Original Research Article

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF CELLULASE FROM A BACTERIUM OBTAINED AT A SAW-MILL SITE IN ILE-IFE, NIGERIA

ABSTRACT

This research characterized cellulase with enviable physicochemical parameters from a bacterium isolated from decaying sawdust heap. Isolated bacteria were screened for cellulolysis using the Congo red plate method. The bacterium with the largest halozone was identified by its 16S rRNA sequence. Optimum growth and cellulase production condition was determined by varying incubation time, pH, temperature, different carbon and nitrogen sources, % substrate concentration and inoculum size. Cellulase was extracted, assayed and partially purified. The kinetic parameters were determined as well as the effect of selected conditions on the activity of the enzyme. Seven isolates showed cellulolytic capabilities. Isolate A8 with 58 mm halozone had 96% sequence identity with *Bacillus subtilis* FJ532063. Optimum activity of 46.18 U/ml at 28 hours was recorded at pH 7, 35 ± 2^{o}C. Yields of 18.5 and 13.5% resulted from ion exchange and gel filtration chromatography respectively. K_m was found to be 0.0108 ± 0.0032 mg/ml with a V_{max} of 119.3 ± 7.4µmol/min. Maximum activity for partially purified cellulase was recorded at pH 9.5 and 55 ^{o}C with stability at 50 ^{o}C; and pH 9, 35 ^{o}C with stability at 45 ^{o}C for crude cellulase. The study revealed that nature is full of cellulolytic bacteria that could be exploited for applications in biotechnology such as hydrolysis of under-utilized lignocellulosic material such as sawdust, to glucose which can further act as feedstock for other value adding products.

Keywords: Decayed Sawdust, Bacteria Isolate, Cellulase, Bacillus.

1. INTRODUCTION

Life thrives on a string of biochemical reactions driven by various enzymes. Enzymes are thus a necessity for the continuous existence of the biological world. Cellulose, which accounts for approximately 1.5×10^{12} tons of biomass produced through photosynthesis annually, is the most abundant organic compound on earth (Guo *et al.*, 2008). This abundance has made cellulase enzyme one of the most sought after in the commercial market as it degrades cellulose. Nature is rich in microbial groups with cellulolytic abilities such as fungi, actinomycetes and bacteria. Higher organisms such as insects, arthropods and plants have been found with various degrees of cellulolytic capability (Fischer *et al*., 2013; Duan and Feng, 2010; Sun and Scharf, 2010). For this study, bacteria have been selected for their profuse growth and shorter generation time when compared with their bio-counterparts. A significant amount of diversity exists among cellulolytic bacteria. Bacteria cells are sources of cellulase irrespective of the gram reaction, oxygen requirements or other basis of classification. Various Gram negative, Gram positive and Gram-variable bacteria produce cellulase (Yan and Wu, 2013; Huang *et al*., 2012). Cellulolytic bacteria could also be aerobic, facultatively anaerobic or anaerobic (Bayer *et al*., 2007). Cellulolytic bacteria have been isolated from a wide diversity of environments; extreme or favourable. *Acidothermus, Bacillus, Clostridium, Pseudomonas, Rhodothermus, Microbacterium, Rhizobioum* and *Escherichia* are genera that have been exploited for cellulase production (Huang *et al*., 2012; López-Contreras *et al*., 2004; Heck *et al*., 2002). This study aimed to find a cellulolytic bacterium capable of producing active cellulase in substantial amounts and with enviable physicochemical parameters from a saw mill.

2. MATERIALS AND METHOD

2.1 *Sample collection and preparation*

Unweighed quantity of sawdust was collected into a sterile bottle from a decaying sawdust heap at the saw mill located at Modakeke, Ile-Ife at a depth of about 2 metres. One gram (1 g) of decaying sawdust was accurately weighed out and dispensed into 10 ml sterile distilled water in a test tube. It was mixed well to ensure even dispersal of the microbial flora in the sample. This made the stock preparation. Aliquots of 1 ml was aseptically pipetted from the stock and transferred into the next tube of 9 ml sterile distilled water and mixed properly. This made the 10^{-1} dilution. The procedure was repeated until the sixth tube (10^{-6} dilution).

2.2 *Bacteria Isolation*

Aliquots of 1 ml of 10^{-4}, 10^{-5}, 10^{-6} dilution were plated out in duplicates using pour plate technique. Pure cultures were subsequently obtained and stored for further use.

2.3 *Screening for Cellulolytic Ability*

Carboxymethylcellulose agar (CMCA) plates incubated with a single streak of pure isolate were flooded with 0.1% Congo red solution after 48 h and de-stained with 1 M NaCl solution. A clear halozone around the line of streak depicted cellulose hydrolysis. The diameter of the halozone was measured and the isolates with considerable large halozones were picked for further studies.

2.4 *Bacterial Identification*

Pure cultures of cellulolytic bacteria were identified by their reactions to biochemical tests and the strain with maximum cellulase activity was further subjected to molecular identification by an analysis of the 16S rRNA sequence.

2.5 *Submerged Fermentation Process*

The cellulolytic bacterial cultures were grown over a period of 48 h in 0.1 M Phosphate buffer, pH 7.0 containing bacteriological peptone (2% w/v), K_2HPO_4, (0.3% w/v), $MgSO_4.7H_2O$ (0.1% w/v), NaCl (0.075% w/v) and high viscosity carboxymethylcellulose (0.2% w/v) as depicted by Kotchoni and Shonukan, 2002. This was done with agitation at 150 rpm in a water bath shaker. The medium was continually assayed for cellulase every 2 h. Thus, the best cellulolytic bacterium alongside its growth pattern was determined.

2.6 *Cellulase Extraction*

The growth medium, after optimal incubation, was centrifuged at 12, 000 rpm for 20 minutes and at a cold temperature of 4^{o}C. The supernatant was used as the crude enzyme.

2.7 *Cellulase Assay*

Cellulase activity was measured by the presence of reducing sugars released by the hydrolysis action of the enzyme on its substrate using Somogyi-Nelson method (Somogyi, 1952; Nelson, 1944). The reducing sugars were determined by incubating 0.1 ml of 0.2% w/v CMC, stabilized by 0.80 ml 0.1 M phosphate buffer, pH 7.0 with 0.05 ml of crude enzyme and inactivated crude enzyme (boiled at 100^{o}C for 15 minutes) at 37^{o}C for 20 mins. The reaction was terminated by the addition of 1 ml alkaline copper tartrate solution and subsequent boiling for 20 minutes. One millilitre (1 ml) of arsenomolybdate solution was added after cooling for colour stabilization. Absorbance was read at 540 nm against a reagent blank by a spectrophotometer and the amount of reducing sugars was interpolated from the glucose standard curve.

2.8 *Optimization of Cellulase Production Conditions*

The pH, temperature, carbon source, nitrogen source, percentage substrate concentration, and inoculum size of the basal medium was varied to observe the effect on enzyme production.

pH was varied from 4-10; temperature from 30-60^{o}C; carbon sources (glucose, sucrose, lactose, maltose, galactose and mannitol); nitrogen sources (tryptone, yeast extract, malt extract and urea for organic nitrogen; NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and $NH_4H_2PO_4$ for inorganic nitrogen); percentage substrate concentration was varied from 0.2-1.0% and inoculum size was varied from 1-5%. In each case, all other conditions were held constant.

2.9 *Cellulase Purification*

Cell Free Supernatant (CFS) was partially purified by precipitation with 80% ammonium sulphate and acetone and then, dialysis. CFS was also concentrated by lyophilization. Further purification was done by Ion exchange chromatograpy on diethylaminoethyl (DEAE)-Sephacel and Gel filtration chromatography on Sephacryl S-200.

2.10 *Determination of Kinetic Properties*

Kinetic parameters (K_m and V_{max}) were determined for the partially purified cellulase by incubating aliquots of the enzyme with CMC to make a final substrate concentration in the range 0.01-0.1 mg/ml and estimating the sugars released. Conditions for cellulase activity were optimized.

2.11 *Effect of Temperature, pH and Heat Stability on crude and Partially Purified Cellulase*

Aliquots of the enzyme was incubated with substrate and reducing sugars estimated as depicted in 2.7 at varying conditions of temperature (30-60 ^{o}C), pH 4 - 10 and 35-70 ^{o}C for heat stability.

3. RESULTS AND DISCUSSION

3.1 Bacteria Isolation and Characterization

As shown in Table 1, *Bacillus cereus, B. subtilis, B. brevis, B. circulans, Serratia marcescens* and *B. megaterium* were the cellulolytic bacteria isolated as A3&A21, A8, A11, A13, A15 and A22 respectively, as compared with the Bergey's Manual of Determinative Bacteriology.

Table 1: Morphological and Biochemical Characteristics of Cellulolytic Isolates

Isolate code	A3	A8	A11	A13	A15	A21	A22
Halozone Diameter (mm)	47	58	26	21	32	25	39
Morphological Characteristics							
Gram reaction	+	+	+	+	-	+	+
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Spore Staining	+	+	+	+	ND	+	+
Motility	+	+	+	+	+	+	+
Biochemical Characteristics							
Catalase	+	+	-	-	+	+	+
Citrate	+	+	-	-	+	+	+
Starch Hydrolysis	+	+	+	+	ND	+	+
Methyl Red	+	-	-	-	-	+	+
Voges Proskauer	+	+	-	-	+	+	-
Nitrate Reduction	+	+	ND	ND	+	+	+
Growth in 6.5% NaCl	ND	+	-	+	ND	ND	+
Oxidase	+	ND	-	ND	-	+	ND
Indole	-	-	-	-	-	-	-
Sulphide	-	ND	-	-	-	-	ND
Urease	-	ND	-	ND	-	-	-
Sugar Utilization							
Glucose	+	-	+	-	+	+	+
Lactose	+	-	-	+	-	+	+
Mannitol	-	+	-	+	+	-	+
L-arabinose	-	ND	-	+	-	-	-

Keys: + = positive reaction, - = negative reaction, and ND = Not Determined

Approximately, 85.7% of the isolates were identified as *Bacillus* species. This shows the predominance of *Bacillus* species as organisms of interest in cellulase production.

3.1 Screening for Cellulolysis

Diameter of halozones recorded vary among the isolates. A8 had the largest diameter of 58 mm (Fig 3.1).

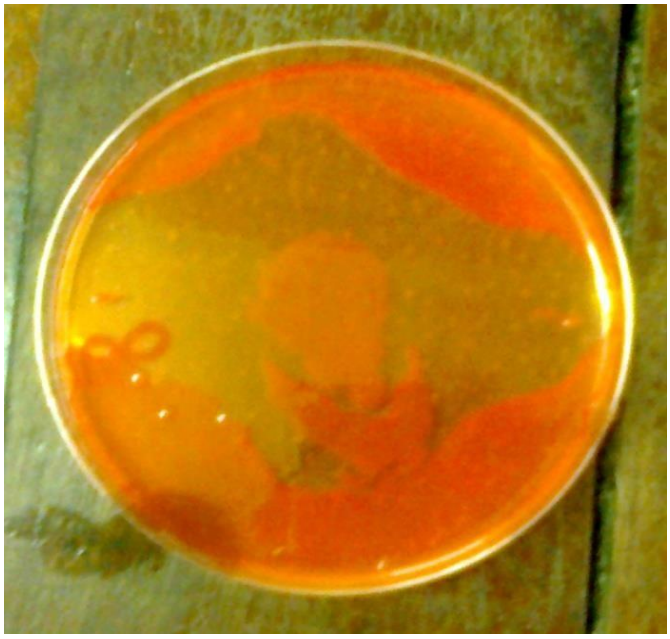


Fig. 3.1: Halozone of Isolate A8

3.2 Molecular Identification

Isolate A8 was further found to have a 96% similarity with the rRNA sequence of B. subtilis with the acession number FJ532063 of the GenBank, hence the isolate was confirmed as *Bacillus subtilis* A8.

3.3 Optimum Conditions for Cellulase Production from *B. subtilis* A8

As depicted in Fig 3.2, the growth pattern of *B. subtilis* A8 revealed a lag phase of about 6 h; logarithmic phase of about 28 h; stationary phase of about 6 h. The peak of cellulase activity was however at 36 h, falling in the stationary phase (34^{th} – 40^{th} hour). This confirms enzymes as secondary metabolites. This however was in contrast with the findings of Shabeb *et al*. (2010) and Mukesh Kumar *et al*. (2012) where maximum cellulase productivity from *B. subtilis* was recorded after 24 h and 72 h respectively. Maximum activity of cellulase at 36^{th}

hour of incubation is of a better advantage. This is because equipment and facilities are tied down in use for shorter periods, lower cost is incurred and less energy consumed.

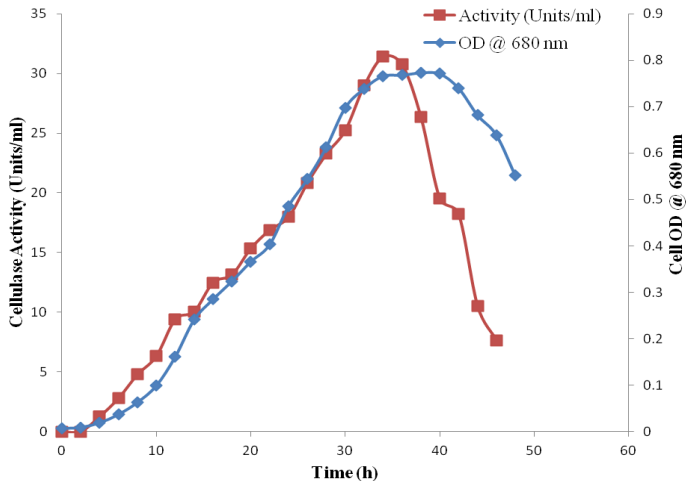


Fig. 3.2: Growth and cellulase activity of *Bacillus subtilis* A8 at 37°C, pH 7

Just as reported by Jayadev, 2014, *B. subtilis* A8 showed the highest cellulase activity at pH 7 (Fig. 3.3). The trend observed however, showed a preference for alkaline over acidic medium. On the contrary, Vijayaraghavan and Vincent (2012) reported the preference of a *Bacillus* sp. for a slightly acidic medium, with optimum pH at 6.5 while there was very low activity at pH 8.0.

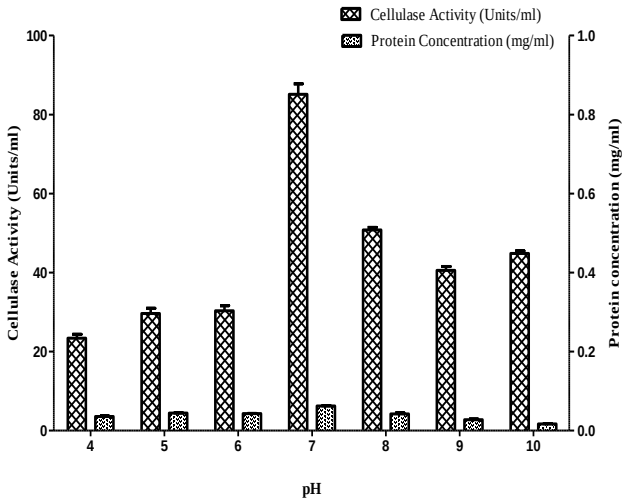


Fig. 3.3: Effect of pH on cellulase production by *Bacillus subtilis* A8 at 37°C

An optimum temperature of 35 ± 2°C was recorded in this present study (Fig. 3.4). This same optimum temperature was reported by Amritkar *et al*. (2004). At this relatively low temperature, not much heat is generated hence, there is little or no need for cooling systems in industries, and less energy is consumed. Shabeb *et al*. (2010) however reported maximum cellulase activity by *B. subtilis* at a higher temperature of 45°C.

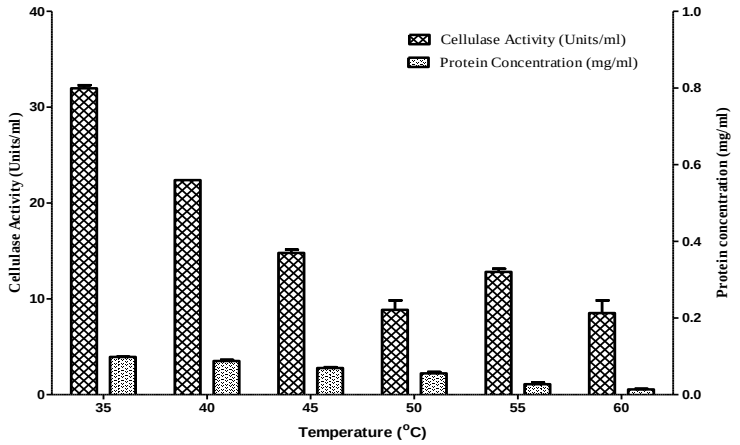


Fig. 3.4: Effect of temperature on cellulase production by *Bacillus subtilis* A8 at pH 7

The preferred choice of carbon source for *B. subtilis* A8 as shown in Fig. 3.5 was CMC and not any of the simple sugars used. This is of benefit in the industrial production of cellulose, as many low-cost carbon sources employed contain carbon in complex forms.

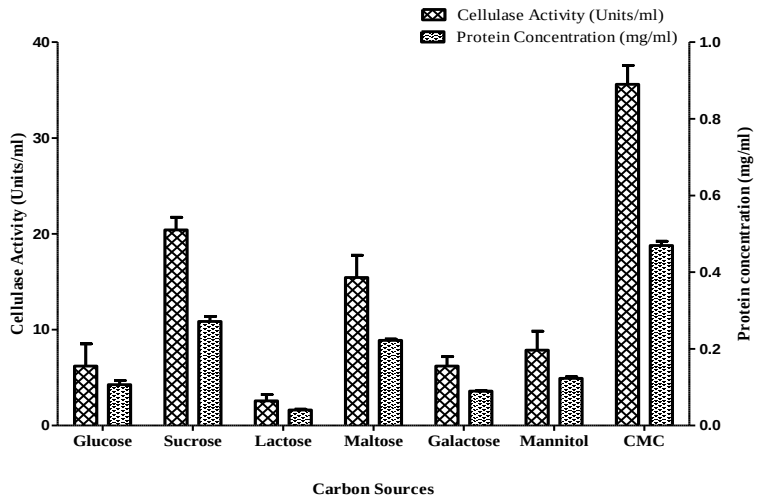


Fig 3.5: Effect of various carbon sources on cellulase production by *Bacillus subtilis* A8 at 37°C, pH 7

Of the nitrogen sources tested, the organic nitrogen sources supported growth and cellulase production better than the inorganic nitrogen sources (Figs. 3.6). This is of immense benefit as organic nitrogen sources abound more in nature.

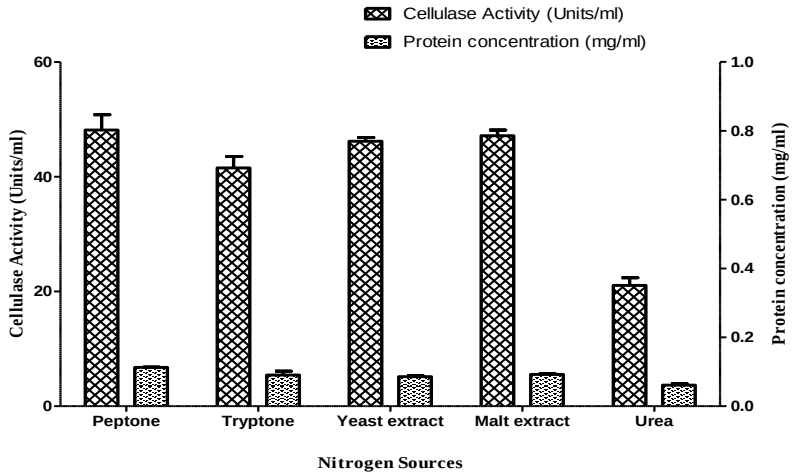
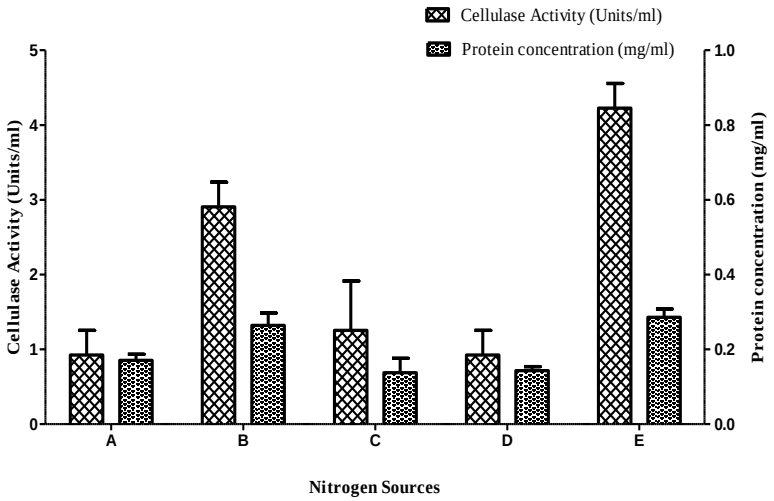


Fig. 3.6a: Effect of various organic nitrogen sources on cellulase production by *Bacillus subtilis* A8 at 37^{o}C, pH 7



Keys: A - NH_4Cl; B - NH_4NO_3; C - $(NH_4)_2SO_4$; D - $NH_4H_2PO_4$; E - $NaNO_3$

Fig. 3.6b: Effect of various inorganic nitrogen sources on cellulase production by *Bacillus subtilis* A8 at 37^{o}C, pH 7

Findings by Gautam *et al*. (2010) showed a maximum yield of cellulase at 1% concentration, a higher figure than that obtained in this study (Fig. 3.7). A lower substrate concentration is

of good economic value to industries and also individual researchers, as it reduces the cost of production.

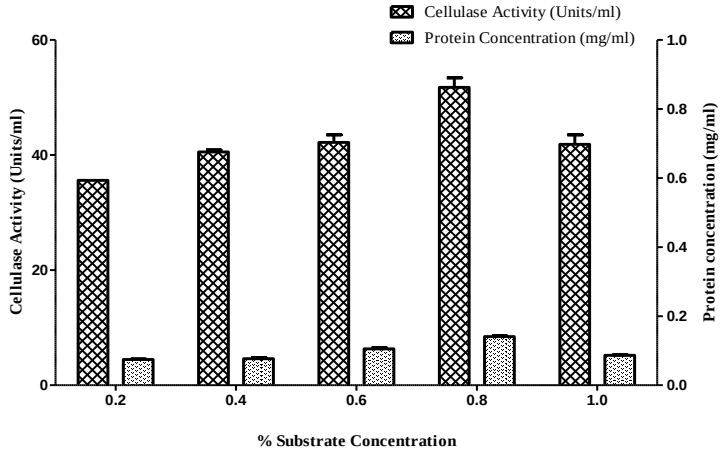


Fig. 3.7: Effect of percentage substrate concentration on cellulase production by *Bacillus subtilis* A8 at 37^{o}C, pH 7

The highest cellulase activity was obtained from the use of 4% inoculum size. This contrasts with the 8 and 10% recorded by Omojasola and Jilani (2009) and Fadel (2000) respectively. A lower inoculum size is better for less competition for resources by the organisms, thereby increasing the production of metabolites.

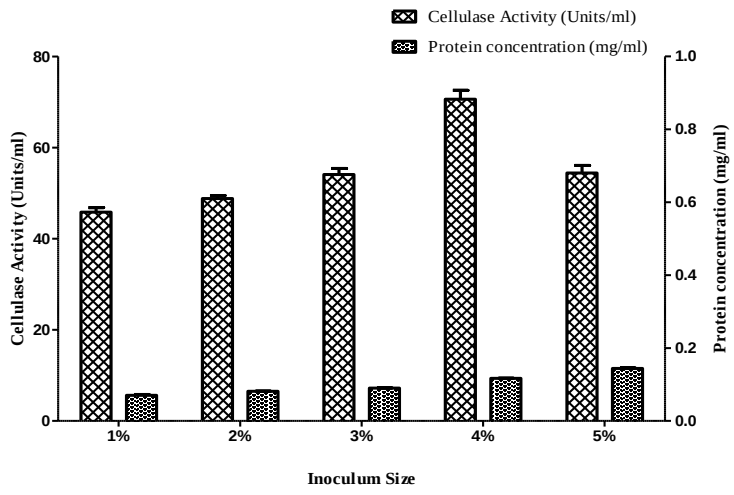


Fig. 3.8: Effect of percentage inoculum size on cellulase production by *Bacillus subtilis* A8 at 37^{o}C, pH 7

Acetone and ammonium sulphate precipitation were partial purification methods employed, both of which resulted in a considerable loss of activity, hence, the decision to lyophilize (Table 2). Concentration of the CFS by lyophilization considerably shortened the time involved in the purification process as there was no further need for dialysis.

Table 2: Comparison of Partial Purification Methods for Cellulase from *Bacillus subtilis* A8

PROCEDURE	VOLUME (ml)	ACTIVITY (Units/ml)	TOTAL ACTIVITY (Units)	PROTEIN (mg/ml)	TOTAL PRROTEIN (mg)	SPECIFIC ACTIVITY (Units/mg)	YIELD (%)	PURIFICATION FOLD
Crude	30	46.18	1385.40	7.24	217.20	6.38	100	-
80% Ammonium Sulphate Precipitation	9	61.20	550.80	9.63	86.67	6.36	39.76	1.00
Acetone Precipitation	4	70.45	281.80	7.29	29.16	9.66	20.34	1.51
Pre-Lyophilized	40	46.18	1847.20	7.24	289.60	6.38	100	-
Lyophilized	5	334.46	1672.30	11.08	55.40	30.19	90.53	4.73

Purification on DEAE - Sephacel resulted in two broad peaks as shown in Fig 3.9, with the second peak having a higher cellulose activity than the first. This probably represents different components of the cellulase complex.

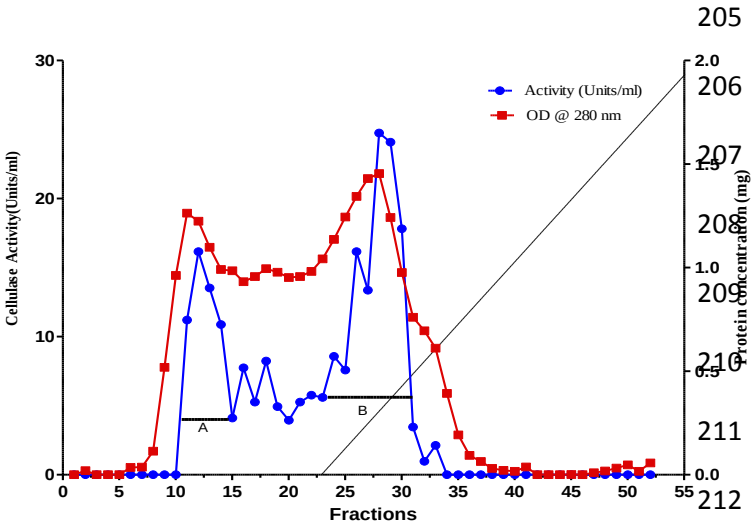


Fig. 3.9: Elution profile of cellulase obtained from *Bacillus subtilis* A8 on DEAE-Sephacel ion exchange column

Further purification by gel filtration on Sephacryl S-200 resulted in a single peak (Fig. 3.10). A yield of 87.8% recorded from the lyophilized cellulase as shown in Table 3. This implies a good suitability for cellulose hydrolysis. A lower yield was however recorded from cellulase partially purified by the chromatographic methods employed.

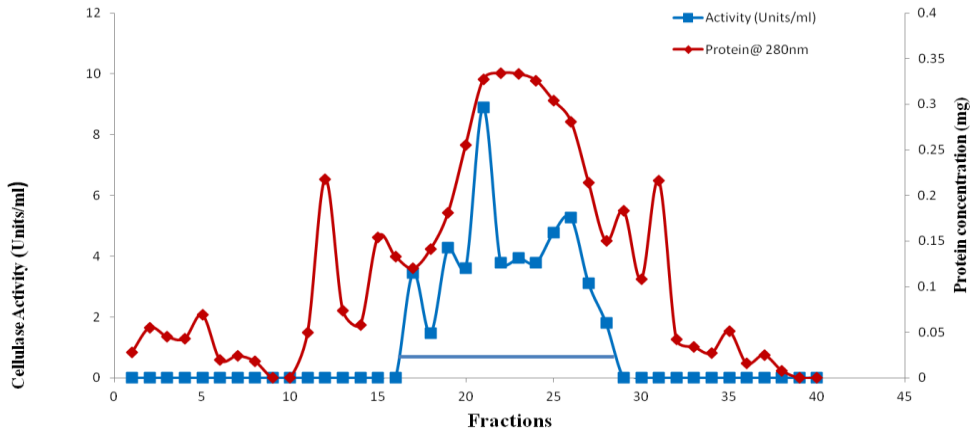


Fig. 3.10: Elution profile of cellulase obtained from *Bacillus subtilis* A8 on Sephacryl S-200 gel filtration column

Table 3: Summary of the Purification Protocol of Cellulase Obtained from *Bacillus subtilis* A8

PROCEDURE	VOLUME (ml)	ACTIVITY (Units/ml)	TOTAL ACTIVITY (Units)	PROTEIN (mg/ml)	TOTAL PRROTEIN (mg)	SPECIFIC ACTIVITY (Units/mg)	YIELD (%)	PURIFICATION FOLD
Crude	50	50.13	2506.28	7.15	357.54	7.00	100	-
Lyophilized	10	220.05	2200.50	12.13	121.30	18.14	87.8	2.59
0 M pooled ion exchange fractions	14.1	8.32	117.31	1.38	19.46	6.03	4.7	0.86
0.5 M pooled ion exchange fractions	8.6	19.47	167.44	2.15	18.49	9.06	6.7	1.29
Lyophilized pooled ion exchange fractions	5	92.76	463.78	6.57	32.85	14.19	18.5	2.03
Gel filtration chromatography	30	11.29	338.70	4.73	141.9	2.39	13.5	0.34

As calculated from Fig. 3.12, the K_m of partially purified cellulase was found to be 0.0108 ± 0.0032 mg/ml with a V_{max} of 119.3 ± 7.4 µmol/min. The low K_m showed high affinity of cellulase from *B. subtilis* A8 for the substrate (CMC) whereas the high V_{max} is an indication of the rapidness of its hydrolytic capability of the produced cellulase from *B. subtilis* A8. Linton and Greenaway (2004) reported a much lower V_{max} of 0.01 µmol/min and 0.03 µmol/min for total cellulase obtained from the foregut of *Gecarcoidea natalis* and *Discoplax hirtipes* respectively.

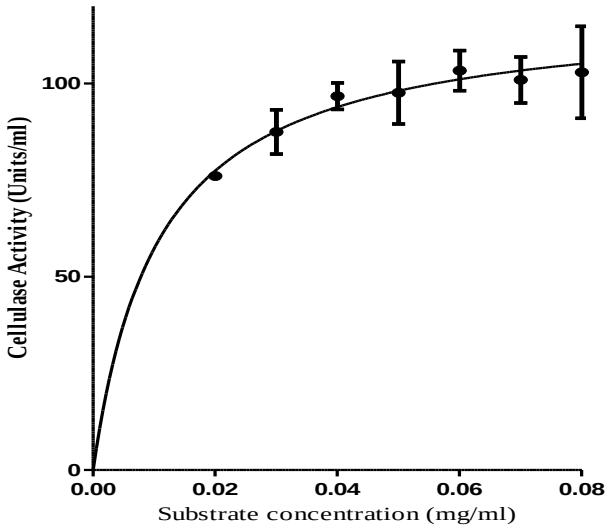
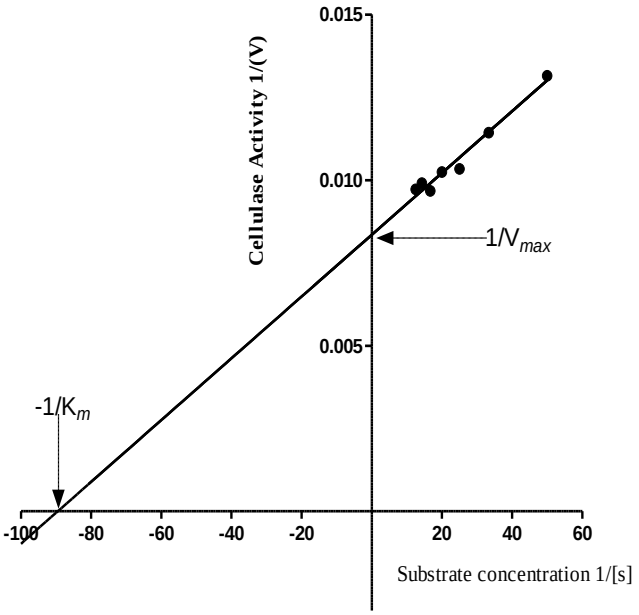


Fig. 3.11: Michealis-Menten plot of partially purified cellulase from *Bacillus subtilis* A8



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232 Fig. 3.12: Lineweaver-Burk plot of partially purified cellulase from *Bacillus subtilis* A8

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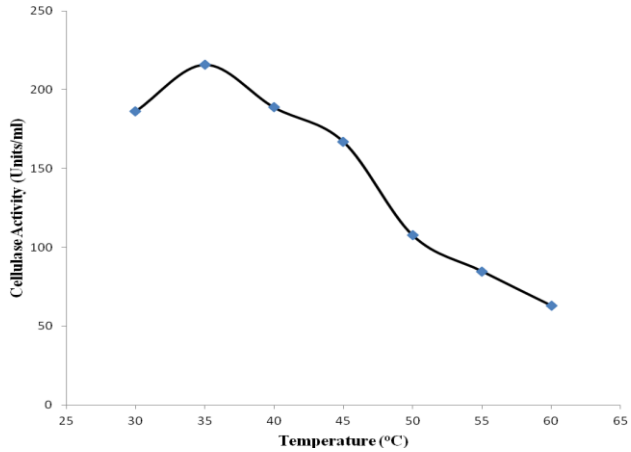

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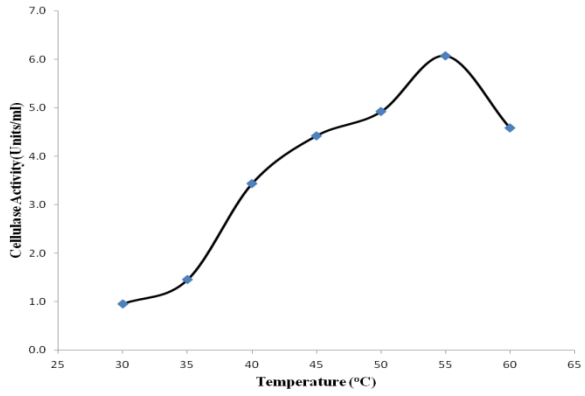
235 Fig. 3.13: Effect of temperature on the activity of crude cellulase obtained from *Bacillus subtilis* A8 at pH 7



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237 Fig. 3.14: Effect of temperature on the activity of partially purified cellulase obtained from *Bacillus subtilis* A8 at pH 7

Crude cellulase had the highest activity at pH 9 (Fig. 3.15) while purified cellulase was more active at pH 9.5 (Fig. 3.16). This is similar to findings by Aygan *et al*. (2011) where optimum for cellulase was pH 10.0. It disagrees with the findings of Yin *et al*. (2010) where maximum cellulase activity from *B. subtilis* YJ1 was recorded at pH 6.0 and that of Linton and Greenaway (2004) which showed an optimum pH of 5.5. Optimum activities at neutral pH values of 7.0 and 7.5 as in the cases of cellulase extracted from *B. coagulans* Co4, *B. amyloliquefaciens* and *Sinorhizobium fredii* have been reported by Adeleke *et al*. (2012).

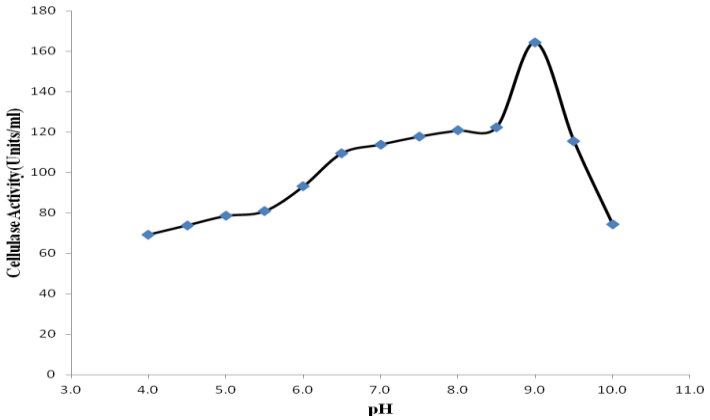

Fig. 3.15: Effect of pH on the activity of crude cellulase obtained from *Bacillus subtilis* A8 at 35^{o}C

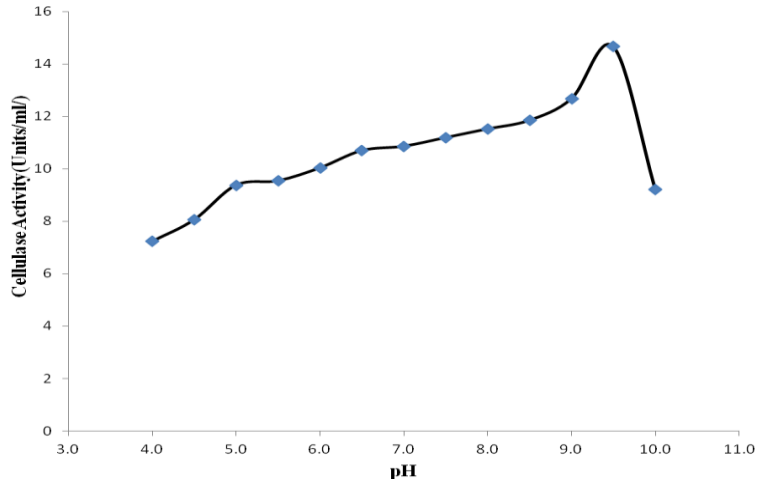

Fig. 3.16: Effect of pH on the activity of partially purified cellulase obtained from *Bacillus subtilis* A8 at 55^{o}C

Crude cellulase from *B. subtilis* A8 showed high activity and stability at 45^{o}C as depicted in Fig. 3.17. From 55-70°C, there was no significant difference in the level of activity. The relatively low temperature at which the crude cellulase is stable might be as a result of the impurities present.

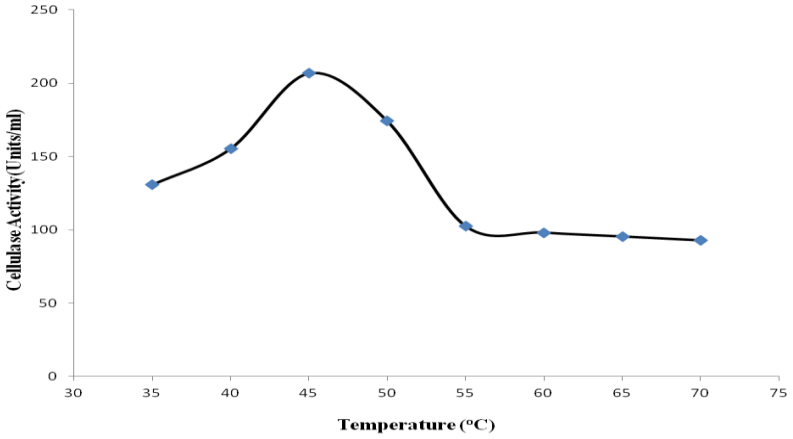

Fig. 3.17: Effect of temperature on the stability of crude cellulase obtained from *Bacillus subtilis* A8 at pH 9

The enzyme was stable at 50-60°C for at least 60 minutes, retaining 89.67% of its initial activity at optimum temperature (Fig. 3.18). Stability is a necessary characteristic of a good industrial enzyme and cellulase produced from *B. subtilis* A8 showed stability at high temperature. Similar results were found with *Bacillus* sp. CH43 and HR68 (Mawadza *et al*., 2000).

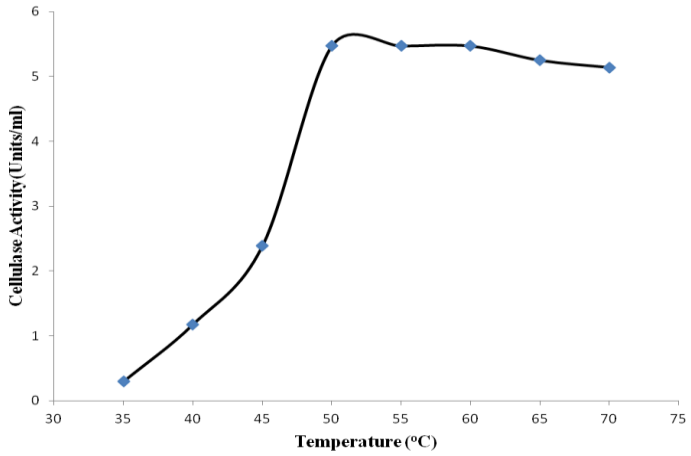

Fig. 3.18: Effect of temperature on the stability of partially purified cellulase obtained from *Bacillus subtilis* A8 at pH 9.5

CONCLUSION

Bacillus subtilis A8 is a bacterium capable of synthesizing cellulase enzyme with a good hydrolysing capability under mild physicochemical conditions. Also, cellulase from *B. subtilis* A8 is thermostable with high activity and could therefore be of immense benefit to industries that rely on the use of cellulase. Also, a different resin aside those employed in this study is recommended for better yield.

REFERENCES

Adeleke, E. O., Omafuvbe, B. O., Adewale, I. O. and Bakare, M. K. (2012). Purification and characterization of a cellulase obtained from cocoa (*Theobroma cacao*) pod-degrading *Bacillus coagulans* Co4. *Turkish Journal of Biochemistry*, 37(2):222-230.

Amritkar, N., Kamat, M. and Lali, A. (2004). Expanded bed affinity purification of bacterial α-amylase and cellulase on composite substrate and analogue-cellulose matrices. *Process Biochemistry*, 39(5):565-570.

Aygan, A., Karcioglu, L. and Arikan, B. (2011). Alkaline thermostable and halophilic from *Bacillus licheniformis* C108. *African Journal of Biotechnology*, 10(5): 789-796.

Bayer, E. A., Lamed, R. and Himmel, M. E. (2007). The potential of cellulases and cellulosomes for cellulosic waste management. *Current Opinion in Biotechnology*, 18(3):237-245.

Duan, C. J., Feng, J. X. (2010). Mining metagenomes for novel cellulase genes. *Biotechnology Letters*, 32(12):1765-1775.

Fadel, M. (2000). Production physiology of cellulases and ß-glucosidase enzymes of *Aspergillus niger* grown under solid state fermentation conditions. *Online Journal of Biological Science*, 1(5):401-411.

Fischer, R., Ostafe, R. and Twyman, R. M. (2013). Cellulases from insects. *Advance Biochemistry, Engineering Biotechnolology*, 136:51-64.

Gautam, S. P., Bundela, P. S., Pandey, A. K., Jamaluddin, Awasthi, M. K. and Sarsaiya, S. (2010). Optimization of the medium for the production of cellulase by the *Trichoderma viride* using submerged fermentation. *International Journal of Environmental Science*, 1(4):656-665.

Guo, R., Ding, M., Zhang, S. L., Xu, G. J. and Zhao, F. K. (2008). molecular cloning and characterization of two novel cellulase genes from the mollusc *Ampullaria crossean*. *Journal of Comprehensive Physiology [B]*, 178(2):209-215.

Heck, J. X., Hertz, P. F. and Ayub, M. A. Z. (2002). Cellulase and xylanase production by isolated Amazon *Bacillus* strains using soybean industrial residue based solid-state cultivation. *Brazillian Journal of Microbiology*, 33(3):213-218.

Huang, S., Sheng, P., Zhang, H. (2012). Isolation and identification of cellulolytic bacteria from the gut of *Holotrichia parallela* larvae (coleoptera: Scarabaeidae). *International Journal of Molecular Science*, 13(3):2563-2577.

Jayadev, A. (2014). Isolation and characterization of cellulolytic bacteria from a biofertilizer generated from municipal solid waste. *International Journal of Biotechnology and Biochemistry*, 10(2):91-98

Klemm, D., Schmauder, H. P. and Heinze, T. (2002). *Biopolymers. Steinb_chel (Wiley-VCH, Weinheim)*, 290-292.

Linton, S. M. and Greenaway, P. (2004). Presence and properties of cellulase and hemicellulase enzymes of the gecarcinid land crabs, *Gecarcoidea natalis* and *Discoplax hirtipis*. *Journal of Experimental Biology*, 207(23):4095-4104.

López-Contreras, A. M., Gabor, K., Martens, A. A., Renckens, B. A. M., Claassen, P. A. M., van der Oost, J. and de Vos, W. M. (2004). Substrate-induced production and secretion of cellulases by *Clostridium acetobutylicum. Applied Environmental Microbiology*, 70(9): 5238-5243.

Mawadza, C., Hatti-Kaul, R., Zvauya, R. and Mattiasson, B. (2000). Purification and characterization of cellulases produced by two Bacillus strains. *Journal of Biotechnology*, 83(3):177–187.

Mukesh kumar, D. J., Saraswati Bai, M., Ravi kumar, P., Balashanmugam, M. D., Balakumaran, P. T. and Kalaichelvan (2014) "Cellulase Production by *Bacillus subtilis* isolated from Cow Dung", Archives of Applied Science Research, 4(1):269-279.

Omojasola, P. F. and Jilani, O. P. (2009). Cellulase production by *Trichoderma longi*, *Aspergillus niger* and *Saccharomyces cerevisiae* cultured on plantain peel. *Research Journal of Microbiology*, 4(2):67-74.

Shabeb, M. S., Younis, M. A., Hezayen, F. F. and Nour-Eldein, M. A. (2010). Production of cellulase in low-cost medium by *Bacillus subtilis* KO strain. *World Applied Sciences Journal*, 8(1):35-42.

Sun, J. Z. and Scharf, M. (2010). Exploring and integrating cellulolytic systems of insects to advance biofuel technology. *Insect Science*, 17:163-165.

Vijayaraghavan, P. and Vincent, S. G. P. (2012). Purification and characterization of carboxymethyl cellulase from *Bacillus* sp. isolated from a paddy field. *Polish Journal of Microbiology*, 61(1):51-55.

Yan, S. and Wu, G. (2013). Secretory Pathway of Cellulase: A mini-review. *Biotechnology for Biofuels*, 6:177.

Yin, L. J., Lin, H. H., and Xiao, Z. R. (2010). Purification and characterization of a cellulase from *Bacillus subtilis* YJ1. *Journal of Marine Science and Technology*, 18(3):466-471.