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Isolation, Identification and Characterization of Cellulase from a Bacterium Obtained at a Saw-Mill Site in Ile-Ife, Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. All the authors designed the study. Author AEA performed the statistical analysis and wrote the first draft of the manuscript. Authors EFA and KOA managed the analyses of the study. Authors AEA, EFA and MKB managed the literature searches. All authors read and approved the final manuscript.

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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°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 ± 7.4 µmol/min. Maximum activity for

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partially purified cellulase was recorded at pH 9.5 and 55°C with stability at 50°C; and pH 9, 35°C with stability at 45°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 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 METHODS

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°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°C for 15 minutes) at 37°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°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°C), pH 4 - 10 and 35-70°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.

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).

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 36th 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.

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

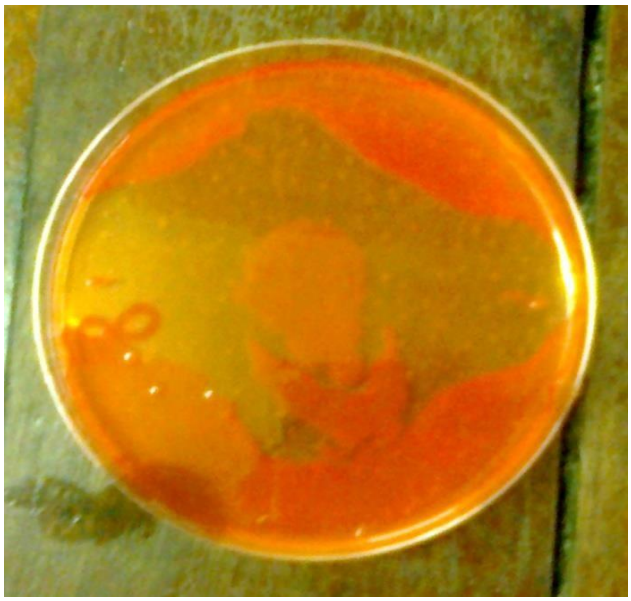

Fig. 1. Picture of the halozone of Isolate A8 on CMCA plate

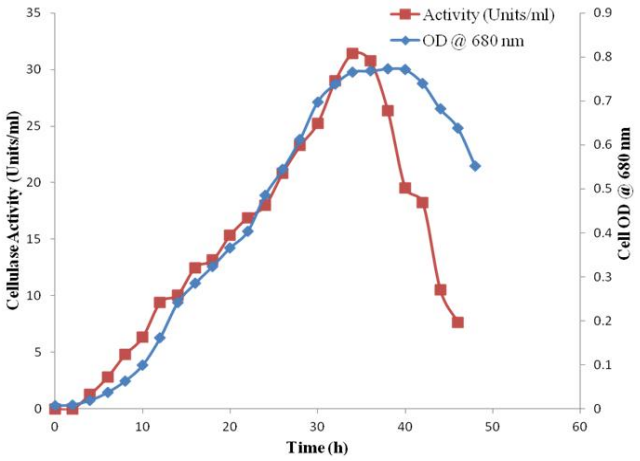

Fig. 2. Graph of the growth and cellulase activity of *B. subtilis A8*

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].

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.

The preferred choice of carbon source for *B. subtilis* A8 as shown in Fig. 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 available contain carbon in the complex form found in CMC.

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

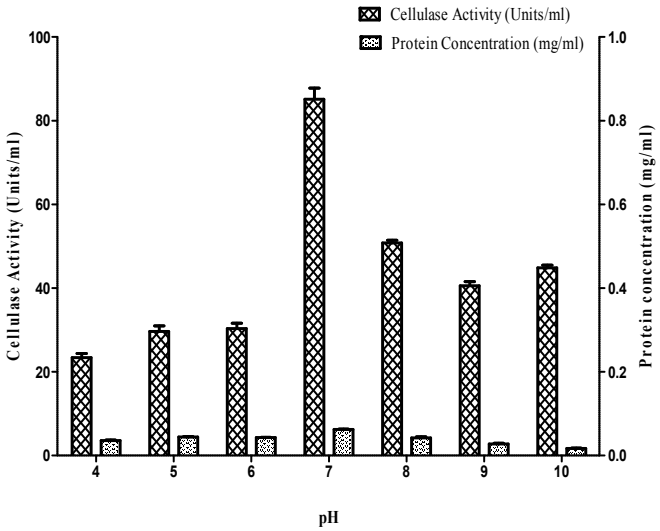

Fig. 3. Effect of pH on cellulase production by *B. subtilis* A8

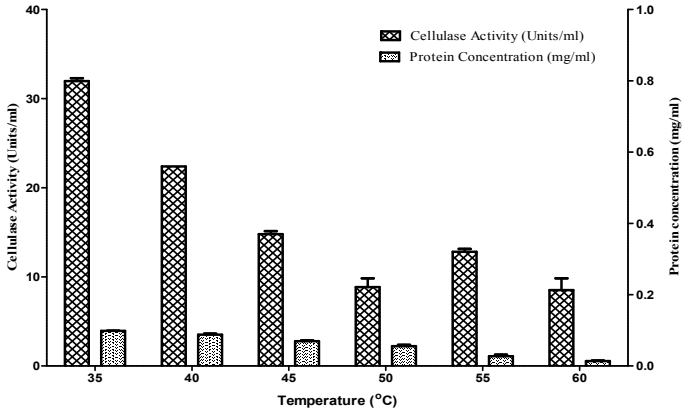

Fig. 4. Effect of temperature on cellulase production by *B. subtilis* A8

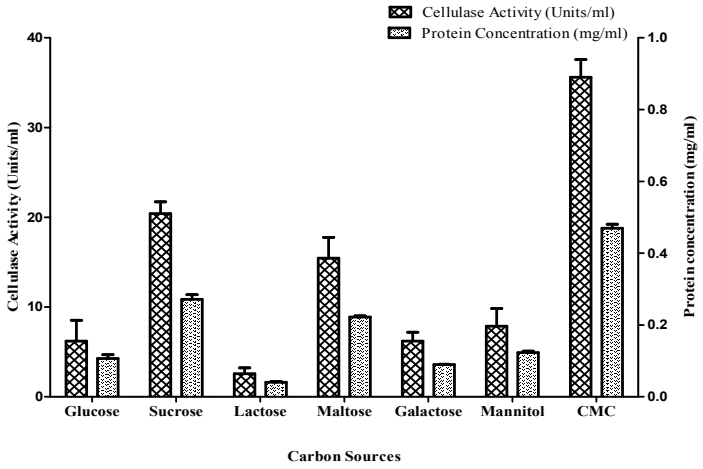

Fig. 5. Effect of various carbon sources on cellulase production by *B. subtilis* A8

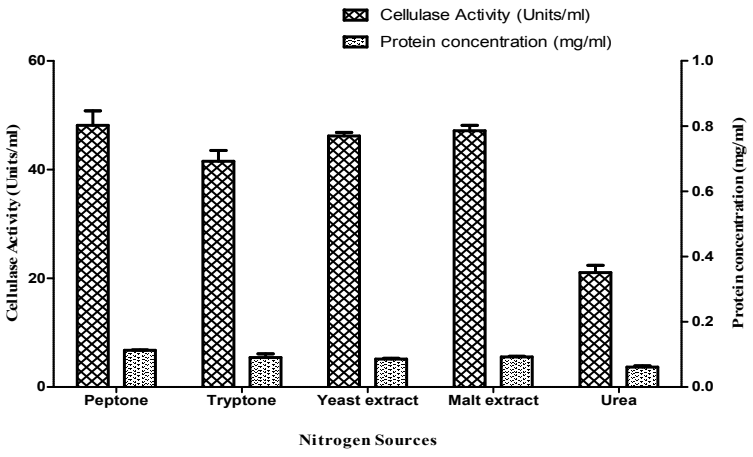

Fig. 6a. Effect of various organic nitrogen sources on cellulase production by *B. subtilis* A8

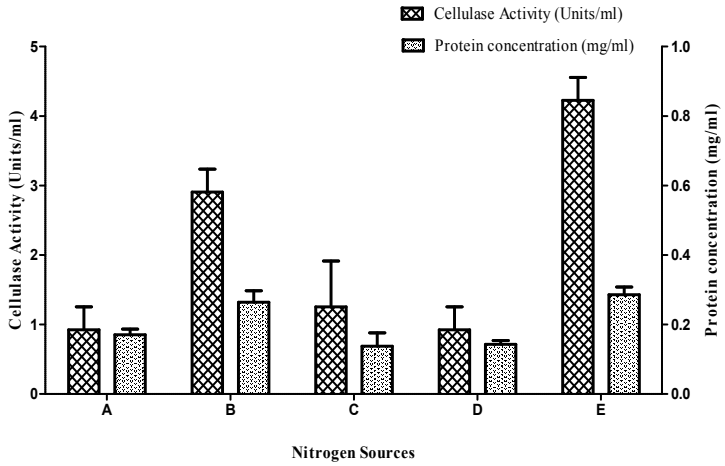

Fig. 6b. Effect of various inorganic nitrogen sources on cellulase production by *B. subtilis* A8
Keys: A - NH_4Cl; B - NH_4NO_3; C - $(NH_4)_2SO_4$; D - $NH_4H_2PO_4$; E - $NaNO_3$

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

In this study, the highest cellulase activity was obtained from the use of 4% inoculums 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.

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.

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.

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.

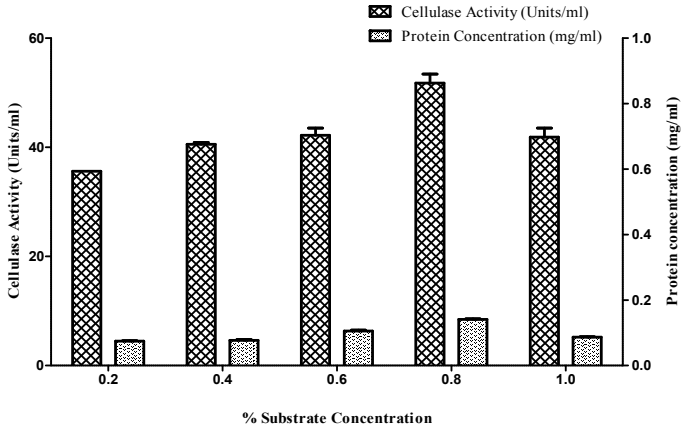

Fig. 7. Effect of percentage substrate concentration on cellulase production by *B. subtilis* A8

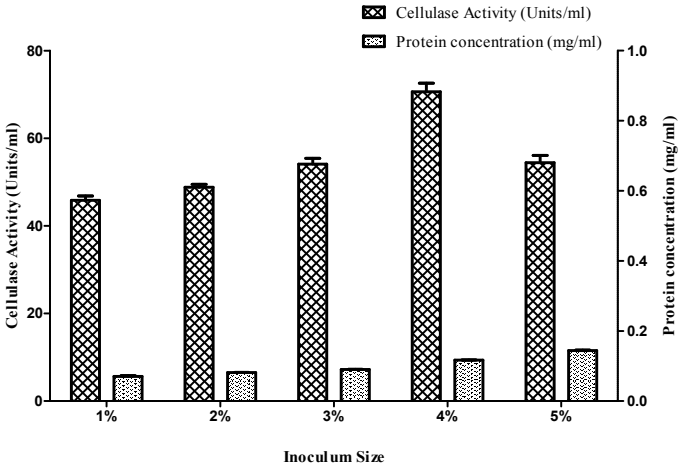

Fig. 8. Effect of percentage inoculum size on cellulase production by *B. subtilis* A8

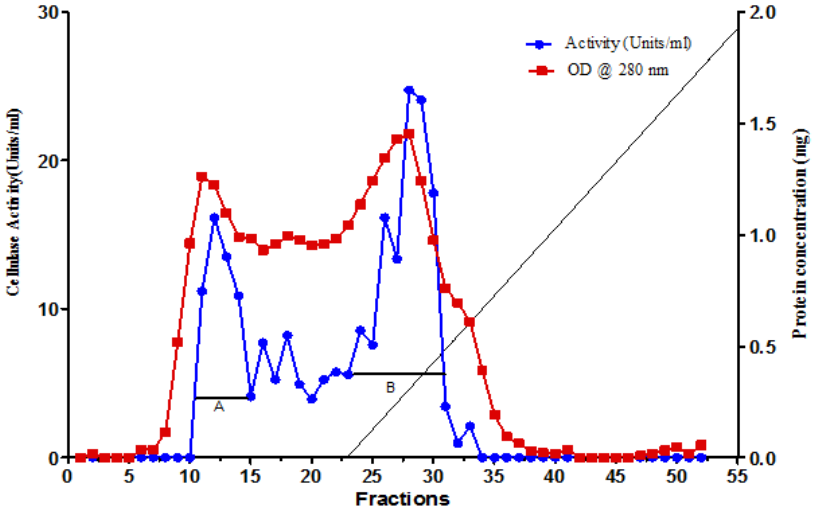

Fig. 9. Elution profile of cellulase obtained from *B. subtilis* A8 on DEAE-Sephacel ion exchange column

As depicted in Figs. 11 and 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. A much lower V_{max} of 0.01 µmol/min and 0.03 µmol/min was recorded for cellulase obtained from the foregut of *Gecarcoidea natalis* and *Discoplax hirtipes* respectively [20].

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.

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 thecases of cellulase extracted from *B. coagulans* Co4, *B. amyloliquefaciens* and *Sinorhizobium fredii* have been reported by Adeleke et al. [23].

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°C, there was no significant difference in the level of activity.

The enzyme was stable at 50-60°C 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.

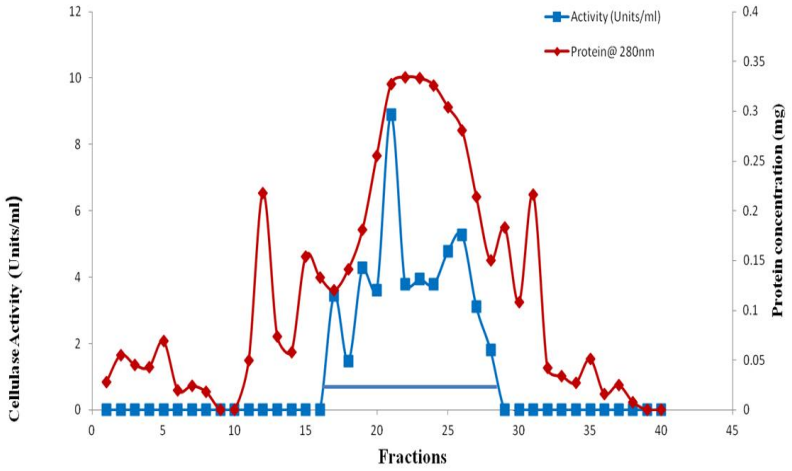

Fig. 10. Elution profile of cellulase obtained from *B. subtilis* A8 on Sephacryl S-200 gel filtration column

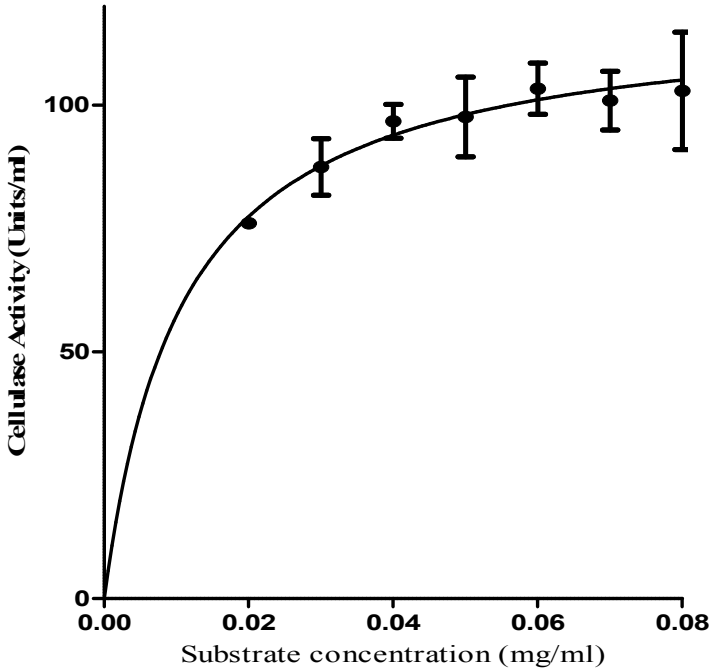

Fig. 11. Michealis-Menten plot of partially purified cellulase from *B. subtilis* A8

Table 2. Comparison of partial purification methods for cellulase obtained from *B. subtilis* A8

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

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

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

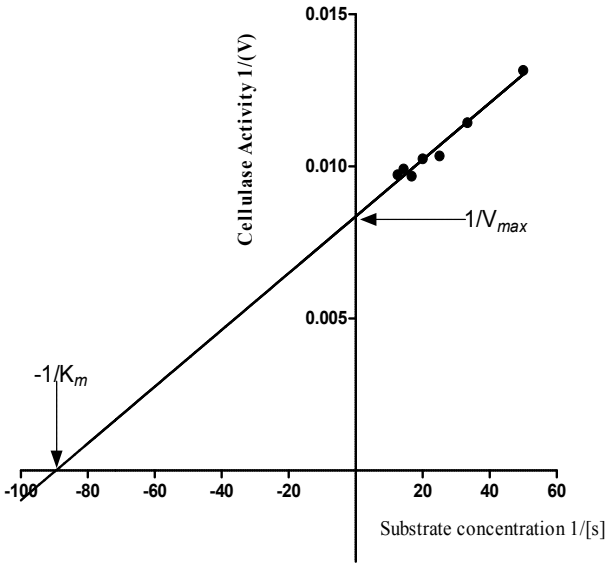

Fig. 12. Lineweaver-Burk plot of partially purified cellulase from *B. subtilis* A8

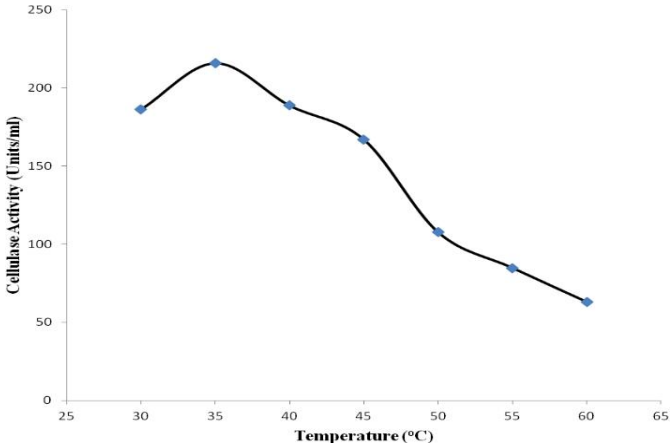

Fig. 13. Effect of temperature on the activity of crude cellulase obtained from *B. subtilis* A8

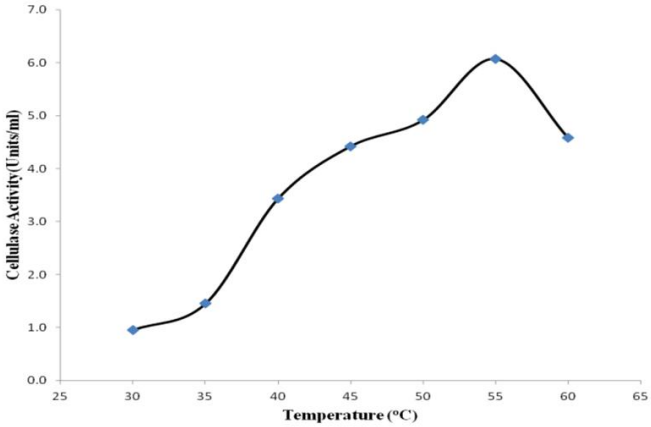

Fig. 14. Effect of temperature on the activity of partially purified cellulase obtained from *B. subtilis* A8

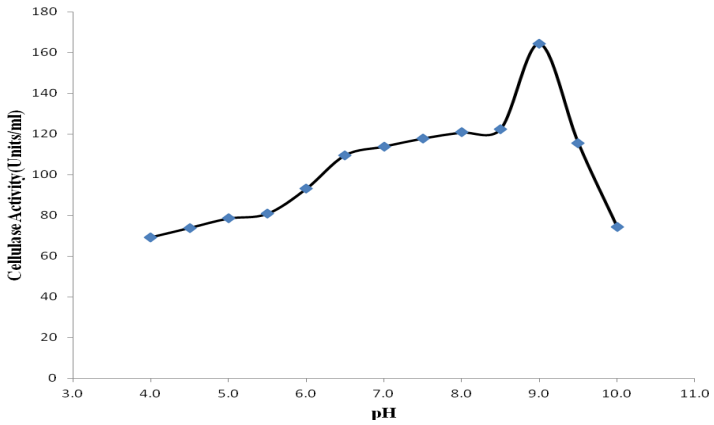

Fig. 15. Effect of pH on the activity of crude cellulase obtained from *Bacillus subtilis* A8

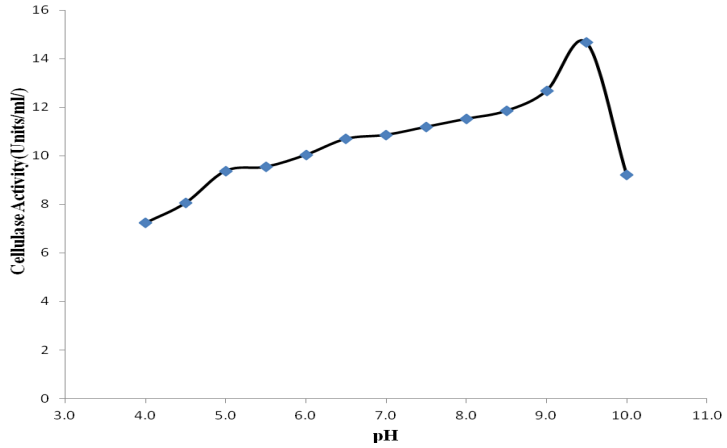

Fig. 16. Effect of pH on the activity of partially purified cellulase obtained from *B. subtilis* A8

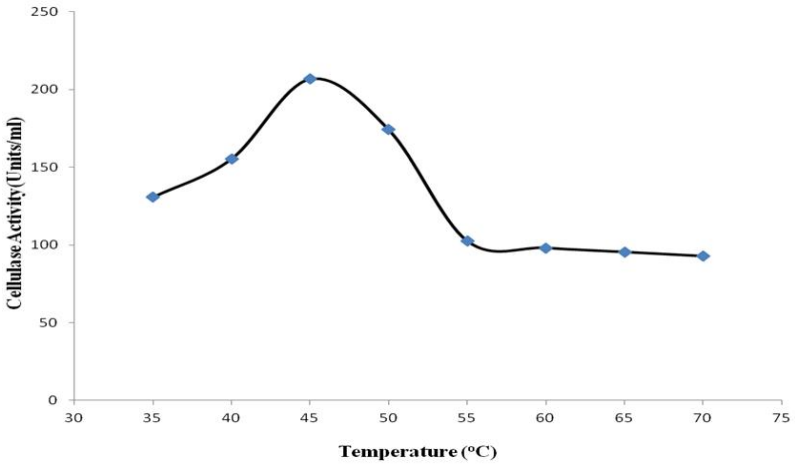

Fig. 17. Effect of temperature on the stability of crude cellulase obtained from *B. subtilis* A8

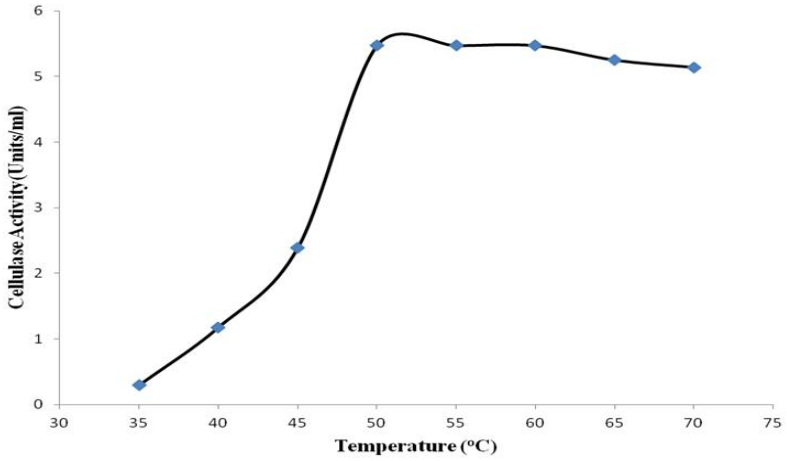

Fig. 18. Effect of temperature on the stability of partially purified cellulase obtained from *B. subtilis* A8

4. CONCLUSION

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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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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