Original Research Article

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

ABSTRACT

Cellulose continues to account for one of earth's most abundant biomass. Cellulase degrades cellulose, thereby making it one of the most sought after enzyme in the commercial market. This research aimed to characterize cellulase with enviable physicochemical parameters from a bacterium isolated from decaying sawdust heap. Isolated bacteria species were screened for cellulolysis. The bacterium with the largest halozone was identified by its 16S rRNA sequence. Optimum growth and cellulase production condition was determined by varying selected factors. Extracted cellulase was partially purified by Ion exchange and gel filtration chromatographic methods. The kinetic parameters were determined. Effect of selected conditions on cellulase activity was studied. Isolate A8 with 58 mm halozone had 96% sequence identity with *Bacillus subtilis* FJ532063. Optimum activity of 46.18 U/ml at 36 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 showed cellulase from *Bacillus subtilis* A8 as active and thermostable enough to be further exploited for industrial applications.

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

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abundant organic compound on earth [1]. 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 varying cellulolytic abilities such as fungi, actinomycetes and bacteria [2]. Higher organisms such as insects, arthropods and plants have been found with various degrees of cellulolytic capability [3-5]. 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 [6, 7]. Cellulolytic bacteria could also be aerobic, facultatively anaerobic or anaerobic [8]. Cellulolytic bacteria have been isolated from a wide diversity of environments; extreme or favourable. *Acidothermus, Bacillus, Clostridium, Pseudomonas, Rhodothermus, Microbacterium, Rhizobium* and *Escherichia* are genera that have been exploited for cellulase production [7, 9, 10]. This study aimed to find a cellulolytic bacterium capable of producing active cellulase in substantial amounts and with enviable physicochemical parameters.

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 Cellulase Production

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) with agitation at 150 rpm in a water bath shaker. The medium was continually assayed for cellulase every 2 h.

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 Nelson-Somogyi method [11, 12]. 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

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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

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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.

3.2 Screening for Cellulolysis

Diameter of halozones recorded vary among the isolates. A8 had the largest diameter of 58 mm (Fig. 1).

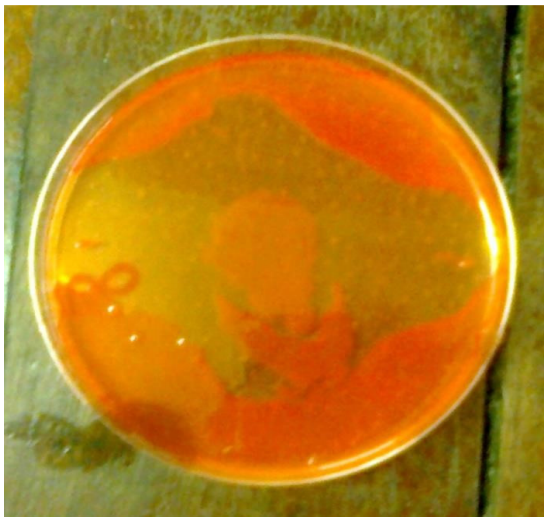

Fig. 1. Picture of the halozone of Isolate A8 on CMCA plate

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3.3 Molecular Identification

Isolate A8 was 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.

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

As depicted in Fig. 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 contrasts with previous studies where maximum cellulase productivity from *B. subtilis* was recorded after 24 h [13] and 72 h [14]. Maximum activity of cellulase at the 36^{th} hour of incubation is of a better advantage. This is because equipment and facilities are tied down in use for shorter periods. This allows less energy consumption and thus, production cost is reduced.

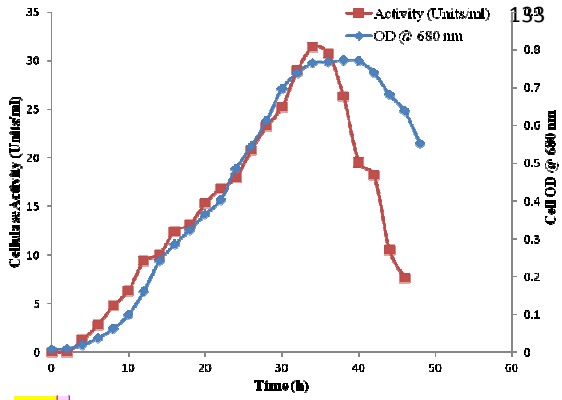

Fig. 2. Graph of the growth and cellulase activity of *B. subtilis* A8

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B. subtilis A8 showed the highest cellulase activity at pH 7 (Fig. 3) as was observed in a previous study [15]. The trend observed however, showed a preference for alkaline over acidic medium. The preference of cellulase from Bacillus has also been reported in a separate study where pH 9 was the optimum recorded [16]. Contrarily, there has been a report of 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 [17].

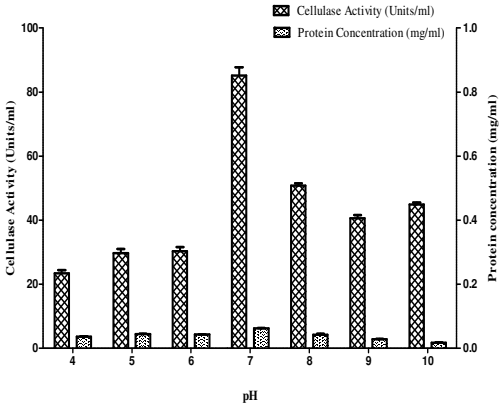

Fig. 3. Effect of pH on cellulase production by *B. subtilis* A8

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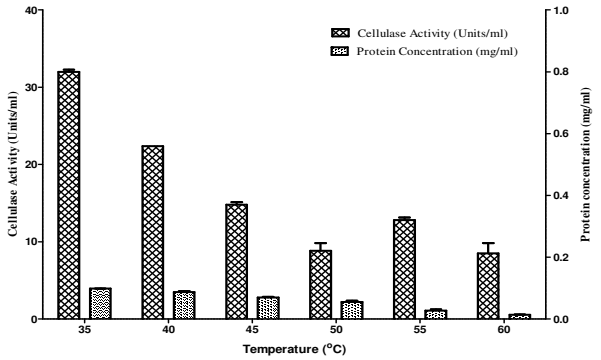
An optimum temperature of 35 ± 2°C was recorded in this present study (Fig. 4). At this relatively low temperature, not much heat is generated hence, there is little or no need for cooling systems in industries, and less energy is consumed.



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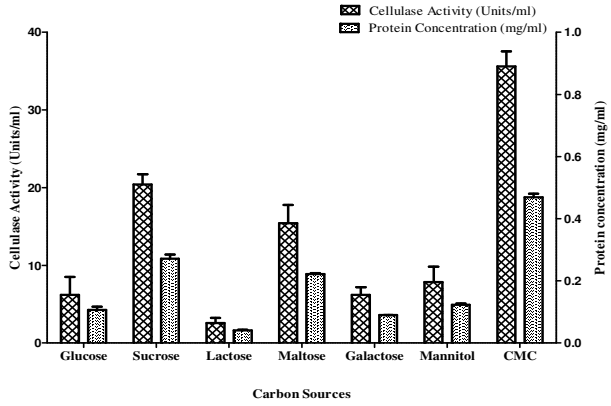
145 Fig. 4. Effect of temperature on cellulase production by *B. subtilis* A8

Comment [N9]: Caption corrected

146 The preferred choice of carbon source for *B. subtilis* A8 as shown in Fig. 5 was CMC and not any of
147 the simple sugars used. This is of benefit in the industrial production of cellulose, as many low-cost
148 carbon sources available contain carbon in the complex form found in CMC.



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150 Fig 5. Effect of various carbon sources on cellulase production by *B. subtilis* A8

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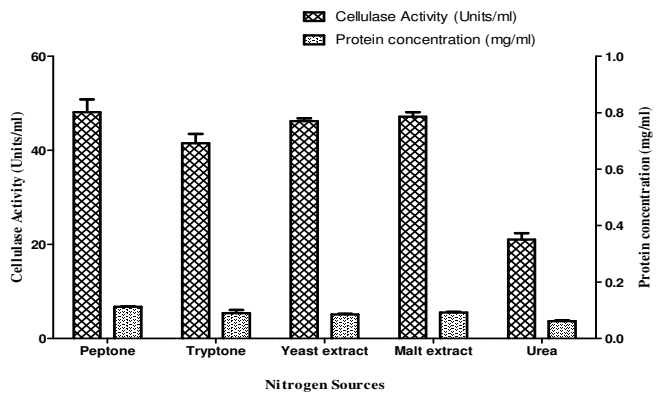
152 Of the nitrogen sources tested, the organic nitrogen sources supported growth and cellulase
153 production better than the inorganic nitrogen sources (Figs. 6). This is of immense benefit as organic
154 nitrogen sources abound more in nature.



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156 Fig. 6a. Effect of various organic nitrogen sources on cellulase production by *B. subtilis* A8

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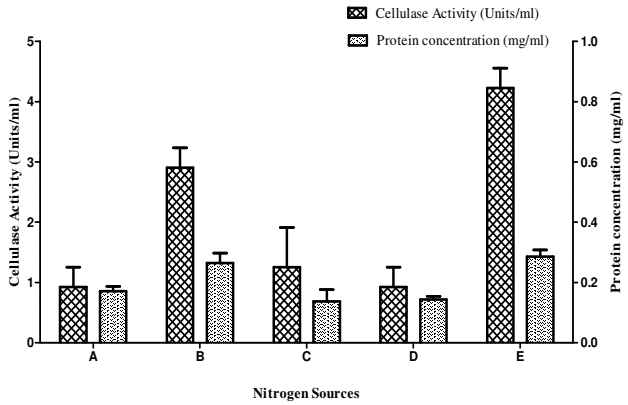

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158 Keys: A - NH_4Cl; B - NH_4NO_3; C - $(NH_4)_2SO_4$; D - $NH_4H_2PO_4$; E - $NaNO_3$

159 Fig. 6b. Effect of various inorganic nitrogen sources on cellulase production by *B. subtilis* A8

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160

161 Maximum cellulase yield was obtained at 0.8% (Fig. 7). Findings by some group of scientists showed
162 a maximum yield at 1% concentration [18], which is a higher figure than that which was obtained in
163 this study. A lower substrate concentration is of good economic value to industries and also individual
164 researchers, as it reduces the cost of production.

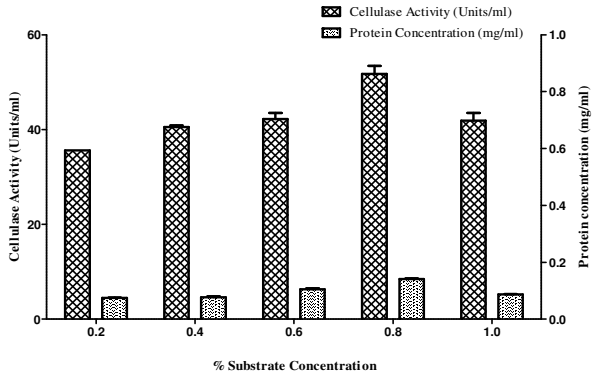

Fig. 7. Effect of percentage substrate concentration on cellulase production by *B. subtilis* A8

Comment [N13]: Caption corrected

In this study, the highest cellulase activity was obtained from the use of 4% inoculum size (Fig. 8). This contrasts with the relatively high value of 10% recorded in a similar study [19]. A lower inoculum size is better for less competition for resources by the organisms, thereby increasing the production of metabolites.

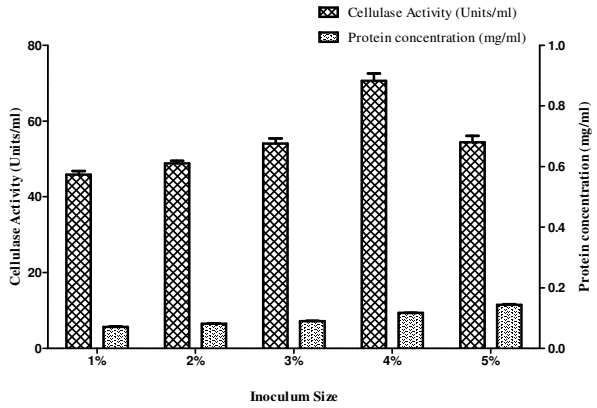

Fig. 8. Effect of percentage inoculum size on cellulase production by *B. subtilis* A8

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 obtained from *B. subtilis* A8

Comment [N14]: Caption corrected

Procedure	Volume (ml)	Activity (units/ml)	Total activity (units)	Protein (mg/ml)	Total protein (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. 9, with the second peak having a higher cellulose activity than the first. This probably represents different components of the cellulase complex.

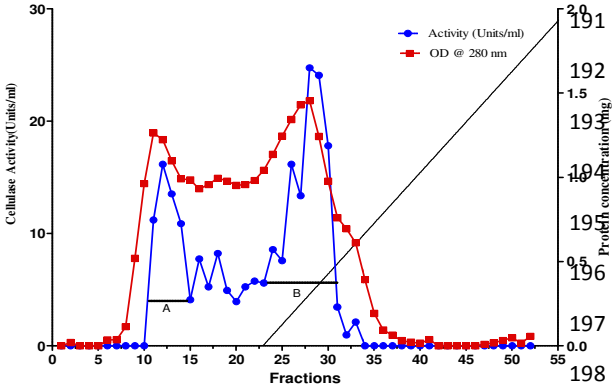

Fig. 9. Elution profile of cellulase obtained from *B. subtilis* A8 on DEAE-Sephacel ion exchange column

Comment [N15]: Caption corrected

Further purification by gel filtration on Sephacryl S-200 resulted in a single peak (Fig. 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.

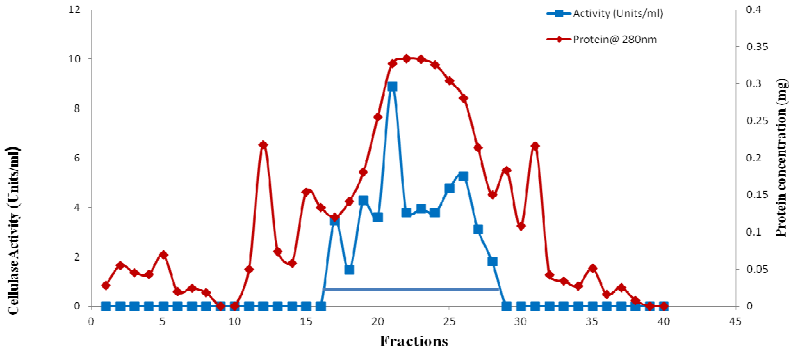

Fig. 10: Elution profile of cellulase obtained from *B. subtilis* A8 on Sephacryl S-200 gel filtration column

Comment [N16]: Caption corrected

212 **Table 3. Summary of the purification protocol of cellulase obtained from *B. subtilis* A8**

Comment [N17]: Caption corrected

Procedure	Volume (ml)	Activity (units/ml)	Total activity (units)	Protein (mg/ml)	Total protein (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 fractions	30	11.29	338.70	4.73	141.9	2.39	13.5	0.34

213

214 As depicted in Figs. 11 and 12, the K_m of partially purified cellulase was found to be 0.0108 ± 0.0032
215 mg/ml with a V_{max} of 119.3 ± 7.4 µmol/min. The low K_m showed high affinity of cellulase from *B.*
216 *subtilis* A8 for the substrate (CMC) whereas the high V_{max} is an indication of the rapidness of its
217 hydrolytic capability of the produced cellulase from *B. subtilis* A8. A much lower V_{max} of 0.01 µmol/min
218 and 0.03 µmol/min was recorded for cellulase obtained from the foregut of *Gecarcoidea natalis* and
219 *Discoplax hirtipes* respectively [20].

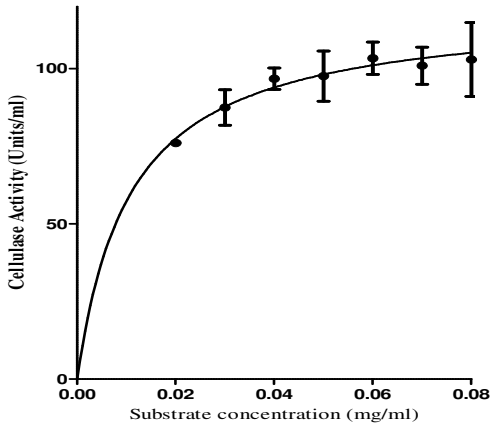

Fig. 11. Michealis-Menten plot of partially purified cellulase from *B. subtilis* A8

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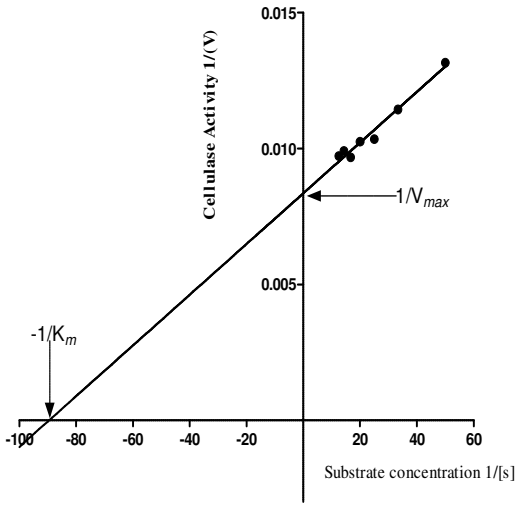

Fig. 12. Lineweaver-Burk plot of partially purified cellulase from *B. subtilis* A8

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The activity of crude cellulase rose to the peak at 35°C but continued to drop as the temperature increased (Fig. 13) while maximum activity was observed at 55°C for partially purified cellulase (Fig. 14). This suggests that cellulase thermostability might increase as the purity increases.

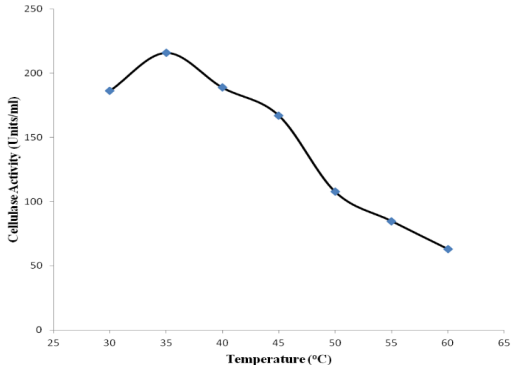

Fig. 13. Effect of temperature on the activity of crude cellulase obtained from *B. subtilis* A8

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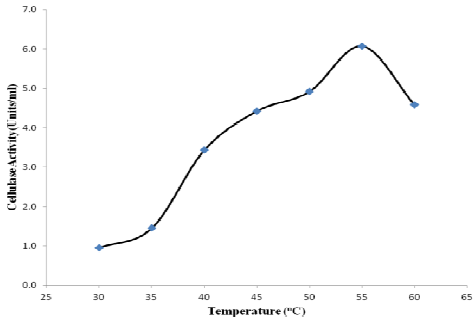

Fig. 14. Effect of temperature on the activity of partially purified cellulase obtained from *B. subtilis* A8

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The highest activity of crude cellulase was obtained at pH 9 (Fig. 15) while purified cellulase was more active at pH 9.5 (Fig. 16). Aygan et al. [21] equally reported significant cellulase activity at pH 10.0. This strongly suggests an affinity of cellulase for alkaline medium. For better hydrolysis thus, substrate must be in an alkaline medium. In a different similar studies, while Linton and Greenaway [20] recorded maximum cellulase activity at pH 5.5, Pang et al. [22] reported very low activity for all components of the cellulase complex. 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. [23].

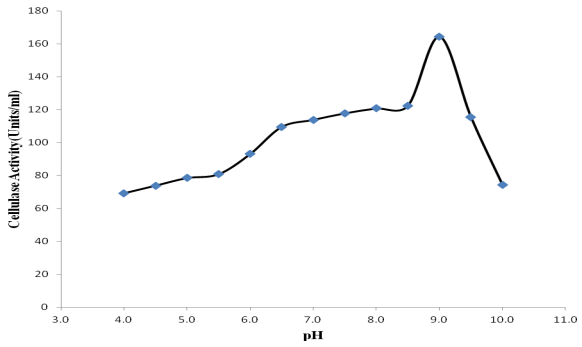

Fig. 15. Effect of pH on the activity of crude cellulase obtained from *Bacillus subtilis* A8

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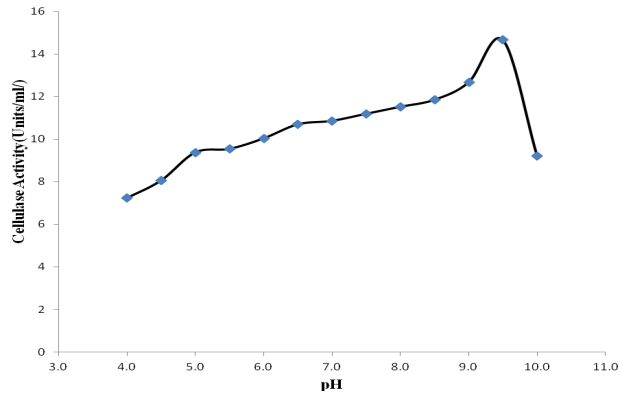

Fig. 16. Effect of pH on the activity of partially purified cellulase obtained from *B. subtilis* A8

Crude cellulase from *B. subtilis* A8 showed high activity and stability at 45°C as depicted in Fig. 17. At temperature range of 55-70℃, there was no significant difference in the level of activity.

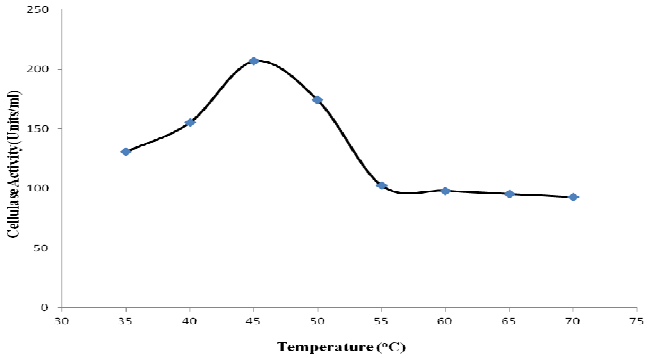

Fig. 17. Effect of temperature on the stability of crude cellulase obtained from *B. subtilis* A8

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The enzyme was stable at 50-60℃ for at least 60 minutes, retaining 89.67% of its initial activity at optimum temperature (Fig.18). Stability is a necessary characteristic of a good industrial enzyme and cellulase produced from *B. subtilis* A8 showed stability at high temperature.

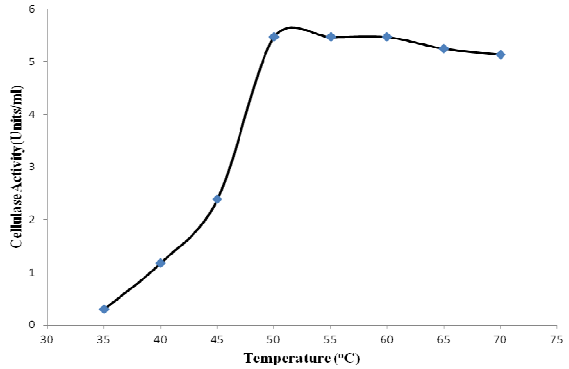

Fig. 18. Effect of temperature on the stability of partially purified cellulase obtained from *B. subtilis* A8

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4. CONCLUSION

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Bacillus subtilis A8 is a bacterium capable of synthesizing cellulase enzyme with a good hydrolysing capability. Under mild physicochemical conditions of pH 7, 35 ± 2°C, 0.8% substrate concentration and 4% Inoculum size for 36 hours with agitation at 150 rpm; *B. subtilis* A8 secretes thermostable cellulase with activity up to 46.18 U/ml and could therefore be of immense benefit to industries that

rely on the use of cellulase. A different resin aside those employed in this study is however recommended for purification in order to obtain a greater enzyme yield.

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