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Partial Purification and Characterization of Cellulase Produced by *Bacillus sphaericus* CE-3

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Abstract

Cellulase is an enzyme produced by fungi, bacteria, protozoa and termite, that hydrolyze cellulose. They are known for their diverse applications in industry and medicine. The aim of this study is to purify and investigate cellulolytic properties of cellulase enzyme produced by *Bacillus sphaericus* CE-3 isolated from refuse dump in Nnamdi Azikiwe University, Awka, Nigeria. Enzyme was produced by submerged fermentation at 30˚C for 30 h. The enzyme was purified to homogeneity by dialysis in 4M sucrose solution, ion-exchange chromatography on Q-Sepharose FF and by hydrophobic interaction chromatography on Phenyl Sepharose CL-4B. The relative molecular mass of the enzyme was estimated using SDS-Polyacrylamide gel electrophoresis. Effects of temperature, pH and metals on enzyme activity and stability and the relative rate of hydrolysis of various substrates were also studied. The Purification fold for the enzyme was 7.8, with 66.4 μ/mg specific activity protein and overall yield of 35.8. The relative molecular mass range of the enzyme was estimated between 22.3 kDa - 26.3 kDa. The enzyme was optimally active at pH 9.0 and 40˚C, stable at pH 9.0 and unusually retained over 90% activity between 50˚C - 100˚C after 30 min incubation. It was strongly activated by Mn^{2+} but inhibited by Ba^{2+}, Co^{2+}, Hg^{2+}, Pb^{2+}, Cu^{2+}, Sr^{2+}, Fe^{2+}, Ca^{2+} and Zn^{2+}. The cellulase displayed high catalytic activity with untreated sawdust, followed by carboxymethyl cellulose, while sodium hydroxide treated sawdust was the least hydrolyzed. Since the enzyme is thermo-stable, alkalophilic and could utilize natural wastes like sawdust as substrate, it is obvious that it would be of great use in textile, starch processing and pulp and paper industries.

Keywords

Cellulase, *Bacillus sphaericus* CE-3, Untreated Sawdust, Catalytic Activity

1. Introduction

Cellulose, a polymer of β-1,4-linked glucose unit, is a major polysaccharide con-

stituent of plant cell walls [1]. Plants produce about 4×10^9 tons of cellulose annually along with other polysacharides [2]. Cellulose is an abundant and renewable energy source which can be converted to useful products (sugars, alcohols), and other industrially important chemicals by enzymatic degradation [3]. It has been reported that effective biological hydrolysis of cellulose into glucose requires synergistic actions of three enzymes, including endo-β-1,4-glucanase (EC3.2.1.4,EG, randomly cleaving internal linkages), cellobiohydrolase (EC3.2.1.91, CBH, specifically hydrolyzing cellobiosyl units from non-reducing ends) and B-D-glucosidase (EC.3.2.1.21, hydrolyzing glucosyl units from cellooligosaccharides) [4]. Celluloses are becoming increasingly important and could provide key opportunity to achieving tremendous benefits in biomass utilization [1] and also serve as substitutes for diminishing fossil energy resource [2].

Cellulases have attracted much attention because of their diverse applications both medically and industrially. They are used in reducing high serum cholesterol level, in improving the nutritional quality and digestibility [3], in animal feed [5], in waste/water management [6] [7], in textile (bio polishing of fabrics and producing stone washed look of denims) [8], in pulp and paper industry, starch processing [9] in brewing and wine making as well as in house hold laundry detergents [10].

Cellulases can be produced by fungal as well as bacterial organisms. Bacterial cellulases, however, are easily obtained, have short generation time and the ability to grow to very high cell density using inexpensive carbon and nitrogen sources. *Bacillus* species are one of the bacterial groups known for their industrial enzyme production, and many have been implicated in cellulase production [9] [11] [12] [13] [14] [15]. The purpose of this study therefore, was to purify a cellulase enzyme produced by *Bacillus sphearicus* CE-3 and to investigate its cellulolytic properties.

2. Materials and Methods

2.1. Microorganism Used

Bacillus sphaericus CE-3 isolated from a refuse dump in Nnamdi Azikiwe University, Awka, Nigeria, was used for the experiment. It was inoculated into Nutrient Agar (BDH) slant and stored at 4˚C.

2.2. Screening of Organism for Carboxymethyl Cellulase Production

Screening *Bacillus sphaericus* CE-3 for carboxymethyl cellulase production was carried out by plate method [16]. Point inoculations of the isolate was made on a Yeast Extract medium (BDH) containing 0.5% carboxymethyl cellulose (CMC). The plate was incubated at 30˚C for 5 days. At the end of the incubation period, the plate was flooded with Congo red (1 mg/ml) and de-stained with 1M $NaCl_2$ for 15 min. Carboxymethyl cellulase production was indicated by clear zones around the colonies.

2.3. Enzyme Production by Submerged Fermentation

A 100 ml Erlenmeyer flask containing 50 ml of the fermentation medium (1% CMC, 0.5% yeast extract, 0.1% $NaCl_2$, 0.1% KH_2PO_4, 0.1% $MgSO_4$, pH7.0 adjusted with NaOH) was inoculated with a 24h old culture of *Bacillus sphaericus* CE-3. The flask was incubated at 30˚C in a rotary shaker (140 rpm) for 30 h. Triplicate flasks were prepared and the broths pooled together for enzyme activity.

2.4. Determination of Enzyme and Protein Activities

The fermentation broth was centrifuged at 5000 rpm for 25 min and 0.5 ml of the supernatant added to 0.5 ml of 0.05% CMC in phosphate buffer. The mixture was incubated at 40˚C for 30 min, and examined for cellulase activity using 3,5-dini-trosalicyclic acid (DNS) [17]. The reducing sugar liberated was measured at 540 nm. One unit of enzyme activity is defined as the amount of enzyme that liberated 1 μg of reducing sugar/ml/minutes from appropriate substrate under assay condition.

The supernatant from the fermentation broth was used to determine the protein activity of the enzyme [18].

2.5. Enzyme Concentration/Purification

Enzyme in the supernatant was concentrated by dialysis in a 4M sucrose solution for 6 h at 4˚C.

2.5.1. Ion Exchange chromatography

The concentrated crude enzyme (45 ml) was applied on a Q-Sepharose column (1.8 × 14.5 cm) equilibrated with 0.02 M phosphate buffer (pH 7.0). Protein was eluted using 0.5 M NaCl in 0.02 M phosphate buffer (pH 7.0) at a flow rate of 1.5 ml/min. A total of 25 fractions (10ml in each test tube) were collected and assayed for enzyme activity and protein. Fractions with high enzyme activities were pooled and concentrated.

2.5.2. Hydrophobic Interaction Chromatography

The recovered enzyme was subjected to hydrophobic interaction chromatography on a Phenyl Sepharose CL-4B column (1.8 × 14 cm) equilibrated with 1.5 M $(NH_4)_2SO_4$. The column was eluted using 1.5 M $(NH_4)_2SO_4$ in 150 ml phosphate buffer for fractions 1 - 7, 1 M $(NH_4)_2SO_4$ in 70 ml phosphate buffer for fractions 8 - 14, 0.5 M $(NH_4)_2SO_4$ in 70 ml phosphate buffer fractions 15 - 21, and phosphate buffer alone for fractions 22 - 34 at a flow rate of 1ml/min. Enzyme and protein activities of the 35 fractions were determined.

2.6. Determination of Molecular Weight

Molecular weight of the purified enzyme was determined by polyacrylamide slab gel electrophoresis in the presence of dodecyl sulphate (SDS-PAGE) [19]. Protein markers used included α-lactoalbumin, trypsinogen, glyceraldehyde 3(p)

dehydrogenase and bovine serum albumin.

2.7. Characterization of the Enzyme

- **Effect of temperature and pH on enzyme activity and stability**

Effects of varying temperatures, 40˚C, 50˚C, 60˚C, 70˚C, 80˚C, 90˚C, 100˚C, on enzyme activity, with CMC as substrate was studied.

Enzyme activity was measured in pH range 3 - 9 using 0.2M citrate-phosphate buffer for pH 3 - 7 and 0.2 M tris buffer for pH 8 - 9 with 0.5% CMC as substrate.

For temperature and pH stability, enzyme was incubated at different temperatures and pH for 30 min and 3 h respectively before used in activity assay.

- **Effect of metal on enzyme activity**

The effects of different metals ($CoCl_2$, $Sr(NO_3)_2$, $MnSO_4$, $CaCl_2$, $FeSO_4$, $CuSO_4$, $BaCl_2$, $HgSO_4$, and $Pb(C_2H_3O_2)_2$) on enzyme activity were investigated. Purified enzyme (0.2 ml) in 0.2 ml of 0.2 M phosphate buffer (pH 7.0) with CMC as substrate was added to 0.2 ml of 5 mM of the metals and incubated at 30˚C for 30min. Residual activity was measured using DNS.

- **Relative rate of hydrolysis of various substrates**

The relative rate of hydrolysis against 1% (w/v) of each of the different β-glucan containing compounds avicel (AV), carboxymethyl cellulose (CMC), filter paper (FP), treated sawdust (TSD), untreated sawdust (USD) and sorghum-β-glucan (SβG))were examined. Substrate (0.2 ml) in 0.2 M phosphate buffer (pH 7.0) was added 0.2 ml of the enzyme and the mixture incubated at 40˚C for 30 min. Enzyme activity was determined using DNS.

- **Effect of substrate concentration on enzyme activity**

Various concentrations (0 - 1 mg/ml) of β-glucan containing compounds in 0.2M phosphate buffer were assayed for enzyme activity.

3. Results and Discussion

3.1. Screening for Carboxymethyl Cellulase

Screening for carboxymethyl cellulase (CMCase) production showed a clear zone around the *Bacillus sphaericus* CE-3 organism, indicating a positive result. The use of *Bacillus* sp for CMC production has been reported by various workers [9] [14] [20]

3.2. Enzyme Production by Submerged Fermentation/Time Course for Enzyme Production

In submerged fermentation with 1% CMC as the sole carbon source, maximum enzyme and protein activities were observed after 30h and at pH 7.0. The time course of enzyme production, pH and growth (**Figure 1**), indicates that maximum activity of CMCase and growth were obtained at 30 h and pH of 7.4.

3.3. Enzyme Concentration/Purification

The enzyme which was concentrated in 4M sucrose solution and further purified

by ion exchange and hydrophobic interaction chromatography showed that the Purification fold for the enzyme was 7.8, with 66.4 μ/mg specific activity protein and overall yield of 35.8 (Table 1).

The elution profile on ion exchange chromatography is shown on Figure 2. Tubes 6 - 12 showed high activity of the enzyme. Figure 3 shows the elution profile on hydrophobic interaction chromatography. Enzyme activity was highest in tube 30.

3.4. Determination of Molecular Weight by SDS-PAGE

The purified enzyme revealed two bands, showing the enzyme to be non-homogeneous, with estimated molecular weights of 22.3 KDa and 26.3 KDa (Figure 4). The result obtained is in line with the work of other researchers [11] [13] [14] [21], who reported molecular weight range of 23 - 65 kDa for cellulases produced by *Bacillus* sp. However, larger sizes (100 - 185 KDa) of CMCase enzyme have also been reported for other *Bacillus* strains [15] [22].

3.5. Characterization of the Enzyme

- **Effect of temperature and pH on enzyme activity and stability**

The effect of temperature on enzyme activity and stability are shown in Figure 5. The CMCase produced demonstrated optimum activity and stability at 40˚C, and maintained over 87% of its activity and stability at 100˚C, after 30min incubation. While Singh 2013 [9], recorded maximum activity at 40˚C for CMCase produced by *Bacillus sphaericus* JS1, which agrees with our work, other researchers recorded varied optimum temperatures for different species of *Bacil-*

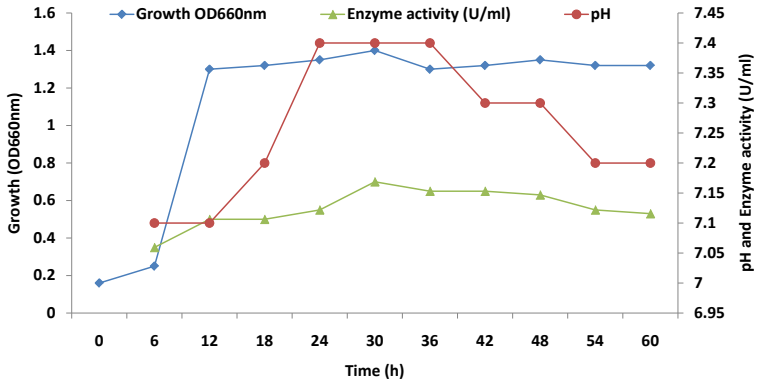

Figure 1. Time course for enzyme activity.

Table 1. Enzyme concentration and purification.

Purification step	Volume	Total activity (U/ml)	Total protein (mg/ml)	Specific gravity (μ/mg/protein)	Yield (%)	Purification
Supernatant	180	612	72	8.5	100	1
4M Sucrose	60	510	30	17	83	2
Q-Sepharose	45	460	10.8	42.5	75	5
Phenyl-Sepharose	15	219	3.3	66.4	35.8	7.8

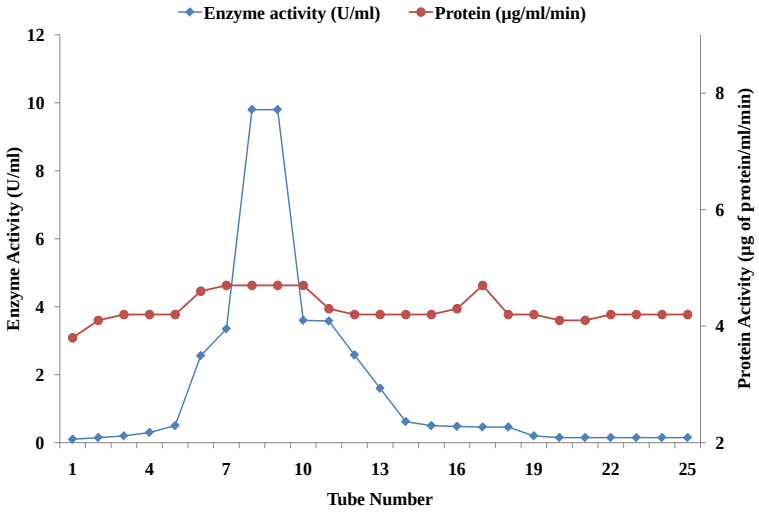

Figure 2. Elution profile of CMCase on Q-sepharose

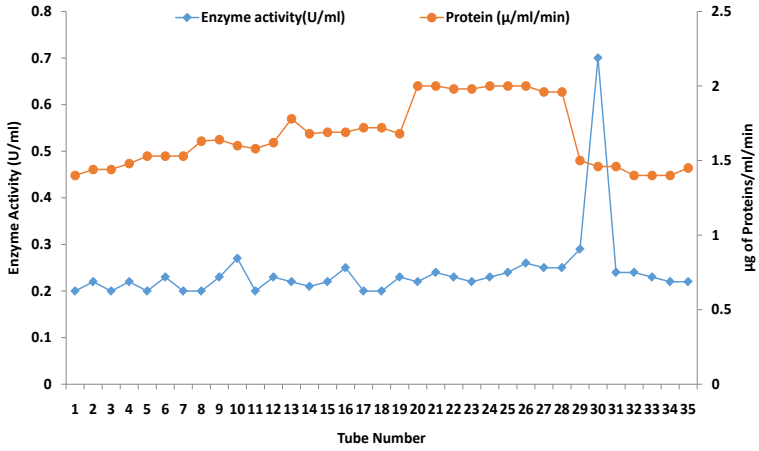

Figure 3. Elution profile on CMCase on phenyl-sepharose CL-4B.

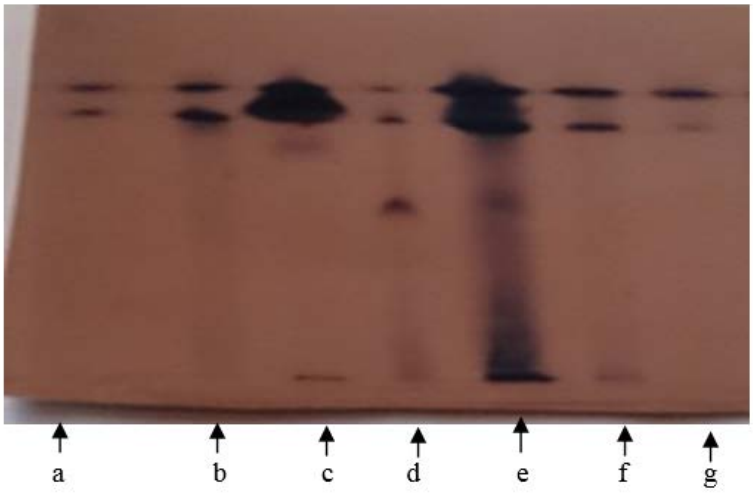

Figure 4. SDS-Polyacrylamide Gel Electrophoresis of CMCase. **a**—purified enzyme sample, **b**—α-lactoalbumin (14.2), **c**—trypsinogen (24), **d**—bovine serum albumin (66), **e**—combined markers, **f**—glyceraldehyde-3(P)-dehydrogenase (36), g—purified enzyme.

lus. Singh and Kumar, 1998 [23] recorded 37˚C for *B. brevis* VS1, Yan *et al.*, 2011 [24] recorded 55˚C for *B. cereus*, and 60˚C was recorded by Araffin *et al.*, 2016 [14] and Aftab *et al.* 2012 [25] for *B. pumilus* and *B. licheniformis* respectively.

The effect of pH on enzyme activity (**Figure 6**), showed very good activity at pH range 6 - 9 with optimal activity and stability at pH 9. The enzyme maintained over 90% stability at pH 8 - 9 indicating that the enzyme is likely an alkaline cellulase. The optimum activity and stability of this enzyme in alkaline pH is in agreement with the work of Singh 2013 [9], Yan *et al.*, 2011 [24] and Aftab *et al.*, 2012 [25], who recorded pH ranges of 6 - 9 for various *Bacillus* sp. However, this is contrary to the work of Li-Jung *et al.* 2010 [2], who recorded a pH of 5 for a *Bacillus subtilis* YJ1 CMCase. This interesting property of the enzyme makes it suitable for use as an effective laundry detergent additive.

- **Effect of metal on enzyme activity**

$MgSO_4$ was the only metal that enhanced the activity of CMCase (**Figure 7**) whereas other metals, $BaCl_2$, $CoCl_2$, $Pb(C_2H_3O_2)_2$, $HgSO_4$, $CuSO_4$, $Sr(NO_3)_2$, Fe

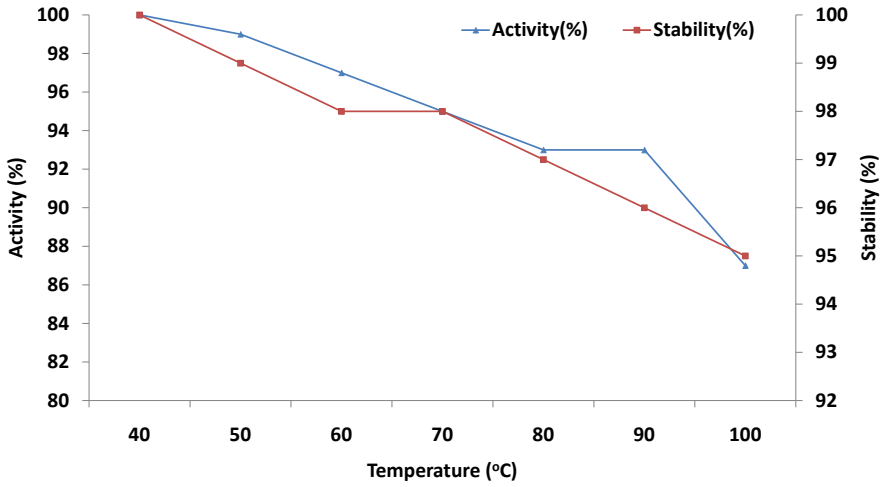

Figure 5. Effect of temperature on activity and stability of enzyme.

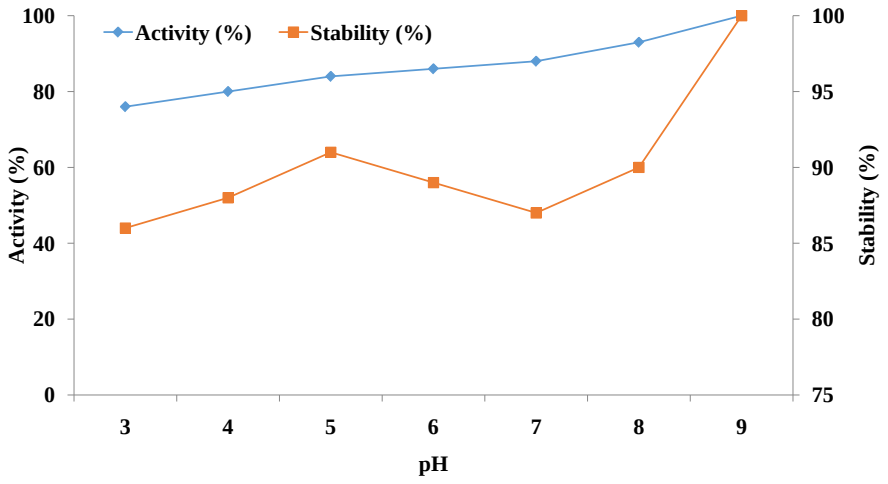

Figure 6. Effect of pH on activity and stability of enzyme.

SO_4, $CaCl_2$, $ZnSO_4$, inhibited its activity. The stimulatory effect of $MgSO_4$ on the activity of CMCase is contrary to the reports of Yan *et al.* 2011 [24] and Chan and Au 1987 [26], who observed a significant loss of CMCase activity with Mg^{2+}. The inhibitory effects of other metals observed in this study is contrary to the work of Padilha *et al.* 2015 [21], who recorded an increase in activity with Co^{2+}, Ca^{2+} and Fe^{2+}. As suggested by Uzii and Sasaki 1987 [27] and Kyami-Horani 1996 [28], the stimulatory effect of Ca^{+} may have been as a result of the fact that microbial extracellular enzymes require Ca^{2+} for their activity and stabilization.

- **Relative rate of hydrolysis of various substrates**

As shown in Figure 8, untreated sawdust was maximally hydrolyzed (100%) followed by Carboxymethyl cellulose (82%) while treated sawdust was the least hydrolyzed (46.6%). The low hydrolysis of treated sawdust may have resulted from the effect of NaOH used in treating the sawdust. Li-Jung *et al.* 2010 [2], recorded no activity against avicel and highest activity against cellulose, which is contrary to our findings.

- **Effect of substrate concentration on enzyme activity**

The effect of different concentrations (0.2 mg - 1 mg/ml substrate) of substrates on enzyme activity is shown in Figure 9. The result shows that the activity of the enzyme increased as the concentration of the substrate increases.

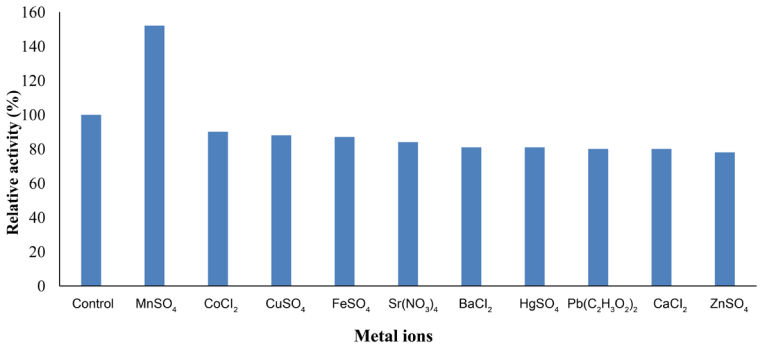

Figure 7. Effect of metal ions on enzyme activity.

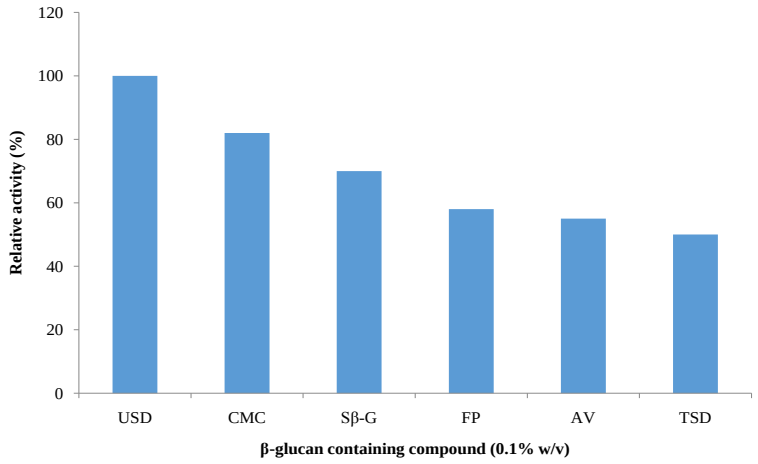

Figure 8. Relative rate of hydrolysis of various β-glucan containing compounds.

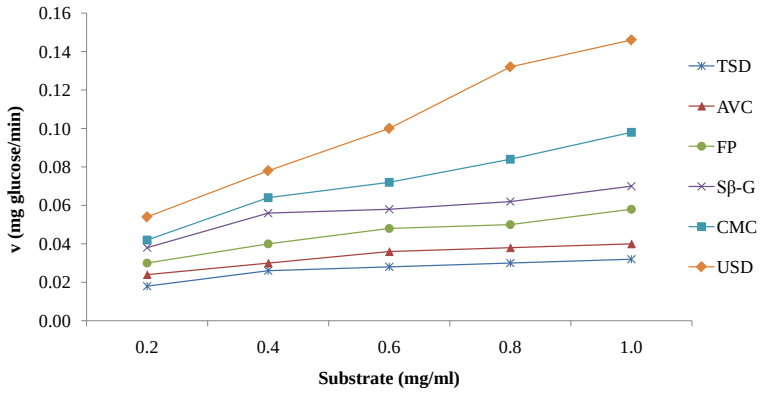

Figure 9. Effect of substarte concentration on enzyme.

4. Conclusion

Bacillus sphaericus CE-3 which was isolated from refuse dump site in Nnamdi Azikiwe University, Awka, Nigeria showed high cellulolytic activity. The enzyme was optimally active at 40˚C and pH 9 and retained over 85% of its activity and stability at 100˚C and pH 6 - 9. This shows the enzyme to be thermo-stable and alkalophilic in nature. The cellulase of *B. sphaericus* CE-3 as obtained in this result could utilize sawdust and other β-glucan containing compounds that are natural wastes and easily available as substrate. These characteristics of this enzyme show that it would be of great use in many industries as the process parameters of the enzyme can easily be manipulated when in use. The by-products of the substrate breakdown could also serve as substitute for renewable energy source.

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Authors Contribution

Odibo, F. J. C. designed the experiment and contributed analysis materials, Ekwealor, C. C. performed the experiment and analyzed the data, and Onwosi, C. O. contributed literature materials.

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