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PRODUCTION AND PARTIAL PURIFICATION OF EXTRACELLULAR CELLULASE BY *BACILLUS CEREUS* ISOLATED FROM AGRICULTURAL WASTE IN ADO-EKITI NIGERIA

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ABSTRACT

Agricultural waste, a widely abundant, virtually free, renewable and important resource serves as major cause of environmental pollution, which can be ameliorated with application of cellulase produced primarily by fungi, bacteria and protozoans that hydrolyzes cellulose. The present study was undertaken to partially purify and optimize the activity of extracellular cellulase produced by *Bacillus cereus* sp.10 isolated from agricultural waste. A total of 20 cellulase-producing bacteria belonging to the genera; *Bacillus*, *Pseudomonas* and *Klebsiella,* were recovered from agricultural waste (sawdust, rice husk, plant waste (grass), palm kernel) collected from three towns (Ifaki-Ekiti, Igbemo-Ekiti and Aare-Afao, Ekiti State, using standard techniques. Among these isolates, *Bacillus cereus* sp.10, *Pseudomonas aeruginosa* sp.2, *Klebsiella pneumonia* sp.1, exhibited highest cellulase activities of 73.29 µM/min, 61.66 µM/min and 57.43 µM/min respectively. Characterization studies showed that partially purified cellulase produced by *Bacillus cereus* sp.10 is optimally active at a temperature and pH of 60^{o}C and 5.0 respectively. The cellulase was stable at 70^{o}C for 120 min retaining 80% of its activity. While the activity was enhanced by K^{+}, it was inhibited by Hg^{+}, Zn^{2+} and Mn^{2+}, inferring the enzyme to belong to the class of metallocellulase. *Bacillus cereus* sp.10 among other cellulase producing bacteria isolated from agricultural waste has proven to be a very consistent and potent cellulolytic bacterium.

INTRODUCTION

Agricultural wastes (either plant-derived or animal derived) is no doubt, most abundant, virtually free and relatively cheap among different by-products of human activities, though serve as major environmental pollutants but also serve as important resources (Ijaz *et al*., 2011; Sadhu and Maiti, 2013; Abu-Gharbia *et al*., 2018; Obi *et al,* 2016; Sadhu and Maiti, 2013; Abu-Gharbia *et al*., 2018). In developing countries such as Nigeria, large portion of these wastes are underutilized as they are abandoned on the farmland to be decomposed by microorganisms or are burnt (serving as source of environmental pollution), except for little that find use in roofing and as soil conditioner to fertilize the soil (Hanapi *et al*., 2013). An interesting point is that, these wastes are enriched with lignocellulosic materials, which is the most abundant renewable organic substrates for biotechnological products of economic importance (Mmango-Kaseke *et al*., 2016). It is therefore crucial to explore this advantage not only to ameliorate the effect of the environmental pollution generated but also to convert these agricultural wastes effectively and economically into valuable products of industrial potential such as; methanol, ethanol, biodiesel, biogas, industrial enzymes (Wang *et al*., 2016). However, the role of lignocellulolytic enzymes (including lignase, hemicellulase and cellulose) in the biotechnological conversion of these wastes cannot be underestimated as it has taken upper edge in numerous industrial applications in brewing and wine, chemicals, food, fuel, animal feeds, pulp and paper, textile and laundry, and agriculture (Howard *et al*., 2004; Obeng *et al.,* 2017).

Cellulase is a complex enzyme system (comprising of exoglucanase, endoglucanase and β-glucanase) that plays an important role in the environment with potential of converting cellulose (major component of agricultural waste) into simpler glucose monomer i.e. useful products (Sadhu and Maiti, 2013). It has also taken a major application in some part of textile industry for "bio-cleaning" of fabrics and making stone washed look of denims, as well as in the household cleansers for enhancing fabric softness and brightness, cotton arrangements, wool, and coloring treatments, effluent treatment, and pharmaceutical purposes (Howard *et al*., 2003; Mmango-Kaseke *et al*., 2016).

Considering the revealing importance of this cellulolytic enzyme in the biotechnological conversion of lignocellulosic biomass (such as agricultural waste), the search for potential sources of the enzymes (cellulase) has continually attracted the interest of numerous researches in the recent time (Ijaz *et al*., 2011; Verma *et al*., 2012; Jadhav *et al*., 2013; Patagundi *et al*., 2014; Mmango-Kaseke *et al*., 2016; Sirohi *et al*., 2018; Isikgor and Becer 2015; Chukwuma et *al*., 2020; Jadhav *et al*., 2013; Patagundi *et al*., 2014; Mmango-Kaseke *et al*., 2016; Sirohi *et al*., 2018). Decayed plants materials, hot springs, faeces of ruminants and composts (Doi, 2008, Hajiabadi *et al*., 2019), agricultural wastes (Jadhav *et al*., 2013) and soil (Patagundi *et al*., 2014) have been reported among the sources of large number of microorganisms capable of producing cellulase.

It is noteworthy that determining various environmental factors that affect the metabolic activities of the cellulolytic bacteria (such as; pH, temperature, carbon source) will also resolve proper application of the enzymes they produce. Effort was therefore made in the present study to partially purify and optimize the activity of extracellular cellulase produced by *Bacillus cereus* sp.10 isolated from agricultural waste.

MATERIALS AND METHODS

Source of plant wastes and sample collection

The plant wastes used in this study included rice husk, palm kernel shaft and saw dust. Rice husk was obtained from a local rice factory at Igbemo-Ekiti, Palm kernel shaft was obtained from Aare-Afao palm kernel factory at Afao-Ekiti and the saw dust was collected from a wood processing factory at Ifaki-Ekiti, Ekiti State, Nigeria.

All samples were obtained from the surface of respective agricultural waste with the aid of pre-sterilized metal hand trowel, into a sterile sampling bottle and transferred in ice-bag to the laboratory within a period of 2hrs for analysis

Isolation of cellulolytic bacteria from agricultural waste samples

Isolation of bacteria

The Bacterial isolates were isolated from each sample following serial dilution techniques using Nutrient Agar (NA). A 10g amount of each sample was homogenized in 90ml of sterile physiological saline, mixed well for 15mins and vortexed. Ten-fold serial dilution of the sample homogenate was made and 1 ml aliquot of dilutions 10^3, 10^4 and 10^5 were plated in duplicates on Nutrient agar (Lab M, UK), using pour plate method and incubated at 37°C for 24hrs (Olutiola *et al*., 2000; Odeyemi *et al.,* 2019). Colonies observed on the plates after incubation period were purified by sub culturing on nutrient agar plates to obtain pure culture of the organisms and subsequently transferred into a nutrient agar slants, which were kept in the refrigerator at 4^oC as stock culture.

Identification of bacterial isolates

Biochemical characterization

The bacterial isolates were tentatively identified by cultural, morphological and biochemical characteristics including microscopic examination, catalase test and oxidase test among others (Olutiola *et al*., 2000; Odeyemi *et al*., 2019; Saha and Santra, 2014). The results were interpreted in accordance with Bergey's Manual of Determinative Bacteriology Edition 8.0 (Holt *et al*., 1994).

Molecular Characterization

Genomic DNA of the isolates was extracted using Bacterial Genomic DNA Isolation Kit (Jena Bioscience, Germany) following manufacturer's instructions. Subsequently, the 16S rRNA gene (~ 1500 bp) was amplified from the extracted genomic DNA by polymerase chain reaction (PCR) using universal bacterial primers; 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1390R (5'-ACGGGCGGTGTGTRCAA-3') (Mao *et al*., 2012). The PCR reaction solution (30µL) consisted of 6µL of RedLoad (Jena Bioscience, Germany) PCR mix, 0.3µL of each primer, 18.4µL of PCR grade water and 5µL of DNA template. PCR was done with GeneAmp 9700 (Applied Biosystems, USA) thermal cycler as follows; 94°C for 3min, 30 cycles of 94°C for 30s, 54°C for 30s, 60°C for 90seconds and a final 7min extension at 72°C, after which product was held at 4^{o}C till terminated. The PCR products were subsequently resolved on a 1% gel stained with ethidium bromide and viewed using a UV transilluminator (Fotodyne Incorporated, USA). Commercial facility (Macrogen Inc., South-Korea) was patronized for purification and Sanger sequencing of the amplicon.

To identify the bacterial isolates, the sequences were compared (using the BLASTn programme) to other publicly available nucleotide sequences in GenBank.

Screening for cellulase-producing bacteria

The agar diffusion method was employed to screen for cellulolytic abilities of the bacterial isolates. Pure cultures of bacterial isolates were individually transferred to Carboxymethylcellulose CMC agar plates. After incubation for 48 hours, CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counterstaining the plates (Teather and Wood, 1982). Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having the largest clear zone were selected for cellulase production.

Cellulase production by cellulase-producing bacteria

Cellulase production was carried out by submerged fermentation procedures in accordance with the method of Sharma and Bajaj (2005) with slight modification, using basal salt medium containing 1% (w/v) CMC as carbon sources. The bacterial isolate was inoculated into the basal salt medium supplemented with CMC and incubated for 48h. Two millilitres of the pre-inoculated culture was inoculated into 100mL of Mandels Salt Medium [Urea 0.3 g L^{-1}, $(NH4)_2SO_4$ 1.4 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, $CaCl_2$ 0.3 g L^{-1}, $MgSO_4$ 0.3 g L^{-1}, yeast extract 0.25g L^{-1} and proteose peptone 0.75 g L^{-1} with 10 g L^{-1} of Carboxymethylcellulose (CMC) in 0.05M citrate buffer (pH 6.0)] as production medium contained in Erlenmeyer flasks (250mL) (Mandels, 1975) and incubated on a rotary shaker 200 rpm at 45^{o}C. The enzyme activity was assayed periodically at different time intervals. Two milliliters of the fermented broth was centrifuged at 10,000× g for 5 min at 4^{o}C to obtain the supernatant and used as the crudr extract for cellulase assay after appropriate dilutions (Mmango-Kaseke *et al*., 2016).

Partial purification of the crude enzyme with ammonium sulphate

Solid ammonium sulphate [$(NH_4)_2SO_4$] was added to the crude enzyme to obtain 20% saturation. The mixture was centrifuged at 10,000 x g, 4^{o}C; for 10 minutes. The precipitate was discarded, and ammonium sulphate was again added to the supernatant to 80% saturation. The mixture was further centrifuged at 10,000 x g for 10minutes at 4^{o}C and the precipitate dissolved in 0.05 M phosphate buffer, pH 7.0 at 10% (w/v). The partially purified enzyme was assayed for cellulase activities following the methods described previously (Sharma and Bajaj, 2005; Sharma and Bajaj, 2017)

Effect of temperature on cellulase activity:

The effect of incubation temperature on cellulase production was assessed at different temperatures, ranging from 20–80^{o}C in accordance to the method of Adhyaru *et al.* (2014). The crude enzyme (0.5 ml) was added to a test tube containing 0.5 ml of carboxyl methyl cellulose (0.2% w/v) in 0.05 M citrate buffer, pH 6.0. The mixture was incubated in a water bath at temperatures of 20, 30, 40, 50, 60, 70 and 80^{o}C for 1 hour.

Effect of pH on cellulase activity:

The effect of initial pH of the growth medium on cellulase activity of the crude enzyme was determined at varying pH values ranging from 3.0 to 9.0. The enzymes were assayed in 0.05 M citrate buffer (pH 3.0 to 6.0) and in 0.05M phosphate buffer (pH 6.5 to 9.0) prior to autoclaving (Ray *et al*., 2007; Adhyaru *et al.*, 2014). All the tubes were incubated at 50^{o}C.

Statistical analysis

All the data were subjected to analysis of variance and the sample means tested for significant differences using the Duncan Multiple Range Test. This was carried out with the statistical package, SPSS 22.0. Graphs were plotted with Microsoft Excel.

RESULTS

Identification of bacteria isolated from the agricultural waste

A total of 20 bacterial isolates were recovered from different samples of the agricultural waste. Based on the cultural characteristics, microscopic examination and biochemical reactions, three genera of bacteria encountered in the agricultural waste and were identified as *Bacillus*, *Pseudomonas* and *Klebsiella*. Table 1 showed the incidences of the isolates. The result showed that *Bacillus* spp. had the highest incidence of 55% followed by *Pseudomonas* spp. with an incidence of 25%, while *Klebsiella* spp. had the least incidence of 20%.

Cellulase Production by bacteria isolated from agricultural waste

All the 20 bacteria isolated from the agricultural waste were subjected to screening for cellulase production after 48hours and the result is depicted in Table 2. The activities of enzymes cellulase produced by each of the bacterial isolates vary, ranging between 10.92mM/min/ml and 73.29µM/min/ml. The least cellulolytic activity of 10.92µM/min/ml was demonstrated by *Klebsiella aerogens*, while *Bacillus cereus* 10 demonstrated the highest cellulolytic activity of 73.29µM/min/ml.

The nomenclature based on molecular characterization of five (5) bacterial isolates that showed higher cellulolytic activities from the result in Table 2 is presented in Table 3.

Figure 1 represents the time course of cellulase production by the five selected isolates at 6 hour interval. All the isolates demonstrated the peak of their cellulolytic activity at 18 hours of incubation after which there was decline and continued until 36 hours of incubation. The production by *B. cereus* 10 was at its peak at 18 hours with 78.75µM/min/ml and slowed down to 60.78µM/min/ml at 36 hour of incubation. The results however showed that *Bacillus cereus* 10 demonstrated highest and more consistent cellulolytic activity.

The production of cellulase in relation to the growth rate of *B. cereus* 10 was separately enumerated and protein in the basal media was also assayed. The result presented in Figure 2 shows that *B. cereus* 10 showed a consistent increasing growth rate between 18 hour and 24 hour of incubation and the cellulolytic activity of this isolate was at its peak at 24 hour of incubation. The protein assay showed that was increase in the protein in the basal media, following a trend similar to that of the enzymatic activity of the isolate, taking its peak (20.50mg/mL) also at 24 hour of the incubation.

Purification of *Bacillus cereus* 10 extracellular cellulase

The elution profile of extracellular cellulose produced by *Bacillus cereus* 10 using ion exchange (DEAE-Sephadex) chromatograph is depicted in Figure 4. The result showed that when cellulase was adsorbed onto a DEAE-Sephadex A-50 chromatography, the cellulolytic activity was recovered as two sharp peaks. The sephadex G-150 gel filtration chromatography analysis of the molecular mass of the purified enzyme, presented in Figure 4, shows the greater proportion of cellulolyitc activity eluting at 40.17mM/min/ml.

The summary of the purification process presented in Table 3 shows that after ammonium sulfate precipitation, a yield of 18.41% was obtained with a purification fold of 0.95. Ion exchange chromatography resulted in 20.88% yield with 3.81 purification fold while gel filtration gave a yield of 9.98% with 6.37 purification fold. The specific activity of the cellulase after ion exchange was 10.21µ/mg protein, while gel filtration gave a partially purified cellulase of 17.09µ/mg protein.

Effect of temperature on *Bacillus cereus* 10 cellulase activity

The effect of temperature on the activity of the extracellular enzymes produced by *Bacillus cereus* 10 is depicted in Figure 5. The optimum cellulase activity of 100% was obtained at temperature 60^{o}C, as reduction in cellulase activity was observed at temperatures below and above 60^{o}C.

The thermostability of the activity of the extracellular cellulase produced by *Bacillus cereus* 10 is also presented in Figure 6. The cellulase activity was stable at 95% at 30^{o}C in 120 min. Also in 120 minutes at 40^{o}C, cellulase retains 90% of its activity. Cellulase retains only 86% of its activity at 50^{o}C in 120 minutes. At 60^{o}C in 120 minutes, cellulase retains about 75% of its activity.

Effect of pH on *Bacillus cereus* 10 cellulase activity

The effect of pH on the activity of the extracellular enzymes produced by *B. cereus* 10 is depicted in Figure 7. The optimum cellulase activity of 82% was obtained at pH 5, as there was reduction in cellulase activity at pH below and above 5.

The pH stability of the extracellular cellulase produced by *B. cereus* 10 is also presented in Figure 8. At pH 5, cellulase retains 83% of its activity after 120 minutes of incubation. Meanwhile, at pH 6, cellulase retains 62% of its activity after incubation for 120 minutes.

Effect of metal ion on *Bacillus cereus* 10 cellulase activity

The effect of metal ions on the extracellular enzyme, cellulase produced by the isolated *Bacillus cereus* 10 was analysed and the result was depicted in Figure 9. The result shows that Iron (Fe), Mercury (Hg), Zinc (Zn) and Manganese (Mn) inhibited the activity of cellulase, while Calcium (Ca) and Lead (Pb) moderately enhanced the activity of cellulase. The activity of *Bacillus cereus* 10 extracellular cellulase was however enhanced by potassium (K).

DISCUSSIONS

Cellulose is among the most abundant plant biomass on earth with an estimated annual production of 4.0×10^7 units (Bakare *et al.*, 200; Aruwajoye *et al.,* 2014), leaving very much others lying down as waste (Tallapragada and Mushimiyimana, 2015). This abundance confers an immense potential as a renewable source of energy to cellulose. In order to convert cellulose into soluble sugars for fermentation, various bioconversion methods such as pyrolysis, acid hydrolysis and enzymatic hydrolysis can be applied (Cooney *et al.*, 1978; Dimos *et al.,* 2019).The latter is more environmental friendly, and it gives a pure product with consumption of less energy (Bakare *et al*., 2005; Aruwajoye *et al., 2014*). Meanwhile, bacteria and fungi are among the leading group of organisms that have been noted for being capable of hydrolysing cellulose to the monomer units, which are highly ubiquitous, easily isolated from the environment. Cellulolytic microorganisms such as fungi and bacteria are however responsible for most of the cellulose degradation in soils, though some insects, crayfish and mollusks produce their own cellulases that are capable of hydolysing cellulose (Ohkuma, 2003). Despite this vast number of cellulase producers, there is a deficiency of microorganisms that can produce significant amount of the cellulase to efficiently degrade cellulose to fermentable products (Maki *et al*., 2011). This has prompted the search for cellulolytic bacteria in the agricultural wastes obtained from different local factories in Ekiti State.

In addition to this, majority of the studies have been focused on fungi with less emphasis on bacterial sources for cellulase production. Meanwhile, Due to their extremely high natural diversity, bacteria have the capability to produce stable enzymes that can be applied in industries (Bhat, 2000; Camassola *et al*., 2004; Haakana et *al*., 2004; Datta *et al.,* 2013). These two factors were the prime motivation in this study; to isolate and characterize cellulolytic bacteria from different agricultural wastes from local factories around Ekiti State.

Isolation and characterization of cellulolytic bacteria

A total of twenty (20) bacterial isolates belonging to three distinct genera; *Bacillus*, *Pseudomonas* and *Klebsiella*, were recovered from different agricultural wastes obtained from local factories around Ekiti State. As expected, attempts to test for their cellulase production showed that all the isolates are capable of producing the enzymes cellulase in varying degree potency with a short period of incubation. The result further supported the hypothesis that microorganisms for enzyme production be assayed in the environment where the substrates are available as suggested by Fossi *et al.* (2005). Several environments have been proven to serve as a novel sources for the isolation of different cellulase producing microorganism including agricultural field, product and waste (Verma *et al*., 2012; Igbinadolor and Onilude, 2013; Singh and Nain, 2014); soil sample (Kumara *et al*., 2012); Coir waste and sawdust (Immanuel *et al*., 2007); paper mill sludges and organic fertilizers (Maki *et al*., 2011); agro waste (Tallapragada and Mushimiyimana, 2015) and several others. The abundance of cellulase producing bacterial isolates experienced in the present study is in support of different research work that has previously established that agricultural wastes harbour several cellulolytic bacteria (Verma *et al*., 2012; Singh and Nain, 2014).

Five among all the isolates were selected as the most proficient cellulase producing bacteria from the agricultural waste and were screened again for their cellulase productivity (Fig 3) within 36 hours of incubation, which was monitored after every 6 hours. All the isolates showed increased and consistent, though relatively varied, cellulase productivity between 12 to 24 hours of incubation. This happens to fall within the log phase of bacterial growth and is possibly responsible for the increase in their productivity. The phenomenon of cellulase synthesis occurring towards the end of the exponential growth phase is a common feature of many bacteria that produce enzymes and is in support of the report of Femi-Ola *et al*. (2014) that also related optimum protease production by *Serratia marcescens* observed between 12 to 18 hours of incubation. Incubation time as affirmed by Tallapragada and Mushimiyimana (2015), affects the enzyme production. Sonjoy *et al*. (1995) reported that, short incubation period of enzyme production offers the potential for inexpensive production and it varies from enzyme to enzyme from single substrate.

Bacillus cereus 10 among other isolates showed significantly high cellulolytic activity of 78.75mM/min/ml at 18 hour of incubation implicating it as a very potent cellulase producer. This goes in line with the report of Schwarz (2003), which presented a review of the known cellulolytic bacteria as species of the genera; *Caldocellulosiruptor*, *Anaerocellum*, *Butyrivibrio*, *Ruminococcus*, *Eubacterium*, *Clostridium*, *Bacteroides*, *Acetivibrio*, *Thermoactinomyces*, *Caldibacillus*, *Bacillus*, *Acidothermus*, *Cellulomonas*, *Micromonospora*, *Actinoplanes*, *Streptomyces*, *Thermobifida*, *Thermomonospora*, *Microbispora*, *Fibrobacter*, *Sporocytophaga*, *Cytophaga*, *Flavobacterium*, *Achromobacter*, *Xanthomonas*, *Cellvibrio*, *Pseudomonas* and *Myxobacter*.

Enzyme cellulase production by bacterial isolates

The enzyme cellulases which are responsible for the hydrolysis of cellulose are composed of a complex mixture of enzyme proteins with different specificities to hydrolyse glycosidic bonds. Cellulases can be divided into three major enzyme activity classes (Goyal *et al.*, 1991; Rabinovich *et al.*, 2002, HU *et al.*, 2013; Collins *et al.*, 2005). These are endoglucanses or endo-1, 4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Howard *et al*., 2003). This enzyme has taken numerous applications and biotechnological potential in various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Beauchemin *et al*., 2003; Kumar *et al.,* 2019)

The selected isolates were cultured in a medium containing 1% CMC; a carbon source to induce the production of cellulases which are inducible enzymes synthesized during the bacteria's growth on cellulosic materials (Kubicek, 1993). The supernatant was found to bear cellulolytic enzymes, supporting other previous studies that indicated the ability by known members of the Bacillus genus to

secrete extracellular proteins (Lin *et al*., 2012).

Determination of optimum temperature and pH for enzyme activity

Enzyme activity assays to determine the optimum pH were carried out in reaction mixtures at varying pH values (3-11) at the predetermined temperature (60°C) using water bath. Each enzyme has its own optimum pH and if the pH increases or decreases beyond the optimum, the ionization groups at the active site may change slowing or preventing the formation of an enzyme substrate complex (Eijsink *et al*., 2005). Optimum pH values of 4.5-8.0 have been reported for different microbial cellulase (Bakare *et al*., 2005; Immanuel *et al*., 2007; Dutta *et al*., 2008). For this isolate (*Bacillus cereus*), there was a significant change in enzyme activity with change in pH. The highest activity was recorded at pH 5 suggesting that the enzyme is an acid cellulase. Acid cellulases act at a pH range of 3.8 and 5.8 (Mosjov, 2012). Similar observations have been reported by Bajaj *et al.*, (2009). There was no significant difference in enzyme activity at pH 6, 7 and pH 3, 11 ($P>0.05$). This finding suggests that these pH pairs have more or less the same effect on enzyme activity.

Cellulase is one of the most important industrial enzymes; it is used in paper, textile, pharmaceutical, food industries, brewing and chemical industries. To reduce cost of production, cellulase was characterized. The characterization protocol involves; determination of effects of temperature and its stability then effects of pH and its stability. Cellulase activity at different pH and temperature were determined as seen in present study above. In the present study maximum enzyme activity observed around pH 5 and the optimum temperature was found to be around 60^0C. According to Tariq *et al.* (2011), optimal pH for CMC from *Aspergillus niger* was found to be 6.0 to 7.0. Mosjov (2012) reported that the production was high at pH 4 and 4.5 by *Aspergillus niger*. Coral *et al*. (2002) reported maximum CMCase activity at pH 4.5 and pH 7.5 by *Aspergillus niger* (Z10, wild type strain) among the tested pH range between 4.0 and 9.0. In this study, there was maximum activity for the effect of temperature was found around 60^0C which show 100% activity as seen in Fig 7 above. and the optimum pH was found around pH 5.0 showing 100% activity.

The structural complexity of pure cellulose and difficulty of working with insoluble substrates has led to the wide use of CMC for endoglucanases studies (Lynd *et al*., 2002; Behera et *al*., 2017). CMC is a soluble cellulose derivative with a high degree of polymerization (DP). The isolate was found to bear CMC activity. This was in line with other studies that have reported *Bacillus licheniformis* strains degrading amorphous substrates such as CMC amongst others (Bischoff *et al*., 2006; Parado and Matulewicz, 2014). DNSA is reduced to 3-amino-5-dinitrosalicylic acid an aromatic compound that absorbs light strongly at 540nm (Miller, 1959). The disadvantage of using this method is loss of some reducing sugars during the analysis but despite that it's a more convenient test compared to other sugar tests.

Determination of the effect of metal ion cellulase activity

The result of the effect of metal ions on the extracellular enzyme, cellulase produced by the isolated *B. cereus* 10 showed that Iron (Fe), Mercury (Hg), Zinc (Zn) and Manganese (Mn) inhibit/impede the activity of cellulase while Calcium (Ca) and Lead (Pb) moderately enhance the activity of cellulase. The activity of Bacillus *cereus* 10 extracellular cellulase was however enhanced by potassium (K). This is an indication that the enzyme cellulase produced by *Bacillus cereus* 10 in this studycould be classified as a metallocellulase, following the report of Aiyappa and Haris (1976) and Tariq *et al*. (2011) which classified protease according to the effect of metal on its activity.

Table 1: Incidence of bacterial isolates in agricultural waste

Bacterial isolate	Number	Incidence (%)
Bacillus spp.	11	55
Pseudomonas aeruginosa	5	25
Klebsiella spp.	4	20
Total	**20**	

Table 2: Cellulase production by isolates from agricultural waste

S/N	Source of waste	*Bacterial isolate	Cellulase activity (μM/min/ml)
1	Grass	*Pseudomonas aeruginosa* sp. 3	3.52
2		*Klebsiella* sp.3	11.27
3		*Bacillus* sp.4	20.08
4		*Bacillus* sp.6	20.43
5		*Pseudomonas aeruginosa* sp.1	23.60
6		*Bacillus* sp.2	28.18
7		*Bacillus* sp.1	35.75
8	Palm kernel	*Pseudomonas aeruginosa* sp.5	19.23
9		*Pseudomonas aeruginosa* sp.4	20.02
10		*Bacillus* sp.9	22.55
11		*Bacillus* sp.10	73.29
12	Rice bran	*Bacillus* sp.3	25.01
13		*Bacillus* sp.5	27.48
14		*Klebsiella* sp.4	57.43
15		*Pseudomonas aeruginosa* sp.2	61.66

16		*Klebsiella* sp.2	10.92
17		*Bacillus* sp.8	14.44
18	Saw dust	*Bacillus* sp.7	15.85
19		*Bacillus* sp.11	24.54
20		*Klebsiella* sp.4	26.07

*Identification of isolates as described by Olutiola *et al*. (2000) and Saha and Santra (2014)

Table 3: **Identification of cellulose producing bacteria from agricultural waste based on 16S rRNA gene amplification**

	Closest type strain in NCBI data base			
Isolate	**Strain name**	**Accession Number**	**16S rDNA identity score (%)**	**[1]Isolate Identity**
Bacillus sp.10	*Bacillus cereus strain* NRC-1	KX301062.1	94	*Bacillus cereus* sp.10
Pseudomonas aeruginosa sp.2	*Pseudomonas aeruginosa* DHS01	CP013993.1	95	*Pseudomonas aeruginosa* sp.2
Klebsiella sp.1	*Klebsiella pneumonia* KP5-1	CP008700.1	88	*Klebsiella pneumonia* sp.1
Bacillus sp.1	*Bacillus cereus* ATCC 14579	NR_074540.1	89	*Bacillus cereus* sp.1
Bacillus sp.2	*Macrococcus carouselicus* strain H8B16	NR_044927.1	85	*Macrococcus carouselicus* sp.2

[1]Identification based on molecular techniques (Mao *et al*., 2012)

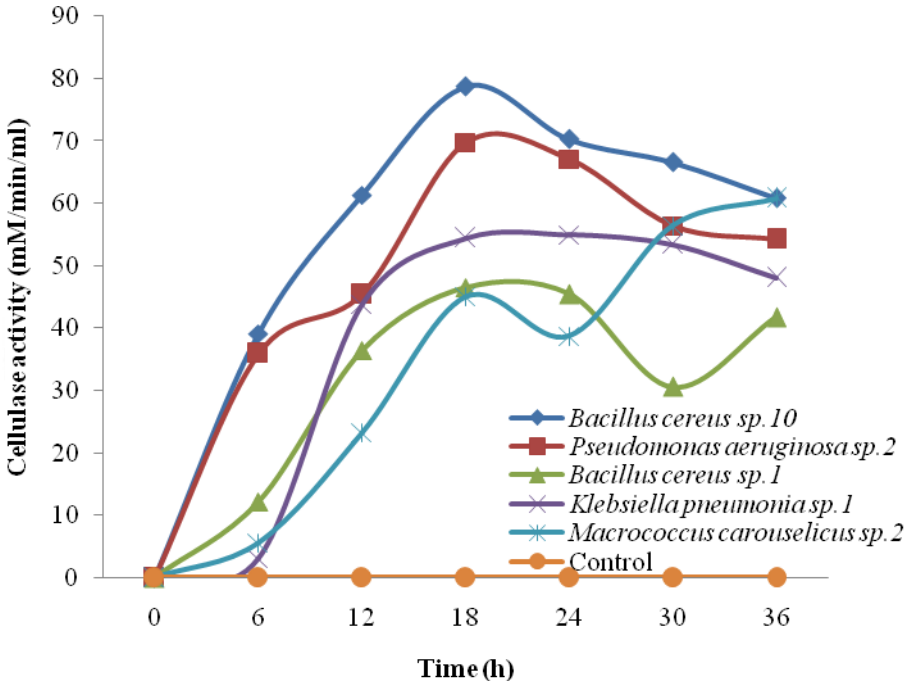

Figure 1: Time-course of cellulase production by five selected bacterial isolates

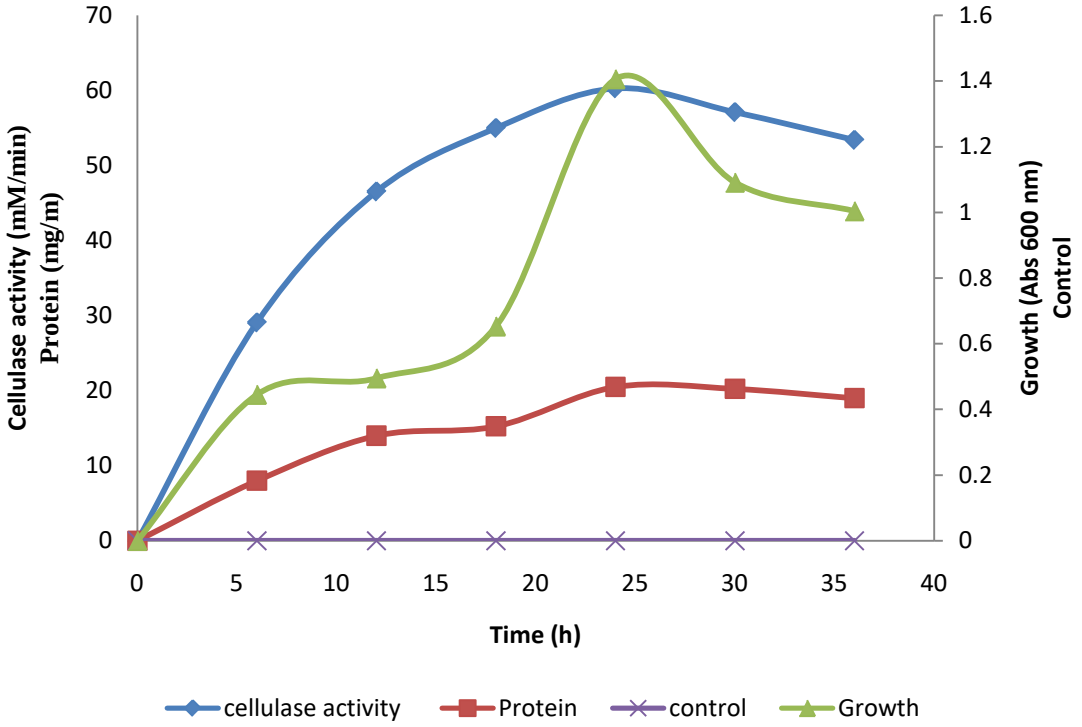

Figure 2: Growth and cellulase activity profile of *Bacillus cereus* sp.10

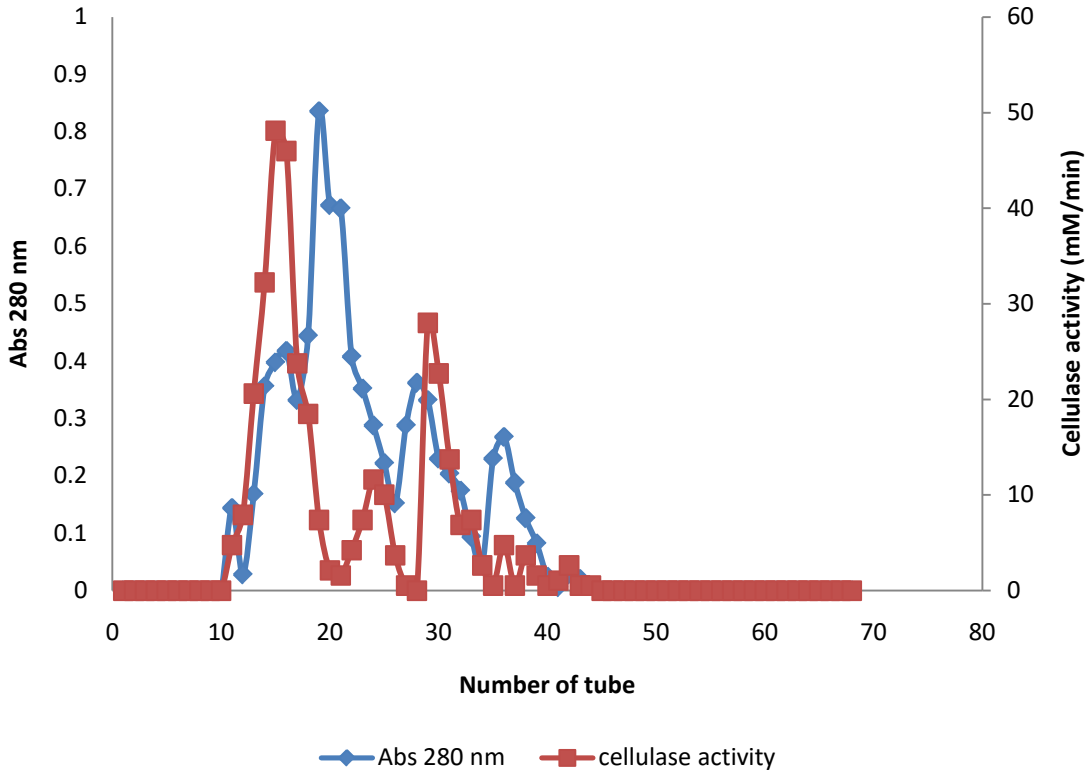

Figure 3: Elution profile of extracellular cellulose produced by *Bacillus cereus* 10 using ion exchange (DEAE-Sephadex A-50) chromatograph

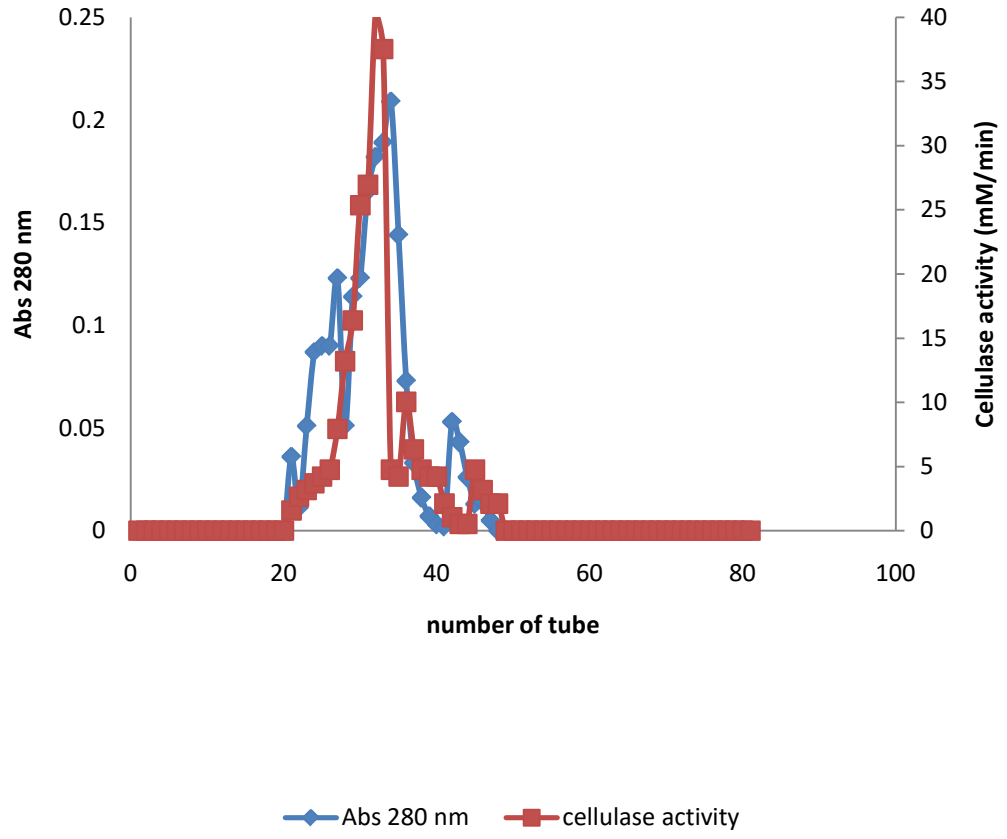

Figure 4: Elution profile of extracellular cellulase *produced by Bacillus cereus* 10 using Sephadex G-150 gel filtration chromatograph

Table 4: Summary of purification of *Bacillus cereus* 10 extracellular cellulase

Fraction	Vol.	Cellulase activity	Protein content	Total cellulose activity	Total protein	Specific activity	Yield (%)	Fold
Crude extract	200	57.35	21.38	11469.34	4275	2.68	100	1
Ammonium sulphate precipitate/Concentration	55.5	38.05	15.00	2112.05	832.50	2.54	18.41	0.95
Ion exchange	35.4	67.65	6.63	2394.93	234.53	10.21	20.88	3.81
Gel filtration	15.3	74.79	4.38	1144.27	66.94	17.09	9.98	6.37

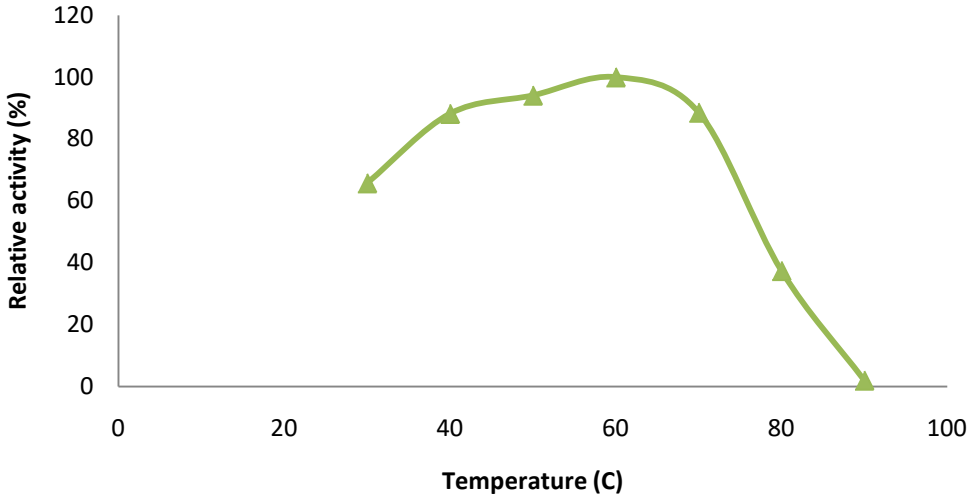

Figure 5: Effect of temperature on the activity of *Bacillus cereus* 10 cellulase

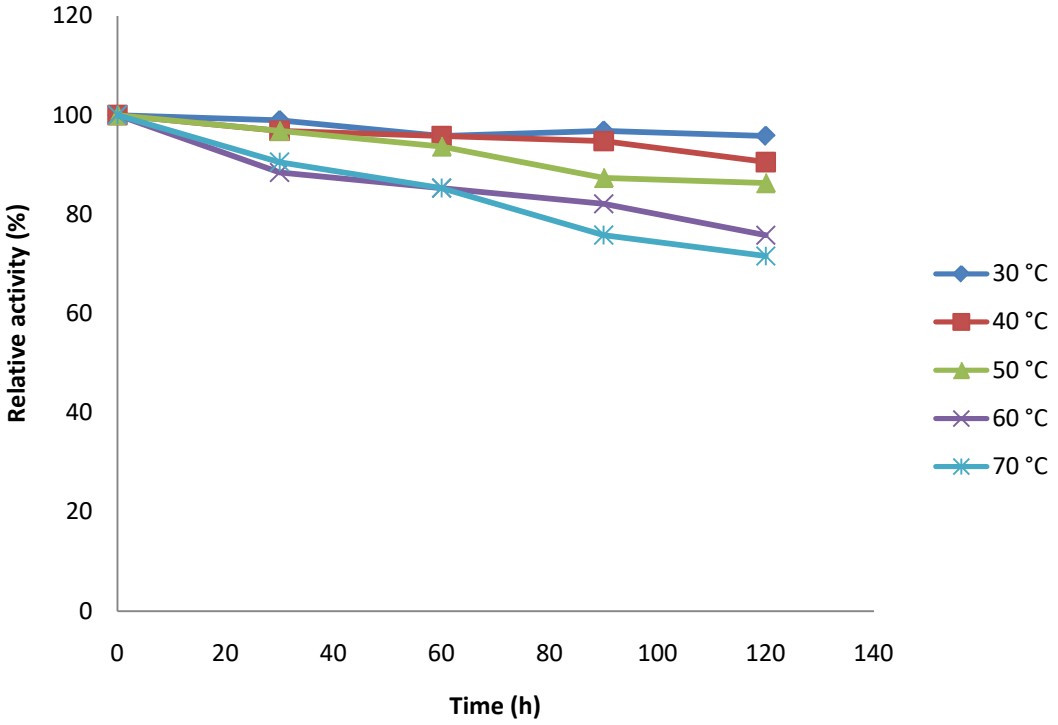

Figure 6: Thermostability of the cellulase produced by *Bacillus* sp.10

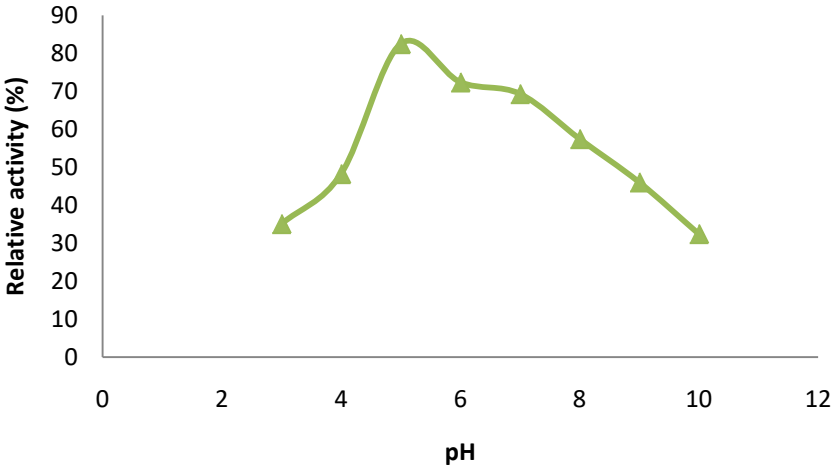

Figure 7: Effect of pH on the activity of *Bacillus cereus* 10 cellulase

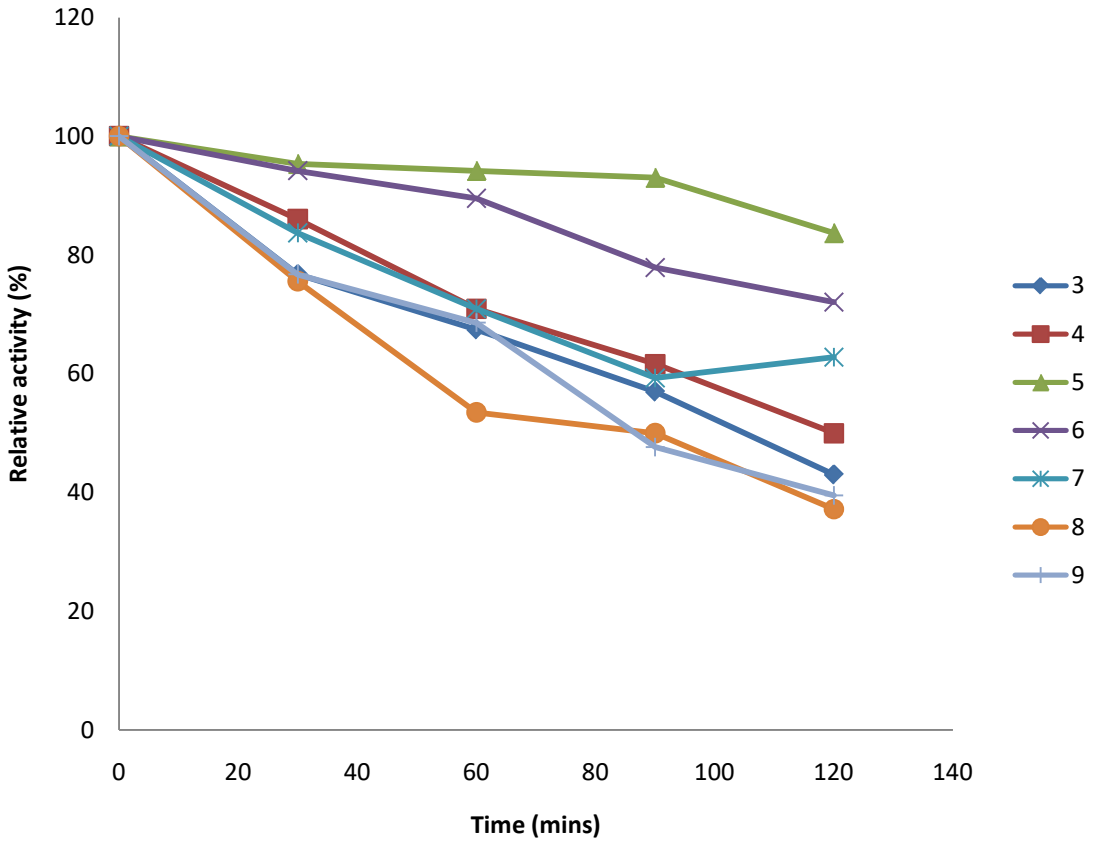

Fig. 8: pH stability of cellulase produced by *Bacillus cereus* 10

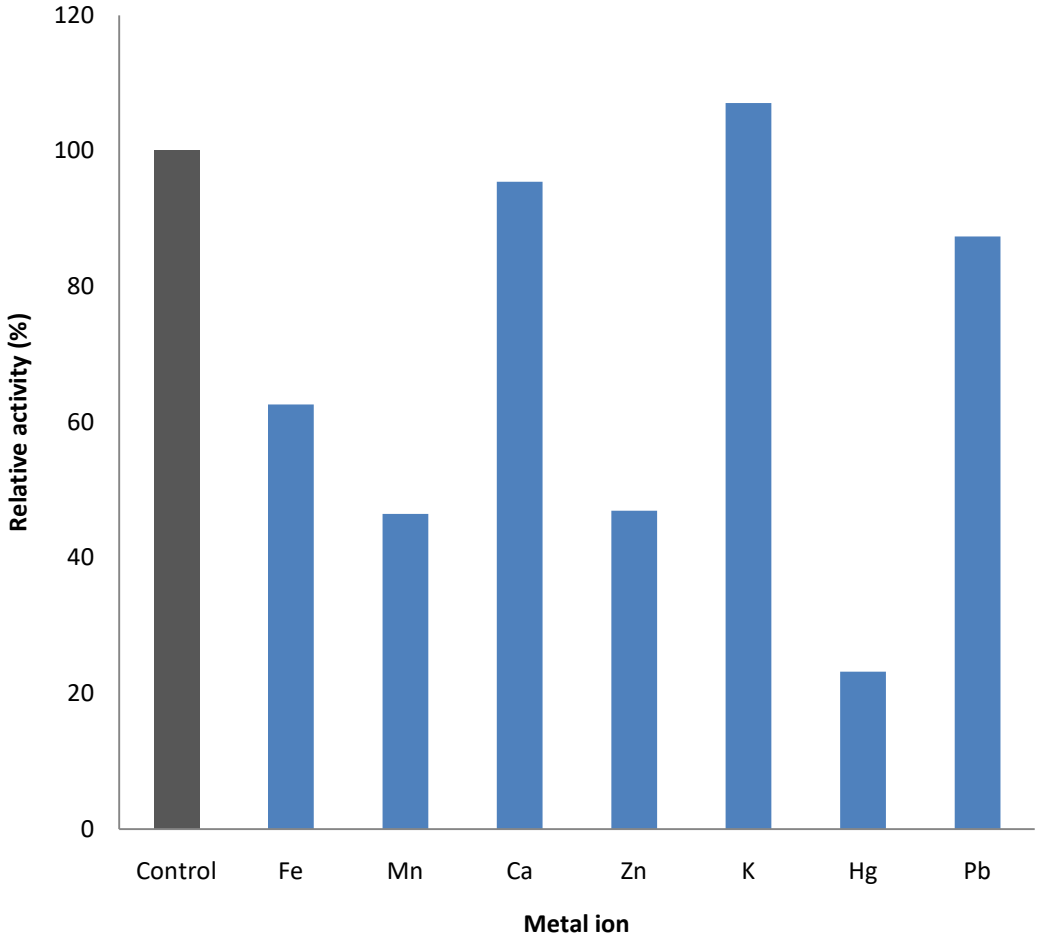

Figure 9: Effect of metal ion on the activity of *Bacillus cereus* 10 cellulase

Conclusion

The current study has revealed that *Bacillus cereus* 10 among other cellulase producing bacteria isolated from agricultural waste around Ekiti State has proven to be a very consistent and potent cellulolytic bacterium. The cellulase produced by this bacterium also showed high activity at pH ranging from 5 to 7 and temperature ranging from 40^{o}C to 70^{o}C. The optimum activity was however obtained at temperature of 60^{o}C and pH of 5, which indicates the enzyme to belong to the group of Endoglucanase and could be classified as being a metallocellulase as it was activate in the presence of potassium. Carbonxylmethylcellulose (CMC) is however a very good source of carbon and nitrogen for the optimization of the production of cellulase by this *Bacillus cereus* 10.

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