the enzyme protein leading to marked reduction in activity. This might explain why there was a sudden rise in activities of the enzymes as pH increased (Fig. 4).

The temperature profile of the CCI enzyme showed an increase in cellulase activity as temperature increased from 25 to 50°C Optimum activity was obtained at 55°C (Fig. 5) The temperature profile of the CCO enzyme also showed an increase in activity with increasing temperature with an optimum temperature of 50°C (Fig. 6). Lowe *et al* (1987) reported temperature optimum of 50°C for CMCase and β-glucosidase activities but 45°C for FPase activity. Several reports have equally claimed different temperature optima. Immanuel *et al* (2007) reported an optimum of 40°C for cellulase from a strain of *Aspergillus fumigatus* whereas Gilna and Khaleel (2011) reported 32°C as temperature optimum for cellulase produced by another strain of *A. fumigatus*. These differences could be attributed to differences in the strain and species of organisms used in the enzyme production.

Temperature differences not only affect enzyme activity but also the stability of the enzyme. High temperatures disrupt or unfold protein structures leading to denaturation. Extremes of temperature apart from causing a loss of activity of water-soluble proteins might also be responsible for precipitating them out of solution. Therefore, the thermal stability of an enzyme refers to the degree to which the secondary, tertiary and quaternary structures are affected by temperature changes. This reflects in an enzyme's activity after its exposure to heat especially in absence of its substrtate. The heat stability study on the CCI enzyme (Fig. 7), revealed a triphasic pattern. In the first phase (35-50°C), it was relatively stable whereas the second phase (50-60°C) showed a pronounced decrease in stability. The last phase (60-70°C) also showed a decrease in stability of the enzyme to heat but the decrease was not as sharp as in the second phase. However, the CCO enzyme was rather stable when stored between the 35 and 45°C for one hour. Its stability however, dropped between 45 and 50°C but was not lowered between 50 and 55°C. Beyond 55°C, the stability of the enzyme continued to decline (Fig. 8). The percentage loss in the activities of the enzymes between 35-70 °C for 1 hr (Figs. 9 and 10) showed that the CCI enzyme lost 9.975% to 26.378% activity between 50 and 55°C, while at 70 °C, as much as 58.137% loss in activity was observed. The loss in activity of CCO enzyme was not as much as in that of CCI enzyme. However, loss in its activity became rapid from 60°C, and at 70°C as much as 45.854% loss in activity was observed. Though the two enzymes were observed to be stable between 35°C and 40°C but as the temperature is increased to 60°C, a steady decline in activity became obvious. These losses in activities of the two enzymes substantiate the fact that storage temperatures affect the shelf life and hence, the activities of enzymes. Other thermostability studies have shown that enzyme preparations are best stored at very low temperatures for them to still retain their activities. Even most cell-free soluble enzyme preparations stored at temperatures as low as 4°C lose some of their activities on long storage since they are no longer in their native environment. This leaves enzyme immobilisation and lyophilisation as better choices for costly enzymes that lose appreciable part of their activity even when stored at low temperatures. However, the temperature of an organism's natural habitat, to a great extent, determines the stability of the enzymes they produce. A thermophile in hot spring for instance, will usually secrete a thermostable enzyme. The temperature of the natural habitat of the organism used in cellulase production affects the thermal stability of the cellulase produced.

Conclusion

This study has shown that the fungus (*Aspergillus fumigatus)*, isolated from the native environment has potential of being a source of cellulases using native carbon sources. Two distinct parts of corn cob (CC-Outer and CC-Inner) were successfully used in the production of the enzyme. Among the two distinct parts of corn cob used, the pulp (CC-Inner), though very light, small in quantity and difficult to separate from the other part, has proven to be a better substrate for fungal cellulase production. Corn

cob, being an agricultural waste littered in the environment can be utilized as a cheap source for the production of cellulases.

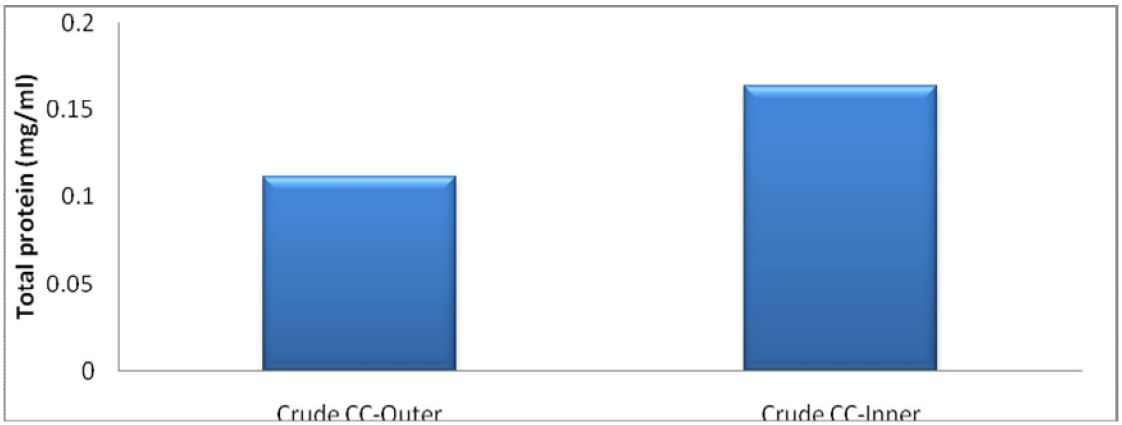

Fig. 1: Comparison of extracellular protein in crude CC-OUTER and CC-INNER. The protein assay was
done in duplicates and total protein given in mg/ml

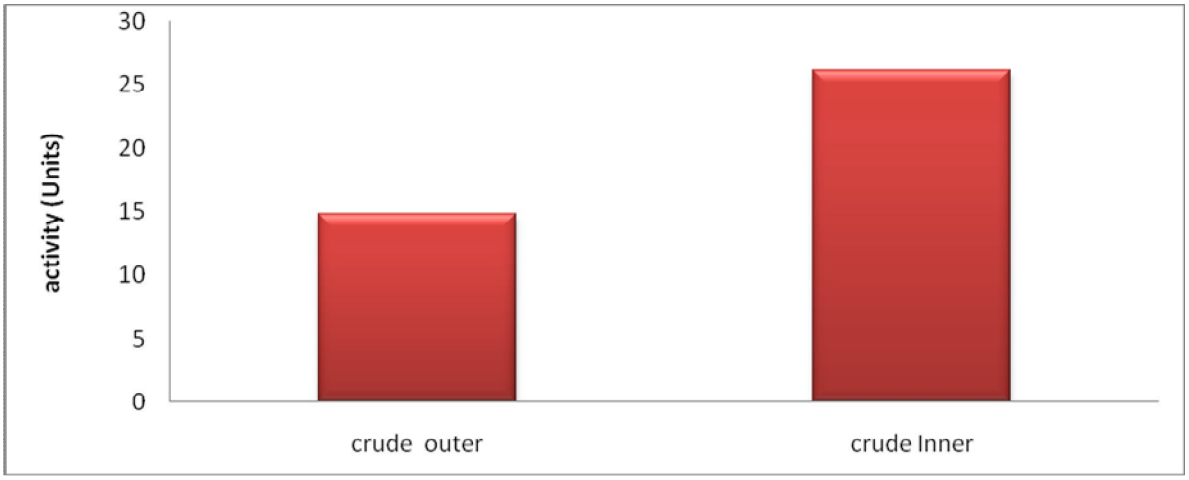

Fig. 2: Comparison of total cellulase activities of crude CC-OUTER and CC-INNER. Total cellulase activity was given in Units. (Units = micromoles/min).

Table 1: Purification for CC-OUTER

Procedure	Total protein (mg)	Total activity (units)	Specific Activity (units/mg)	Purification factor
Crude cellulase	56	14.861	0.265	1
Undialysed ppt at 50% salt	4.14	13.056	3.154	11.190
Dialysed cellulase	5.964	45.833	7.685	29

Ppt: precipitated

Table 2: Purification for CC-INNER

Procedure	Total protein (mg)	Total activity (units)	Specific Activity (units/mg)	Purification factor
Crude cellulase	82	26.111	3.18	1
Undialysed ppt at 50% salt	11.24	13.472	1.20	0.377
Dialysed cellulase	14.882	92.917	6.24	1.960

Ppt. : precipitated

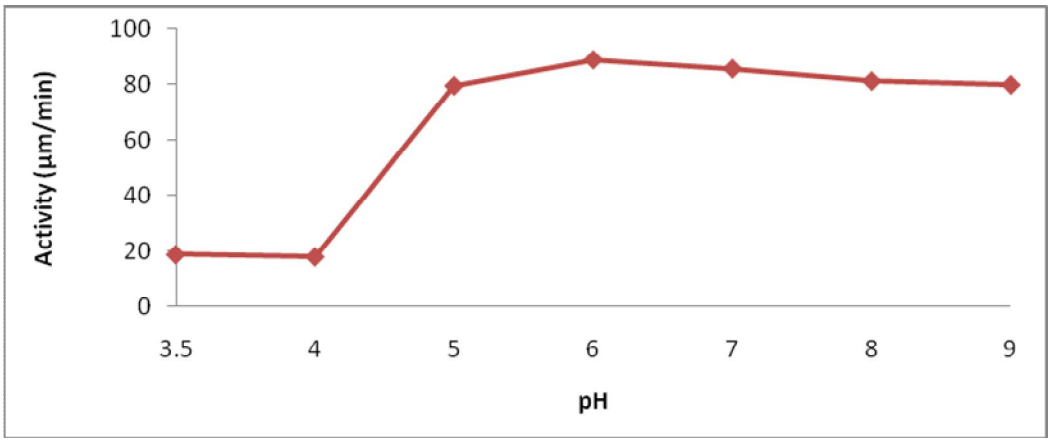

Fig. 3: pH profile of dialysed CC-INNER. The assays were carried out in duplicates. (Units = micromoles/min).

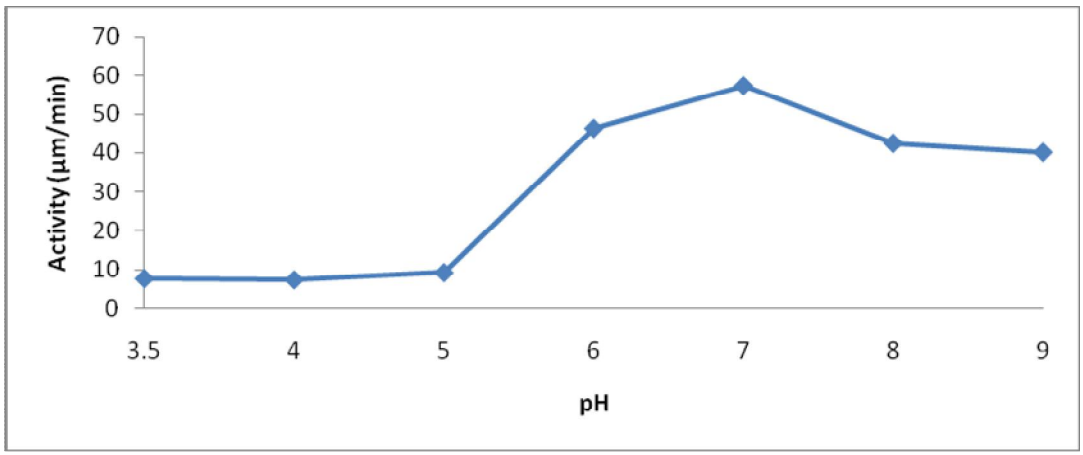

Fig. 4: pH profile of dialysed CC-OUTER. The assays were carried out in duplicates.

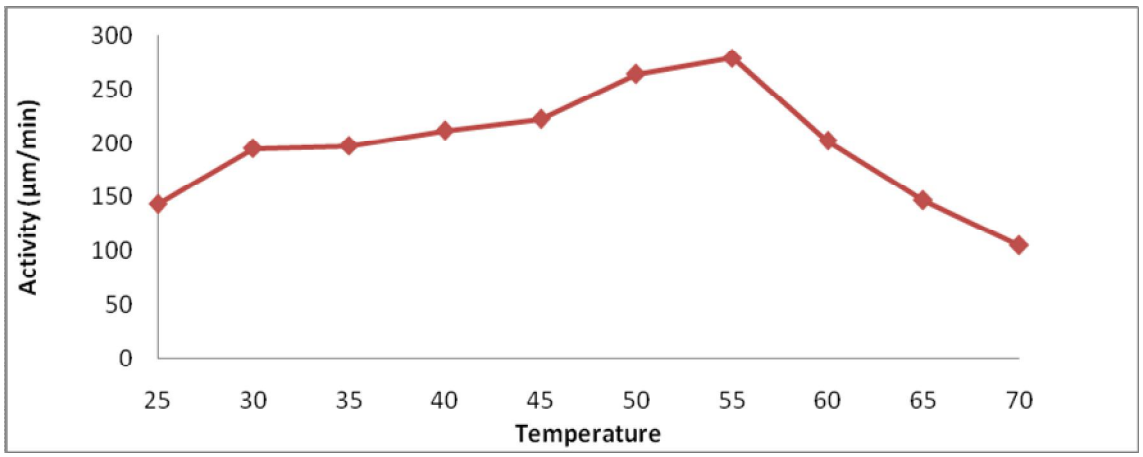

Fig. 5: Temperature profile of dialysed CC-INNER.

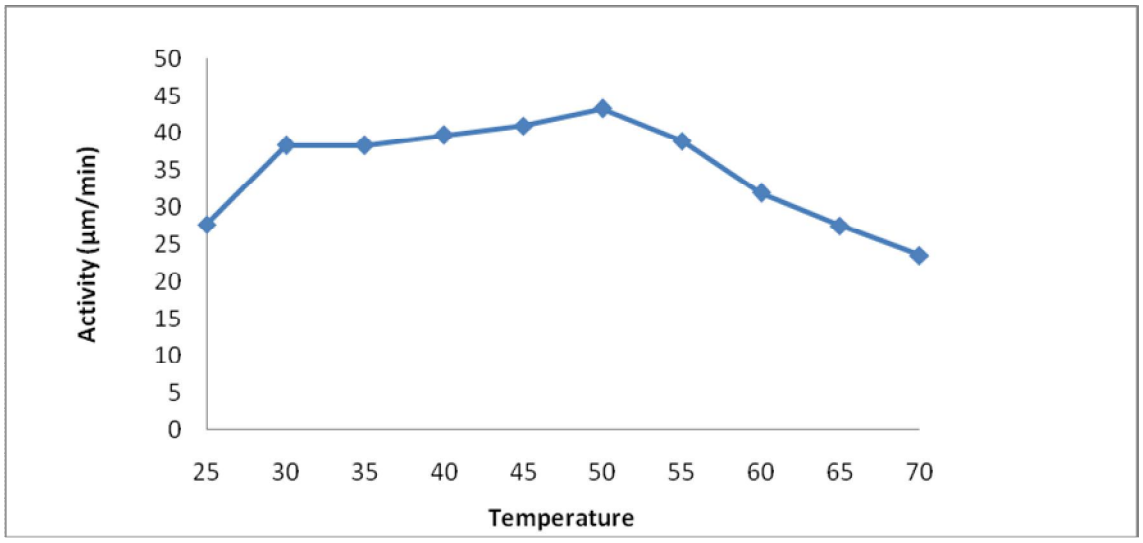

Fig. 6: Temperature profile of dialysed CC-OUTER.

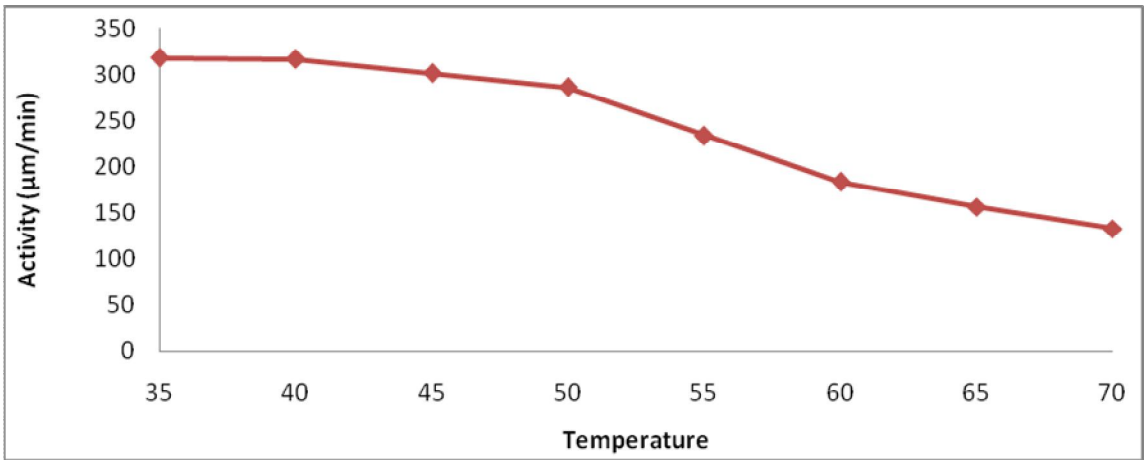

Fig. 7: Heat stability of dialysed CC-INNER

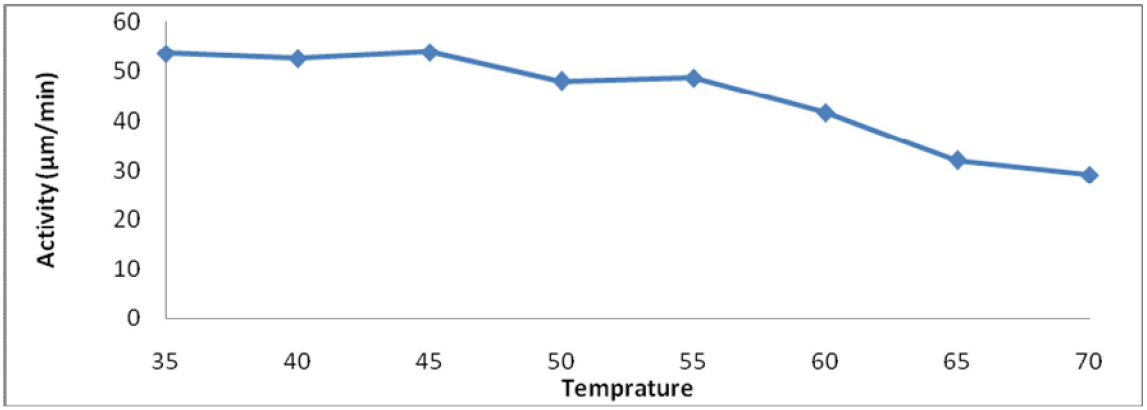

Fig. 8: Heat stability of the partially purified CC-OUTER. The assay was done in duplicates.

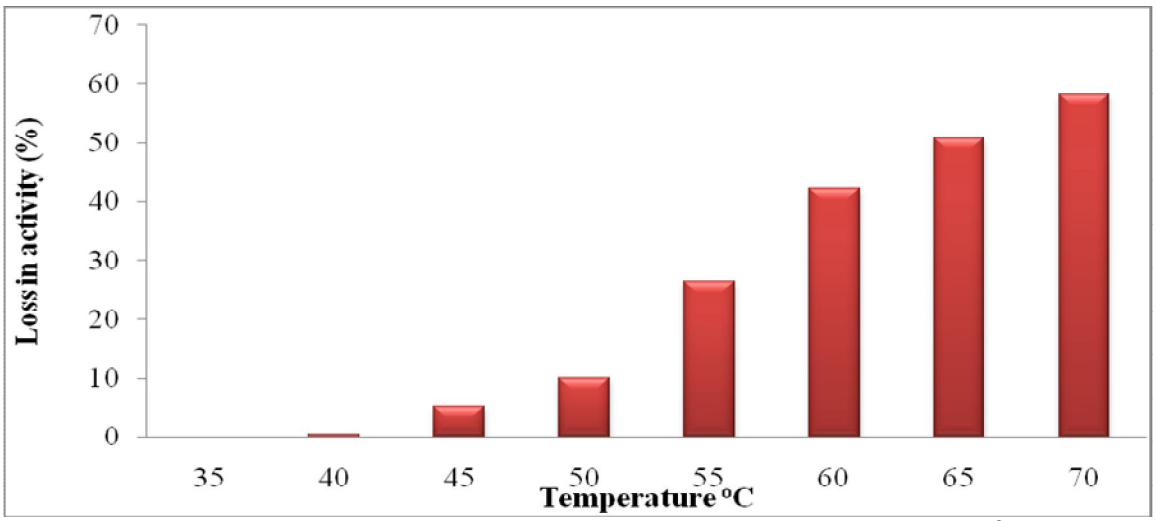

Fig. 9: Percentage loss in CC-INNER activity under varying temperatures (35 - 70°C). The assays were carried out in duplicates and mean values used.

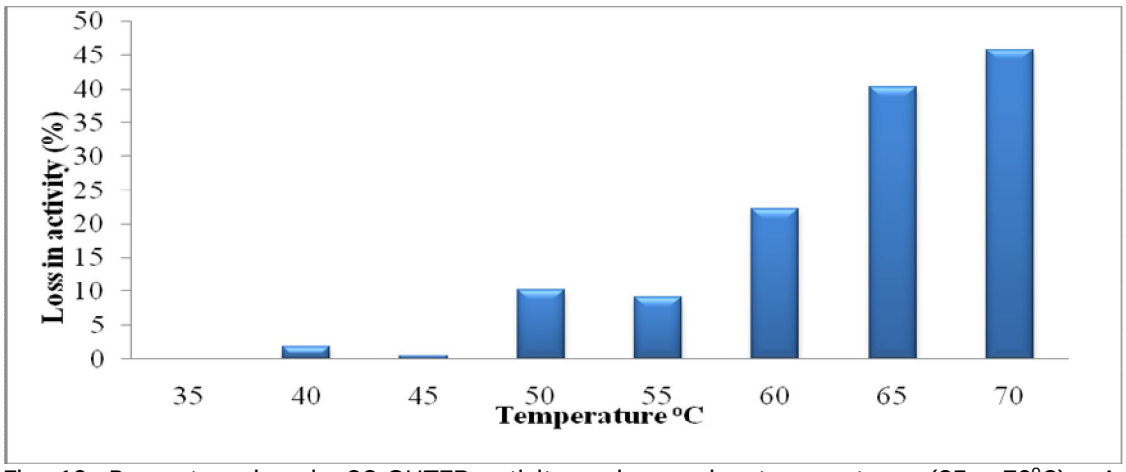

Fig. 10: Percentage loss in CC-OUTER activity under varying temperatures (35 - 70°C). Assays were carried out in duplicates and mean values used.

REFERENCES

Abdelnasser, S.H.I. and El – diwany, A.I. (2007). Isolation and identification of new cellulases producing thermophilic bacteria from an Egyptian hot spring and some properties of the crude enzyme. *Australian Journal of Basic and Applied Sciences.* 1 (4): 473 – 478.

Abo–State, M.A.M., Swelim, M., Hammad, A.I. and Gannam, R.B. (2010). Some critical factors affecting Cellulase(s) production by *Aspergillus tereus* Mam – F23 and *Aspergillus flavus* Mam – F35 under solid – state fermentation of wheat straw. *World Applied Sciences Journal.* 9 (10): 1171 – 1179.

Ahmed, S., Bashir, A., Saleem, H., Saadia, M. and Jamil, A. (2009). Production and purification of cellulose–degrading enzymes from a filamentous fungus *Trichoderma harzianum. Pakistan Journal of Botany*. 41 (3): 1411 – 1419.

Alef, K. and Nannipieri, P (eds)(1995). *Methods in Applied Soil Microbiology and Biochemistry.* Academic Press, London.

Balasaravanan, T., Rathnan, R. K. and John, D. (2013). Isolation, screening, identification and optimised production of extracellular cellulase from *Bacillus subtilis* using cellulosic waste as carbon source. *Journal of Microbiology, Biotechnology and Food Sciences*. 2(6) 2383-2386.

Betts, W.B., Dart, R.K., Ball, A.S. and Pedlar, S.L. (1991). *Biosynthesis and Structure of Lignocellulose*. In Betts (eds) Biodegradation: Natural and Synthetic Materials, Springer – Verlag, Berlin, Germany. P. 139.

Coral, G., Arikan, B., Naldi, M.N. and Venmez, H.G. (2001). Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 Wild-Type strain. *Turkish Journal of Biology.* 26: 209-213.

Gilna, V.V. and Khaleel, K.M. (2011). Cellulase Enzyme activity of *Aspergillus fumigatus. Recent Research in Science and Technology.* 3(1): 132-134.

IImen, M., Saloheimo, A., Onnela, M. and Penttila, M.E. (1997). Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei. Applied and Environmental Microbiology.* 63: 1298 – 1306.

Immanuel, G., Bhagavath, C.M.A., Raj, P.I., Esakkiraj, P. and Palavesam, A. (2007). Production and partial purification of cellulase by *Aspergillus niger* and *Aspergillus fumigatus* fermented in coir waste and saw dust. *The Internet Journal of Microbiology*. 3 (1).

Immanuel, G., Dhansha, P., Prema, P. and Palavesam, A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *International Journal of Environmental Science and Technology*. 3: 25-34.

Kato, S., Haruta, S. and Cui, Z. J. (2006). Effective cellular degradation by a mixed culture system composed of cellulolytic Clostridium and aerobic cellulolytic Bacillus. *Journal of Applied Environmental Microbiology and Technology.* 15(8): 388-398.

Lowe, S.E., Theodorou, M.K. and Trinci, A.P.J. (1987). Cellulases and xylanases of an aerobic rumen fungus grown on wheat straw, wheat holocellulose, cellulose, and xylan. *Applied and Environmental Microbiology.* 53: 1216-1233.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with Folin Phenol Reagent. *Journal of Biological Chemistry*. 193:265-275

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry.* 31: 426 – 428.

Ojumu, T.V., Solomon, B.O., Betiku, E., Layokun, S.K. and Amigun, B. (2003). Cellulase production by *Aspergillus flavus* Linn Isolate NSPR 101 fermented in saw dust, bagasse, and corncob. *African Journal of Biotechnology.* 2: 150-152.

Peij, N., Gielkens, M.M.C., Verles, R.P., Visser, K. and Graff, L.H. (1998). The transcription activator XinR regulates both xylanolytic endoglucanase gene expression in *Aspergillus niger. Journal of Applied Environmental Microbiology*. 64 (10): 3615-3617.

Ponpium, P., Ratanakhanokchai, K. and Kyu, K.L. (2000). Isolation and properties of a cellulosome – type multienzyme complex of the thermophilic *Bacteroides Sp* strain P – 1. *Enzyme and Microbial Technology.* 26: 459 – 465.

Sherief, A.A., El-Tanash, A.B. and Atia, N. (2010). Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran. *Research Journal of Microbiology*. 5 (3): 199 – 211.

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic material for ethanol production: *Annual Review of Bioresources Technology.*

Suto, M. and Tomita, F. (2001). Induction and catabolite repression mechanisms of cellulase in fungi. *Journal of Bioengineering.* 92(4):305-311.

Zhang, Y.P. and Lynd, L.R. (2005). Regulation of cellulase synthesis in batch and continuous cultures of *Clostridium thermocellum. Journal of Bacteriology.* 187: 99-106.