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Xylanolytic and cellulolytic enzymes extracellular characterization on organic waste degrading bacteria

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Abstract. *Ochieng OR, Njagi EN, Orinda GO, Otieno MF. 2019. Xylanolytic and cellulolytic enzymes extracellular characterization on organic waste degrading bacteria. Bioteknologi 16: 62-73.* The disposal of untreated municipal wastes is a global problem that challenges public and environmental health. In Kenya, composting, a microbial process with minimal environmental impact, has been given very little attention as an alternative waste treatment technology. Although bacteria can easily be genetically manipulated and therefore are good candidates for industrial use, very few have been researched. Furthermore, the optimum conditions for biodegrading solid wastes are not well understood. This study aimed at extracting and characterizing the biodegradative enzymes from 15 bacterial isolates obtained from dumpsite soil. The optimum period for extracellular enzyme production, pH, temperature, and the influence of selected ions on the activities of the enzymes was studied. The isolates were grown in four different growth media, and their enzymes were purified using ammonium sulphate precipitation. The substrate utilization pattern of the individual isolates was investigated using spectroscopic methods. Most of the isolates were bacterial of the genusbacillus. All the isolates were secreting extracellular proteins into their growth media. Most isolates had optimum protein production between 48-96 hours. Enzymes produced by most isolates were acting on all three commercial substrates (CM- cellulose, xylan, and cellobiose) [P>3.23 (F=3.88)]. Cellulases, xylanases and cellobioses had activities at broad temperature ranges 27°C ([P< 3.23 (F=0.21)], 50°C [P<3.23(F=0.14)] and 80°C [P<3.23 (F=0.06)], respectively). From the study, cellulases and cellobioses were acting within a large pH range (pH 4.8) (P> 3.23 (F=3.24] and (pH 6.8) [P>3.23 (F= 6.33)], respectively, while xylanases were acting within a narrow pH range (pH 4.8) [P< 3.23 (F=1.91]). Magnesium and calcium ions had a stimulatory effect on cellulases and cellobioses, while Silver and Copper ions inhibited the enzymes. *Bacillus clausii* (293), *Bacillus* sp. NER (117) and *Bacillus* sp. CSS-8 strain (108) had highactivities on all the substrates and biodegrades at high pH and temperature ranges and canbe used to facilitate biodegradation of waste when used with the correct ion concentration.

Keywords: Composting, degrading bacteria, extracellular cellulolytic, organic waste, xylanolytic enzyme

INTRODUCTION

Due to urbanization, rapid industrialization, and economic development, the population grows with the generation of large quantities of heterogeneous solid waste. Sustainable management of solid waste is a major challenge faced by municipal authorities worldwide, both in the North and South. In developing countries, the urban waste remains a serious problem that causes contamination of soil and water bodies and endangers human health and the environment. In Africa, Asia, and Latin America, sustainable waste management is a major challenge for municipal authorities (Qizilbash 2002). Municipal authorities have insufficient financial, technical, and institutional capacities to collect, transport, and safely treat and dispose of municipal wastes.Consequently, waste management remains one of the major urban problems (Drechsel and Kunze 2001). In Ghana, for example, 58 percent of solid waste generatedis dumped by households in designated dumping sites, 25 percent is dumped elsewhere in non-designated sites, and only 5 percent s actually collected (Asumani-Boateng and Haight 1999). The situation in other African cities is hardly different. In many city households, waste collection is restricted to wealthy neighborhoods, while in the remaining areas, wastes are dumped along roadsides, illegal dumps, and in stormwater drains (Mbuyi 1989). The city authorities in Tanzania collect only 24 percent of the refuse, while in Nigeria, 35 percent of Ibadan's households, 33 percent of Kaduna's, and44 percent of Enugu's do not have access to the waste collection (Asumani-Boateng and Haight 1999). In India, about 50 percent of the refuse generated is collected. As much as 90 percent of the MSW collected in Asian cities end up in open dumps (Medina 2002).

The situation in Kenya's capital city, Nairobi, is no exception. A report by NEMA reveals that Nairobi generates approximately 2000 tonnes of daily waste. Of this, 68% is household waste (East African Standard 2004). About 50 percent of the Nairobi residents live below the poverty line and are concentrated in Peri-urban and slum areas characterized by limited amenities and unhygienic living conditions (Republic of Kenya 2001b). Such rapid urban growth has caused deterioration of the city's solid waste management services, resulting in environmental pollution (Kibwage 2002). The Nairobi City Council, the legal authority responsible for waste management, has no capacity because only 40 percent of the amount of solid waste generated by the city is collected and disposed of. This poor state of solid waste management services in Nairobi is attributed to insufficient financial outlays, a shortage of equipment, and unfavorable institutional and organizational arrangements. Furthermore, there is an absence of a systematic and integrated approach to tackling the waste management problem. The attitudes of poorer city residents towards environmental cleanliness are also a contributing factor (Slavin et al. 1981). An urgent need exists for new methods of waste handling and promoting fuller environmental awareness.

As a city with critical waste management problems and a burgeoning informal sector, Nairobi possesses both the need and potential for an innovative approach to its waste problems. Composting technology, in contrast to the above-mentioned conventional techniques, has been shown, where feasible, as cost-effective and environmentally friendly. The process is best achieved by providing optimal conditions for the microorganisms, primarily fungi, and bacteria, through the best combination of air, moisture, temperature, and organic materials (Taylor 2003). Although bacteria are considered easier to manipulate genetically than fungi, most researchers are biased toward the latter. Their ability to secrete extracellular enzymes into their growth media is little known. Moreover, the incubation period the microorganisms require for maximum enzyme secretion is largely unknown. Although enzymes are known to be substrate-specific, several bacteria have been found to secrete enzymes that degrade more than one substrate. Determination of such bacteria would therefore be of great benefit for the selection of decomposers. Further, the natural conditions under which the bacteria biodegrade solid wastes at the dumping sites are largely uncontrolled. The rapid pH and temperature changes witnessed under such conditions influence the rate of enzymatic activities and need to be fully investigated. Similarly, there is a need to understand the role of ions that forms the ecosystem where the biodegrades thrive. Some of these ions may directly affect the enzyme's active site, whereas others may cause mutations in the organisms. Based on the strength of these limitations, the study was conducted to determine the most appropriate organism and the best conditions for biodegradation of solid organic wastes. The study will therefore contribute to the millennium development goal number 7, which aims at "ensuring environmental sustainability."

MATERIALS AND METHODS

Collection, isolation, and identification of bacteria

Two sets of soil samples were collected from dumping sites at Uthiru and Dandora of Nairobi, Kenya. The soils were stored at room temperature in axenic conditions and protected from direct sun and light until the isolation was performed. Bacteria were extracted by suspending 1 g of soil sample in 20 mL saline solution, Ringer (2.25 g NaCl, 0.105 g KCl, 0.12 g $CaCl_2$ per liter, pH 7.2, ADSA Micro, Pharmafaster SA, Barcelona, Spain). After 10min vigorous stirring and additional sedimentation, two samples of 8 mL of the aqueous phase were collected. One of the samples was treated for 10 mins at 80°C to isolate only spore-forming bacteria. The other sample remained untreated to isolate both spore-forming and non-spore-forming micro-organisms (Ruiz et al. 2005).

These samples were used to isolate aerobic and facultative micro-organisms by serial dilutions. 0.1 mL of each dilution was spread on agar plates containing four different culture media, and the resulting plates were incubated in duplicate at different temperatures (27°C, 42°C, and 50°C). The four culture media used were: Luria Bertani (LB) agar plates [10 g bactotryptone, 5 g yeast extract,10 g NaCl, 15 g agar per liter, pH7.0]; Nutrient broth(NB) agar plates [5 g peptone, 2 g yeast extract, 5 g NaCl, 1 g meat extract, 15 g agar, per liter, pH 7.4]; Horikoshi-1- Agar (H1A) plates [10 g D(+) glucose, 5 g yeast extract, 5 g bactotryptone,1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 10 g $NaCO_3$, 20 g agar per liter, pH 7.0]; and polycarboxymethyl Sodium salt (PCS) plates [10 g Cellulosic material-Sodium salt, 2.5 g NaCl, 2.5 g $CaCO_3$, 2.5 g peptone, 2.5 g yeast extract, 10 g agar, per liter, pH 8] (Sorano et al. 2000).

A total of 308 bacterial colonies were isolated. Identification of the colonies was performed morphologically and physiologically. Further identification based on full 16S rRNA sequence length was made to ascertain the safety of the isolates (Shirai et al. 1997). The fifteen safe bacterial isolates with high hydrolytic activities on the solid media considered for further characterization were as shown in Table 1.

Table 1. Selected bacterial isolates were incubated on different solid media (H1A, PCS, NB, and LB) at 27°C, 42°C, and 50°C

Organism S/no.	Name	Incubation temp (°C)	Media
31	*Bacillus cohnii*	42	H1A
35	*Bacillus* sp. YASI	27	H1A
108	*Planococcus* sp.	27	H1A
117	*Bacillus* sp. NER	27	H1A
119	*Bacillus flexus* strain KSC SF. 9C	27	H1A
195	*Bacillus clausii* KSM - K16	27	H1A
160	*Planomicrobium* sp. EP22	27	PCS
145	Bacillaceae bacterium KVD - 1982	27	PCS
203	*Bacillus* sp. YAS 1	27	PCS
207	*Bacillus psychrodurans* strain	27	PCS
217	Uncultured bacterium clone Y2	27	PCS
262	*Bacillus* sp. YY	27	NB
267	*Bacillus subtilis* strain JM4	27	NB
293	*B. clausii* strain	50	NB
153	*Bacillus* sp. CSS -8	27	LB

Protein production and purification

Each bacterial isolate was harvested and used to inoculate a 6 mL liquid media at previously identified temperature conditions (Table 1) for 30 hours in a centrifuge tube. Two (2) mL of each inoculated media was centrifuged for 10 minutes, and 6 mL of Acetone was added to pellet the proteins (Harris and Angal 1994). The pellets were then redissolved in 50 mM citrate buffer (pH 4.8) enriched with Tween-20 to reduce the adsorption of proteins by glassware. Soluble protein was measured by the Biuret method, using bovine albumin as the standard. Uninoculated media was used as a control (Harris and Angal 1994).

Period for optimum protein production

Freshly grown bacterial isolates were harvested and used to inoculate 14.5 mL of semi-solid media in 15 mL plastic centrifuge tubes. The inoculated media were left to stand for 6 hours to allow the bacteria to start growing. The tubes were then shaken vigorously using a vortex mixer to obtain a uniform bacterial distribution. The mixture was then divided into seven 2 mL portions to be used every 24 hours interval. The protein concentration was determined for each isolate for seven consecutive days using the Biuret method with bovine albumin as the standard. Uninoculated media was used as a control.

Substrate specificity

Characterizing the extracted extracellular enzyme(s) that formed part of the protein was done using commercial substrates (CM cellulose, larch wood xylan and cellobiose). Six (6) mL of concentrated ammonium sulphate was added to 2 mL of supernatant to precipitate the proteins. Ammonium sulphate helps retain enzyme activity (Harris and Angal 1994). The precipitate is redissolved in buffer at the ratio of 3:1 to form enzyme preparation. The tests were duplicated and averaged across to minimize experimental error. Controls were prepared using the substrates without enzyme preparation. Buffers were used as blanks in each case. Enzyme concentration was determined for each treatment for each isolate. The activities of the enzymes were expressed as micromoles of glucose and xylose equivalence liberated per minute per mg protein for cellulases and xylanases, respectively (Pratimaba 1997).

Cellulases

With CM cellulose as the substrate, 1 mL of enzyme preparation was incubated in 1 mL of 50 mM citrate buffer (pH 4.8) containing 10 mg of CM- cellulose for 30 minutes at 50°C. The reaction was halted, and the reducing sugars were determined by adding 3 mL of DNS acid reagent (Miller 1959; Chaplin 1986). The absorbance at 540 nm values was read with glucose as the standard (Domingues et al. 2000).

Xylanases

With xylan as the substrate, 1 mL of enzyme preparation was incubated with 1 mL of 50 mM citrate buffer (pH 4.8) containing 10 mg of Larchwood xylan for 30 minutes at 50°C. The reaction was halted, and the reducing sugars were determined by adding 3 mL of DNS acid reagent. The xylose liberated was measured spectrophotometrically with absorbance at 540 nm using xylose as the standard (Chaplin 1986).

Cellobioses

With Cellobiose as the substrate, 1 mL of enzyme preparation was incubated with 1 mL of 50 mM citrate buffer (pH 4.8) containing 2 mM cellobiose for 30 minutes at 50°C. The reaction was halted, and the reducing sugars were determined by adding 3 mL of DNS acid reagent. The liberated glucose was determined spectrophotometrically with absorbance at 540 nm using xylose as the standard (Chaplin 1986).

The activity of the extracted extracellular enzymes at different temperatures

The activity of the extracted and purified extracellular enzyme(s) from the bacterial isolates was determined by incubating 1 mL of enzyme preparation with 1 mL of each substrate (CM cellulose, larchwood xylan, and cellobiose as described previously) at different temperatures (27°C, 50°C, 80°C) for 30 minutes. The tests were carried out in duplicate and averaged across. Controls were prepared using the substrates without enzyme preparation. Each case used Buffers as blanks (Bischoff et al. 2006).

The activity of the extracted extracellular enzymes at different pH values

The activities of the extracted and purified extracellular enzymes from the bacterial isolates were determined by incubating 1 mL of enzyme preparations with 1 mL of the substrates (CM cellulose, Larch wood xylan, and Cellobiose) dissolved in citrate buffers (pH 4.8, 6.8 and 8.6) at 50°C for 30 minutes (Shikata and Nisizawa 1975). The tests werecarried out in duplicate and averaged across. Controls were prepared using the substrates without enzyme preparation. Each case used Buffers as blanks (Singh et al. 2001a,b). The liberated monosaccharides were estimated as outlined earlier.

Influence of metal ions on the activities of the purified extracellular enzymes

The activities of the extracted and purified extracellular enzymes from the bacterial isolates were determined by incubating 1 mL of enzyme preparation with 1 mL of the substrates (CM cellulose, Larch wood xylan, cellobiose) containing 1mM of the metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Ag^{+}) at 50°C for 30 minutes. The buffer used in this test was supplemented with EDTA (metal ion scavenger) to chelate the metal ions (Elling 1995). The products were estimated as outlined previously. The tests were carried out in duplicate and averaged across controls made up of substrates lacking the identified metal ions were also set up.

Data management and statistical analysis

The spectrophotometer readings for treatments, controls, and standards were recorded, tabled, and averaged across the three samples to minimize experimental error.

The spectrophotometric analysis relies on the interaction of electromagnetic radiation (light) with the matter of interest. Strictly speaking, every compound has a distinct absorption spectrum which allows its identification, in many cases, in the presence of other compounds. In addition, it is also possible to determine the concentration of that compound quantitatively.

A standard curve was generated by measuring the absorbance of a series of samples for which the concentration was known. Since the Beer-Lambert Law shows a linear relationship between absorbance and concentration, a linear least-squares fit of the standard curve yielded a mathematical relationship between absorbance and concentration. This relationship, in turn, was used to determine the concentration of the unknown samples.

Laboratory analysis

Determination of total protein-Biuret Method

The method relies on the complexation of Cu^{2+} by the functional groups involved with the peptide bond. A minimum of two peptide bonds is needed for the complexation to occur. Upon complexation, purple color is observed. Relatively few substances interfere with the method. The characteristic wavelength at which the colors are read is 540 nm.

Determination of concentration of sugar- DNS colorimetric method

The method tests for the presence of a free carbonyl group (C=O) called reducing sugars. It involves the oxidation of the aldehyde or ketone functional groups in reducing sugars. Simultaneously, 3, 5-dinitrosalicylic (DNS) is reduced to 3-amino, 5- nitrosalicylic acid under alkaline conditions. The reaction leads to the development of color whose optical density (OD) depends on the reducing sugar type and concentration.

RESULTS AND DISCUSSION

Extraction and purification of proteins

All the bacterial isolates secreted extracellular proteins in their growth media after 30 hours. There was a relation between the growth media and the protein level produced in the study period, as shown in Figure 1. Bacterial isolates grown on low molecular weight (HIA) media (*Bacillus cohnii*, *Bacillus* sp. YASI) recorded higher protein production than those from high molecular weight media (PCS, NB, and LB). However, Uncultured bacterium clone Y2 (207) grown on PCS produced the highest protein contentduring the 30-hour growth period.

Time for maximum protein production

All the bacterial isolates had secreted proteins into their growth media after 24 hours. The *B. cohnii* (31), *Bacillus subtilis* strain JM4 (267), and *Bacillus flexus* strain KSC SF. 9C (119) had two distinct production peaks, while the rest had single peaks. Most bacterial isolates had peaks falling between 48 hrs and 96 hrs except the *Bacillus clausii* strain (293), which peaked at 24 hrs. There was a gradual drop in the concentration of protein produced after 120 hrs except in *B. cohnii* (31) and *B. subtilis* strain JM4 (267). *Bacillus* sp. YAS 1 had very low protein production throughout the growth period, as shown in Figure 2.

Substrate specificity

All bacterial isolates secreted cellobioses except *Planomicrobium* sp. EP22 (160). All bacterial isolates biodegrade CM cellulose substrate at a relatively low rate. Uncultured bacterium clone Y2 (217), *Bacillus* sp. YY (262), *B. subtilis* strain JM4 (267) and *B. clausii* KSM K16 (195) did not secrete xylanases. *Planococcus* sp. (108), *Bacillus* sp. YASI (35) and *B. flexus* strain KSC SF. 9C (119) secreted enzymes with moderate activities on Cellobiose, while enzymes from *B. clausii* (293) and *Bacillus* sp. CSS-8 strain (153) had exceptionally high activity on the substrate, as shown in Figure 3.

Effect of temperature changes on the activity of cellulases

All the bacterial isolates secreted cellulases with activities at 27°C except Bacillaceae bacterium KVD-182 (145), *Bacillus* sp. CSS-8 (153), *Bacillus psychrodurans* strain (207), and *Planomicrobium* sp. EP22 (160). The *B. cohnii* (31) cellulases had very activity at this temperature. All bacterial isolates produced cellulases with activities at 500C. *Planococcus* sp. (108) and *Planomicrobium* sp. EP22 (160) produced cellulases with high activities.

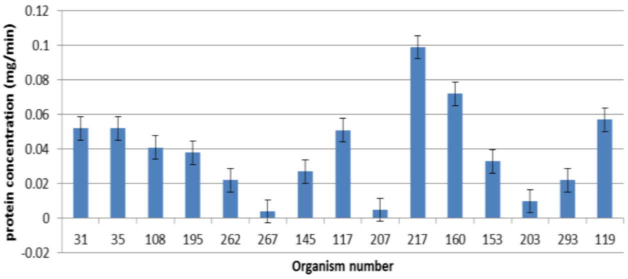

Figure 1. Proteins (mg/mL) produced by bacterial isolates grown in liquid media for 30-hour

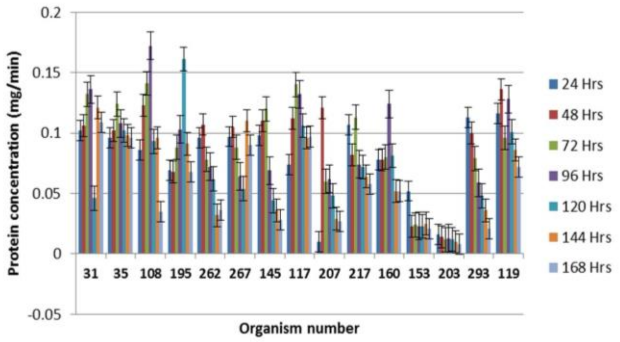

Figure 2. The concentration of proteins produced (mg) from 1 mL of enzyme extract when bacterial isolates were grown on liquidmedia at the 24-hour interval for seven days

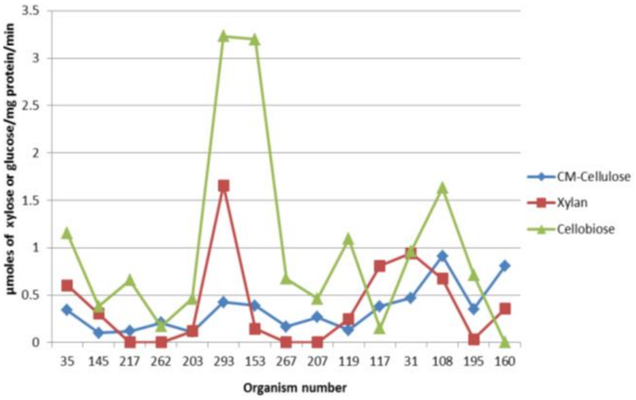

Figure 3. Activities of enzymes from each bacterial isolate on three substrates. BothCM cellulose and cellobiose biodegraded to glucose while xylan forms xylose (µmole/mgprotein/min) at 50°C and pH 4.8

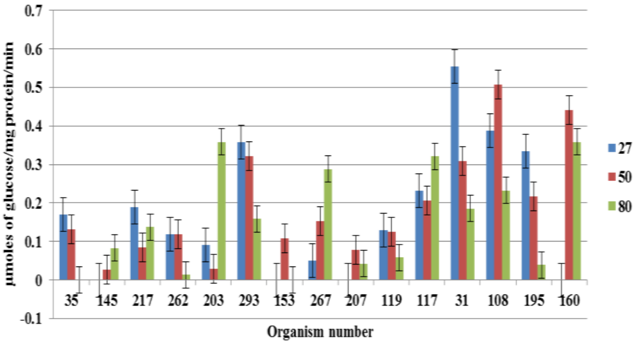

Figure 4. Enzyme activity (µmole glucose/mg protein/min) on CM cellulose at 27°C, 50°C, and 80°C and ph 4.8

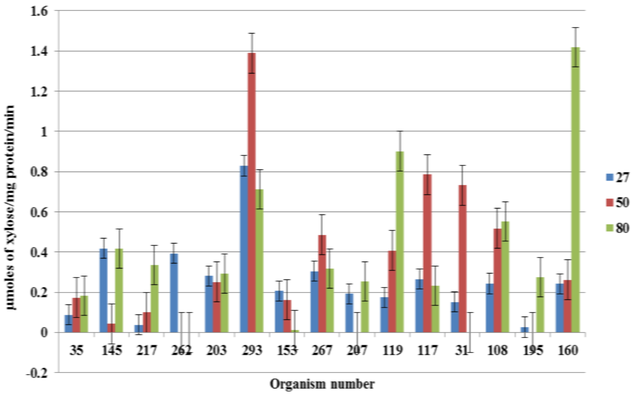

Figure 5. Enzyme activity (µmole xylose/mg protein/min) on xylan producing xylose at27°C, 50°C, and 80°C and pH 4.8

However, enzymes from Bacillaceae bacterium KVD - 182(145) and *Bacillus* sp. YASI (203) showed exceptionally low activities at this temperature. *Bacillus* sp. CSS- 8 produced enzyme activity at 50°C only, and 13 (86 %) organisms produced cellulases with activity at 80°C. Enzymes produced by *Bacillus* sp. YAS 1 (35) and *Bacillus* sp. CSS-8 (153) showed no activities at this temperature. *Planomicrobium* sp. EP22 produced cellulases with high activities at the said temperature, as outlined in Figure 4.

Effect of temperature changes on the activity of xylanases

All organisms produced xylanases with low activities at 27°C except the *B. clausii* strain (293). Xylanases from the isolate showed relatively high activities at all temperatures. Enzymes from 12 (80%) isolate had activities at 50°C. Of these, 3 (20%) had very low activities. *Bacillus sp* YY (262), *B. psychrodurans* strain (207), and *B. clausii* KSM-16 (195) had no activity at this temperature. Enzymes from 12 (80%) isolate had activities at 80°C. Enzymes from *Bacillus* sp. YY (262), *Bacillus* sp. CSS-8 (153), and *B. cohnii* (31) had no activity on xylan, while those from 7 (47%) isolates had minimal activity at this temperature. *Planomicrobium* sp. EP22 (160) produced xylanases with high activity at this temperature, as shown in Figure 5.

Effect of temperature changes on the activity of cellobioses

Most isolates (86%) secreted enzymes with very low activities on cellobiose at all temperatures except *B. clausii* strain (293) and *Bacillus* sp. CSS-8(153), which showed relatively high activities. Enzymes from the two isolates showed comparatively high activities at 27°C. Cellobioses secreted by *Planomicrobium* sp. EP22 (160), and Uncultured bacterium clone Y2 (217) had no activity at the said temperature. All isolates secreted cellobioses with low activities at 50°C except *Planomicrobium* sp. EP22 (160) and Uncultured bacterium clone Y2 (217). Cellobioses from 9 (60 %) isolates showed relatively higher activities at this temperature than at 27°C and 80°C. Only 2 (13 %) isolates produced enzymes with high activities at 80°C. Enzymes secreted by *B. clausii* strain (293) and *Bacillus* sp. CSS-8 (153) showed activities at all the experimental temperatures, as in Figure 6.

Effect of pH changes on the activity of cellulases

All isolates produced cellulases with activities at a pH of 4.8. Cellulases from *Bacillus* sp. YAS 1(203), *Bacillus bacterium* KVD-1982 (145), and *B. psychrodurans* strain (207) had minimal activities, while those from *Planoccocus* sp. (108) and *Planomicrobium* sp. EP22 (160) had high activities at this pH. Enzymes from Bacillaceae bacterium KVD-1982 (145) and *B. psychrodurans* strain (207) had low pH values. Cellulases secreted from *B. subtilis* strain JM4 (267) showed high activity, those from *Bacillus* sp. NER (117) had moderate, and the rest had minimal activities at a pH of 6.8. Forty percent of the isolates secreted enzymes that had activities at pH 8.6. Enzymes from *Bacillus* sp. NER (117) expressed an outstandingly high activity, while the rest secreted cellulases with low activities at the pH, as expressed in Figure 7.

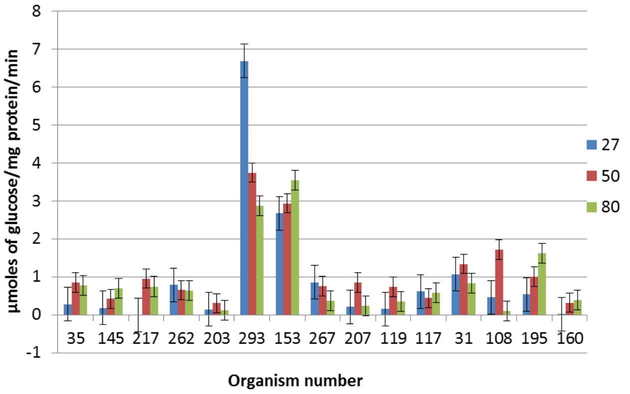

Figure 6. Enzyme activity (µmole glucose/mg protein/min) on cellebiose producingglucose at 27°C, 50°C, and 80°C and pH 4.8

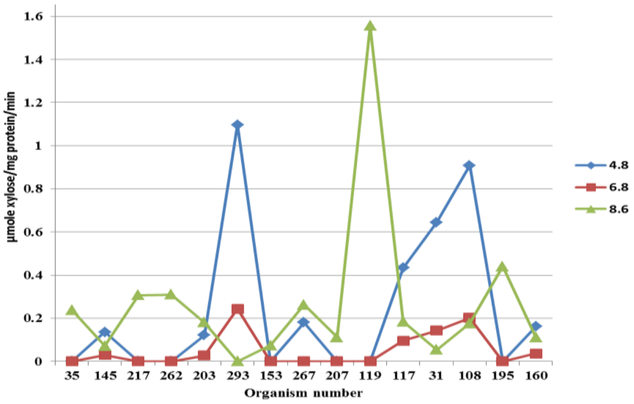

Figure 8. Activity of xylanases (µmole xylose/mg protein/min) xylan at 50°C under pHof 4.8, 6.8, and 8.6

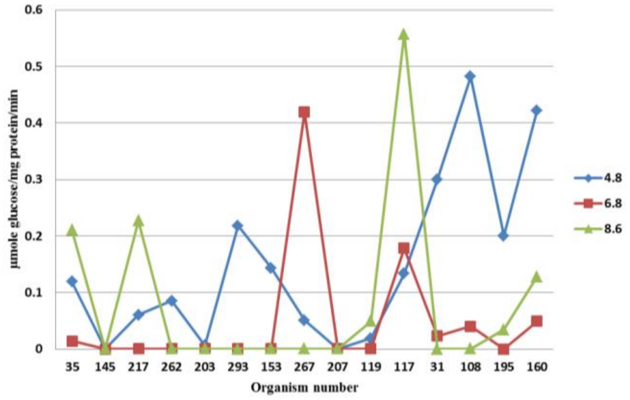

Figure 7. The activity of cellulases (µmole glucose/mg protein/min) on CM-cellulose at 50°C under pH of 4.8, 6.8, and 8.6

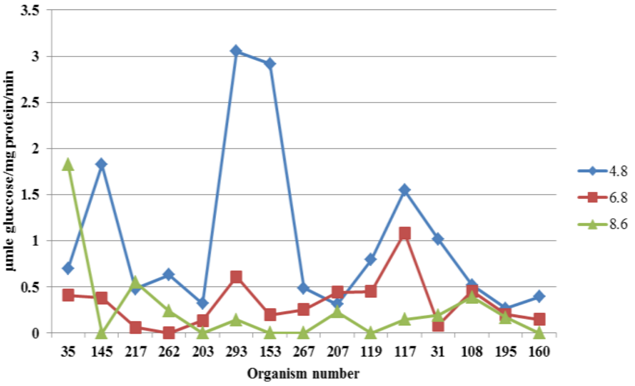

Figure 9. The activity of cellobioses (µmole glucose/mg protein/min) on cellobiose at 50°C under pH of 4.8, 6.8, and 8.6, producing glucose

Effect of pH changes on the activity of xylanases

High xylanase activities were found at pHs 4.8 and 8.6; however, enzymes from all the isolates had very low activities at a pH of 6.8. The *B. clausii* strain (293) and *Planococcus* sp. (108) produced enzymes with relatively high activities at a pH of 4.8. Enzymes from *B. flexus* strain KSC SF. 9C (119) showed very high activities at a pH of 8.6. Bacillaceae bacterium KVD-1982 (145), *Bacillus* sp. CSS-8 (153), and *B. clausii* KSM-K16 (195) had low xylanase activity at all pH, as shown in Figure 8.

Influence of pH on cellobioses

Nearly all isolates produced enzymes with activities within a wide pH range. Enzymes from all the organisms showed activities at a pH of 4.8. The *B. clausii* strain (293) and *Bacillus* sp. CSS-8 (153) produced enzymes with very high activities at this pH. Enzymesfrom all the isolates showed minimal activities at a pH of 6.8. Enzymes obtained from all isolates showed very low activity at a pH of 8.6. Only *Bacillus* sp. YASI (35) produced enzymes with moderate activity at this pH, as shown in Figure 9.

Influence of metal ions on cellulases

Calcium ion (Ca^{2+}) stimulated the activities of enzymes from 11 (73 %) isolates but inhibited enzymes from the rest. The stimulatory effect was moderate on *B. subtilis* strain JM4 (267) and very low on the rest. Magnesium ions (Mg^{2+}) stimulated enzymes from 67 percent of the isolates, while the rest were inhibited. The stimulatory effect was pronounced in *B. subtilis* strain JM4 (267), *B. flexus* strain KSC SF. 9C (119) and *B. psychrodurans* strain (207). Copper ions (Cu^{2+}) inhibited cellulases from most isolates with limited stimulation on enzymes from a few isolates. Eighty percent of the isolates produced enzymes stimulated by silver ions (Ag^{+}), while the ion inhibited enzymes produced by the rest. Enzymes from *B. flexus* strain KSC SF. The ion highly stimulated 9C (119). All the tested metal ions stimulated cellulases from the *B. subtilis* strain. Enzymes from *Bacillus* sp. CSS-8 (153), *Planococcus* sp. (108), and *Planomicrobium* sp. EP22 (160) were inhibited by all the ions except Calcium ions (Ca^{2+}) as in Figure 10.

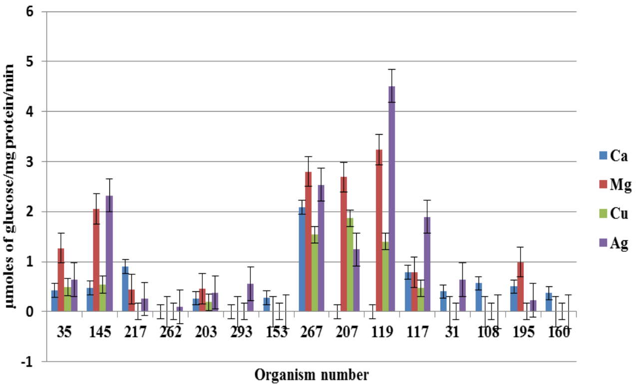

Figure 10. The activity of cellulases (µmole/mg protein/min) on cellulose substrate dissolvedin a buffer containing 50 mM of four metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Ag^{+}) at 50°C and pH of 4.8

Influence of metal ions on xylanases

All tested metal ions highly stimulated xylanases from *B. subtilis* strain JM4 (267) and *B. clausii* KSM-K16 (195) . Calcium ions (Ca^{2+}) stimulated enzymes from 6 (40 %) isolates, while enzymes from other bacteria showed low activities. A remarkable stimulation was shown by xylanases from *B. subtilis* strain JM4 (267). A similar trend was expressed by Magnesium ion ($Mg^{2+)}$. The highest stimulation by the ion was seen in enzymes from *B. subtilis* strain JM4 (267). Xylanases from 12 (80%) organisms showed low activities under the influence of copper ions (Cu^{2+}) with minimal stimulation on enzymes obtained from the rest of the isolates. Xylanases from most isolates had very low activities when treated with silver ions (Ag^{+}) except those from *B. subtilis* strain JM4 (267) and *B. clausii* strain (195), which were slightly stimulated. Xylanases from Bacillaceae bacterium KVD-1982 (145), uncultured bacterium clone Y2 (217), *Bacillus* sp. YAS and *B. clausii* strain (293) had low activities when treated with all the metal ions, as shown in Figure 11.

Influence of metal ions on cellobioses

Cellobioses from 10 (67%) isolates were minimally stimulated by Calcium ions (Ca^{2+}). However, enzymes from the rest showed low activities when incubated by the ion. Magnesium ions (Mg^{2+}) stimulated enzymes from 10 (67%) isolates with Uncultured bacterium clone Y2 (217) and *Bacillus* sp. NER (117) showed moderate stimulation while other isolates produced enzymes with very low activity under the influence of the ion. Cellobioses from *Bacillus* sp. CSS-8 (153) were highly stimulated by copper ion (Cu^{2+}), while six produced cellobioses with low activity. Seven (47%) isolates produced enzymes that were moderately stimulated by silver ions (Ag^{+}), while those from 5 (20%) isolates had very low activities. Very high stimulation by the ion was shown by enzymes from the *B. clausii* strain (293), as shown in Figure 12.

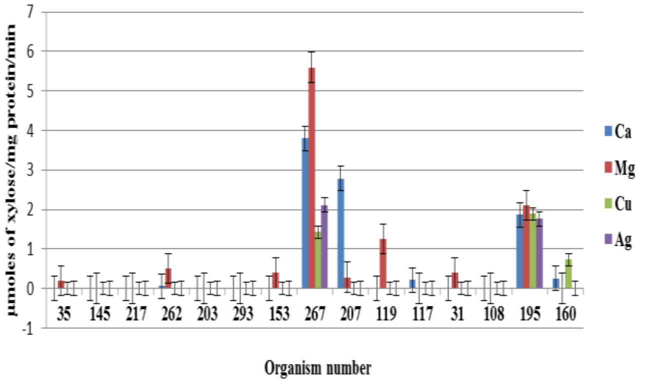

Figure 11. Activity of xylanases (µmole xylose/mg protein/min) on xylan substrate dissolved in buffer containing 50 mM of four metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Ag^{+}) at 50°C and pH of 4.8

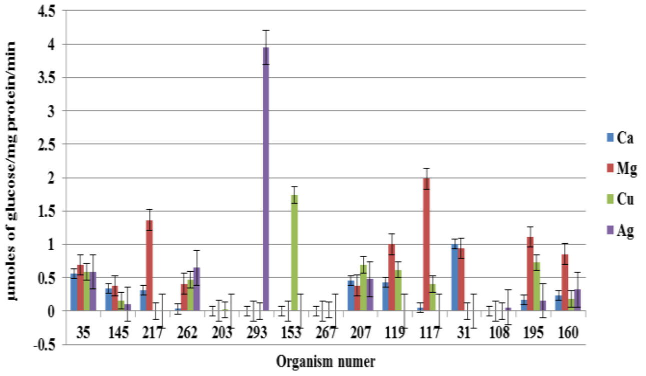

Figure 12. The activity of cellobioses (µmole glucose/mg protein/min) on cellobiose substrate dissolved in a buffer containing 50 mM of four metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+},Ag^{+}) at 50°C and pH of 4.8

Discussion

Microorganisms decompose organic wastes to generate energy and nutrients for their growth by producing intra and extracellular enzymes. A great significance for the process of composting represents the cell wall of microorganisms through which mass transfer is possible. Low molecular weight and water-soluble molecules can easily pass through the cell wall where they take part in the cell metabolism, providing energy and being built into larger polymers with the help of intracellular enzymes. To attack high molecular weight components, which cannot pass through the cell wall, microorganisms secrete extracellular enzymes, which break molecules down into fragments that can beassimilated. At the same time, the rest is converted into a stable product, humus or compost (Haug 1980).

The results showed that all bacterial isolates used in the study secreted extracellular enzymes into their growth media within 24 hours period. The production was higher in theisolates grown on high molecular weight media than those grown in the low molecular weight media. Isolates grown on high molecular weight media secretes enzymes earlier tobreak the substrates to generate soluble products that are catabolized to produce energy (Deshpande et al.

1978). However, isolates grown in low molecular weight media obtain soluble products that readily diffuse into the cell for assimilation hence delayed enzyme production (Biddlestone and Gray 1985).

Several previous researchers have reported the production of extracellular cellulolytic enzymes by Bacillus bacteria into their growth media (Priest 1977; Robson and Chambliss 1984). James and Barko (2004) reported that 115 bacilli grown on Horikoshi-I medium for 24 hours at 37°C using eight different substrates: birchwood xylan, carboxymethylcellulose, casein, citrus pectin, polygalacturonic acid, soluble starch, and Tween 20 and 80 produced extracellular enzymes. These strains were identified as producers of extracellular proteases (Singh et al. 2001a,b) and can be cultivated under extreme temperatures and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments (Han and Damodaran 1997).

Bacterial isolates grown on H1A (containing readily available carbon source; glucose) showed maximum enzyme production between 72-120 hours. However, isolates grown on media where free carbon and Nitrogen (CMC and NB, respectively) lack secretes their enzymes early (24-48 hours) to digest the complex materials to obtain the raw materials for growth. The maximum enzyme production stage by different bacterial species is quite variable. It largely depends on the type of microbial strains and their genetic make-up on cultural and environmental conditions employed during the organism’s growth. Easily metabolizable and utilizable substrates induce lower enzyme production (due to catabolite repression) than the complex and slowly utilizable substrates. Isolates grown on CM cellulose media secretes cellulases digesting the substrate to an insoluble disaccharide (cellobiose). This is followed by the production of cellobioses that finally breaks the sugar into soluble glucose. *Bacillus* sp. mostly produces two groups of proteases, alkaline and neutral (Rao et al. 1998), of which the neutral proteases are active in a narrow pH range (pH 5.0-8.0) and have relatively low thermotolerance (Barrett 1994). For most isolates, the level of enzyme production diminished towards the end of the seven days. This may be attributed to the accumulation of proteases that degrades the proteins or product inhibition (Shevelev and Hubscher 2002). The products of exoglucanases and cellobiohydrolases that are cellobiose and cellodextrans, respectively, are inhibitory to their activity (Miranda et al. 2009).

The results showed that most bacterial isolates' enzymes secreted into the growth media biodegrade more than one type of substrate. Given the mixed composition of the solid wastes in the field, most isolates produce enzymes that biodegrade across a section of substrates as a survival strategy. In addition, plant tissues are formed from several different monomers covalently linked. The isolates must secrete corresponding enzymes to biodegrade the plant tissues to obtain energy. In cellulase synergism, the reaction between endoglucanases and exoglucanases was considered a mechanism in which endoglucanases initially nicked the crystalline cellulose surface, followed by exoglucanases liberating cellobiose from these nicks (Henrissat et al. 1985). Finally, beta-glucosidase is required to hydrolyze cellobiose into individualmonosaccharides for use by the isolate. A novel thermophilic, a cellulolytic bacterium isolated from swine waste, *Brevibacillus* sp. Strain JXL used a broad spectrum of substrates such as crystalline cellulose, carboxymethyl cellulose, xylan, cellobiose, glucose, and xylose (Liang et al. 2009). A bifunctional endoglucanase/ endoxylanase isolated from *Cellulomonas flavigena* had cellulase and xylanase activity (Pérez-Avalos et al. 2008). Similarly, in 2007, a multifunctional enzyme was produced by *Terendinibacter turnerae* T7902, a bacterial symbiont isolated from the wood-boring marine bivalve *Lydrodus pedicellatus*. According to Moo-Young (1992), the actions of cellulases and xylanases are synergetic over substrates, especially for microorganisms isolated from environments where wood and agro-residues are biodegraded.

These results suggest that some isolates may have secreted enzymes that produce promiscuous secondary metabolites that act on a relatively broad range of different substrates, which is important for the evolution of new biosynthetic pathways (Firn 2006). Cellulases from strains like *C. flavigena* and *T. turnerae* have been cited to produce multifunctional cellulases with broad substrate utilization (Firn 2006). Some purported xylanases, for example, also appear to have activity against substituted carboxymethyl cellulose. When present, they can reduce cellulose viscosity by attacking the amorphous regions (Miranda et al. 2009). Some degree of cross-specificity was reported from numerous fungal and bacterial 1, 4-β glucanases and xylanases (Shikata and Nisizawa 1975; Tods et al. 1975; Kanda et al. 1976; Hurst et al. 1978; John et al. 1979; Pettipher and Latham 1979: Uchino and Nakane 1981; Peiris et al. 1982).

Temperature affects the speed of molecules, the catalytic reaction's activation energy, and the enzyme's and substrate's thermal stability. Generally, the enzyme reaction rate is very slow at low temperatures as the molecules have low kinetic energy, and collisions between them are less frequent. Even if they collide, the molecules do not possess the minimum activation energy required for the reaction. It can be said that the enzymes are deactivated at low temperatures (Abrahams and Katherine 2011). An increase in temperature increases the enzyme activity since the molecules now possess greater kinetic energy. The rate of enzyme activity is highest between 0-40°C, and this increase is almost linear. After 40°C, the rate of reaction starts to decrease. This is because the increase in temperature after 40°C does not increase the kinetic energy of the enzyme but instead disrupts the forces maintaining the molecule’s shape. The enzyme molecules are gradually denatured, causing the active site’s shape to change. Temperatures above 65°C completely denature the enzymes (Abrahams and Katherine 2011).

The study showed that cellulases and xylanases and cellobioses were acting within large temperature range ([$P< 3.23$ ($F=0.21$)], [$P<3.23$ ($F=0.14$)] and [$P<3.23$ ($F=0.06$)] respectively). Temperature increases with depth at the dump site, and isolates at the lower depths need to produce thermophilic enzymes that tolerate high temperatures. The stability of xylanase at high temperatures might be due to the protection caused by any compatible solute, such as the polymeric substrate xylan and/or xylooligosaccharides resulting from the hydrolysis, which could exert a protective effect on the enzymes (Damaso et al. 2000). These protection effects related to disaccharides and trisaccharides and also polymeric ficoll on the restriction enzyme PstI was described by Colaço et al. (1992). Kumar et al. (2000) observed that both thermophilic and mesophilic proteins have similar hydrophobicities, oligomeric states, and hydrogen bonds. On the other hand, salt bridge numbers are higher in most thermophilic proteins. This fact can be explained since salt bridges, and their networks rigidify protein structures. A higher concentration of salt bridges, particularly networks, "stitches" the protein structure, making it more resistant to local deformation/melting or unfolding at high temperatures. Previous studies reported that xylanase was active over a wide range of temperatures and pH values, with the optimum at 75ºC and 6.0, respectively (Uchino and Nakane 1981). Xylanase from thermophilic *Bacillus* exhibited a temperature profile with a sharp peak of maximal activity at 90°C and showed activity between 40-100°C (Cordeiro et al. 2002).

Cellulases from thermophilic fungi showed optimum activities at 50°C (Cooney and Emerson 1964). Similarly, the optimum temperature for cellulase activity with carboxymethyl cellulose substrate varied between 58-77°C with 10 hrs incubation time (Eriksen and Goksoyr 1976). *Bacillus* sp. 3M exhibits cellulase retained high activity at 90^0C, making it attractive in the pulp and paper industry (Marques et al. 1997). Effect of temperature on endoglucanase activity showed that the enzyme was highly active over a broad temperature range (50-100°C). Cellulases from different *Bacillus* spp. have been reported to possess optimum activity at 40-50°C. However, *Bacillus licheniformis* cellulase was found to be moderately thermostable with optimum activity at 65°C and retained 90% of the original activity for 1 hr at 60°C (Bischoff et al.1987). The highest mean activity of *Bacillus* sp. C14 endoglucanase enzyme was observed as 88 and 96% between 20-60°C and had a thermal stability average of 71 and 60% for 15 and 30 min, respectively, between 20-90°C (Oyekola et al. 2007).

Most enzymes are sensitive to pH changes and have specific activity ranges and an optimum pH. The pH change can stop enzyme activity by denaturation (altering) the three-dimensional shape of the enzyme by breaking weak bonds such as ionic and hydrogen. The isolates are subjected to drastic pH changes. Anaerobic decomposition of organic matter increases the acidity of the soil. This is compounded by the acidic industrial wastes, including sulphuric acid from used batteries. This condition is neutralized by stone debris from construction sites leaving the pH to stabilize around a neutral value. These abrupt pH changes at the dump sites can only be tolerated by isolates that secrete enzymes acting within a large pH range (Saini 2007).

From the research, cellulases and cellobioses were acting within a wide pH range (P>3.23 (F=3.24] and [P>3.23 (F= 6.33)]) respectively, while xylanases were acting within a narrow pH range [P< 3.23 (F=1.91]). Most enzymes function between a pH of 6 and 8 (Fukumori et al. 1985). Bacteria that thrive at lower pH values are acidophilic (acid loving) (Robson and Chambliss 1984). *Bacillus* sp. JB-99 was also able to grow over a wide range of pH (6-12), and it required an alkaline pH (8-10) for growth and enzyme secretion (Johnvesly and Naik 2001). Alkaliphilic Bacillus strains often produce various alkaline enzymes, including alkaline cellulases (Horikoshi and Akiba 1982; Horikoshi 1996). Horikoshi and colleagues found that Alkaliphilic Bacillus strains N-4 and NO. 1139 (Horikoshi et al. 1984), both of which had first been described in Japanese patent, produced alkaline cellulases (Carboxymethylcellulose (CMC) hydrolyzing enzymes CMCase). A bifunctional endoglucanase/ endoxylanase isolated from *C. flavigena* was found to have optimum cellulase and xylanase activity at pH 6 and 9, respectively (Miranda et al. 2009). Enzymes stably work in a wide range, including an alkaline side. Their activity is shown even at low temperatures (cellulase K, CMCase I, and CMCase II) were isolated from a culture product of *Bacillus* sp. KSM-635 (Japanese patent Application Laid-open No. 61-19483). *Bacillus* sp. C14 showed growth at a wide range of pH from 6 to 12. *Bacillus* sp. JB-99 was also able to grow over a wide range of pH (6-12), and it required an alkaline pH (8-10) for growth and enzyme secretion (Johnvesly and Naik 2001). Seven strains of *B. subtilis* group, isolated from fermented African locust bean (iru), compared based on growth and extracellular enzyme production in media with and without locust bean, had their optimum pH for growth between 7.0 and 9.0 (Aderibigbe and Odunfa 1990).

Enzymes produced by the isolates at the dump sites are subjected to various metal ions derived from household wastes. Calcium ions are readily obtained from stone wall debris, while magnesium ion is abundant in the dumping soil. Copper ions constitute 80% of the electrical wastes, while silver ions come as impurities from industries' oil discharge. The survival of such organisms depends on the ability of their enzymes to circumvent the adverse effects the ions have on their structure. Many reports are available about different activation and the inhibition pattern of the metal ions on enzymes. Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) ions had a stimulatory effect on cellulases as they might be involved in the protection of the enzyme or strengthening of the active site, thereby maintaining the conformation of the enzyme in its active state. Metal ions have been reported to influence enzyme production by increasing their activity in microorganisms (Rani and Nand 2000; Rani et al. 2004). Cu^{2+}, $Cd^{2+,}$ and Ni^{2+} have been reported as heavy metals which are generally toxic to some organisms (Sunkar et al. 2003; Rani et al. 2004). According to Mawadza et al. (2000), most metal ions such as K^+, Na^+, Ca^{2+}, and Zn^{2+} did not actually influence the enzyme activity.

However, Singh reported an increased enzyme activity in the presence of Na^+ (Singh et al. 2001a,b; Singh et al. 2004). The inhibition of *Bacillus* sp. C14 by Zn^{2+} (27 percent) concur with other work due to the inhibitory effects of heavy metals on enzymes. The obstructive activity of Zn^{2+} on cellulases was also reported by Voget et al. (2006) and Huang and Monk (2004). Mercuric and cupric ions have been found to inactivate the cellulolytic activity of the cellulase (1, 4- (1.3; 1, 4)-β-d-glucan-4-glucanohydrolase; endoglucanase; EC 3.2.1.4) from *Schizophyllum commune* in a time-dependent manner (Anthony and Lenley 1987). Inhibition of cellulase activity by the metal ions Ag^{+} and Hg^{2+} was also reported and was ascribed to interaction with tryptophan residues rather than with thiol groups (Hurst et al. 1977). Of the numerous metal ions examined, Ca^{2+} and Co^{2+} at 0.1 mM concentration were slightly activated under the assay conditions, while 1.0 mM Pb^{2+} and Hg^{2+} were the most inhibiting ions (Ferchak and Pye 1983). Ca^{2+} ions have earlier been reported to be required by cellulosome enzymes, with the former enhancing the substrate binding affinity of the enzyme and stabilizing the conformation of the catalytic site (Mansfield et al. 1998). These results suggest that the metal ions such as Ca^{2+}, Na^{+}, and Zn^{2+} apparently protected the enzyme against thermal denaturation and played a key role in continuing the enzyme's active conformation at high temperatures (Donagy and Mckay 1993). A study by Wang and his colleagues showed that Fe^{2+} might activate cellulase even in lower concentrations, while Mg^{2+} showed an inhibitory effect in higher concentrations. The other seven kinds of metal ions all showed the inhibitory character, and the inhibitory effect of Hg^{2+} was the most potent, even inlower concentrations (Wang et al. 2009). Ca^{2+} ion has long been known to promote the formation of active trypsin from the inactive trypsinogen and stabilizes trypsin against autolysis (Zoltan et al. 2003).

Mark and his colleagues found that the activity of a purified enzyme shown to be exclusively xylanolytic was significantly enhanced following treatment with manganese and potassium chlorides but significantly reduced by calcium, cobalt, and iron (Mark et al. 2009). Annamalai et al. (2008) also reported the activity of xylanases produced by *B. subtilis* isolated from the marine environment, which MgSO4 enhanced, $CaCl_2$, $FeCl_2$, and $FeSO_4$ in both 1 and/or 10 mM concentrations. While $NiSO_4$ and $ZnSO_4$ showed slight inhibition in the enzyme activity, $CuCl_2$, $CoCl_2$, $CoSO_4$, $CuSO_4$, $MnSO_4$, and $ZnCl_2$ showed moderate inhibition, and $HgCl_2$ expressed severe inhibition at 10 mM concentration (Annamalai et al. 2008). Similarly, Monica (2002) noted that 5 mM of Zn^{2+}, Ca^{2+}, and Ba^{2+} caused slight inhibition of the xylanase activity. Yet the ions of Mg^{2+} and Fe^{3+} at the same concentration led to an inhibition of approximately 25 percent. On the other hand, NaCl concentrations at 3 mM or 5 mM resulted in slight enzyme activity stimulation.

Mohammed identified three cellobioses from *Aspergillus niger* A20, A, B, and C. Ca^{2+} ions were found to stimulate cellobioses B and C, while Co^{2+} and Mg^{2+} ions stimulated A (Fareeha et al. 2011). The activity of β-glucosidase from thermophilic fungus *Melanocarpus* sp. microbial type culture collection (MTCC) 3922 was positively influenced by metal ions such as Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} but inhibited by the presence of $CuSO_4$ (Jatinder et al. 2001). The studies on two extracellular β-glucosidase from *A. niger* USDB 0827 and *A. niger* USDB 0828 showed that both enzymes were relatively unaffected by Ca^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (Yin et al. 1992). β-glucosidase from *A. ornatus* has also been inhibited by Ag^{+} and Fe^{2+} (Yeoh et al. 1986), whereas β-glucosidase from *A. terreus* was relatively unaffected by metal ions (Workman and Day 1982). In the study of unique inhibitors and inducers of the protease by Siddalingeshwara et al. (2010), Cu^{2+}, Mn^{2+}, Ca^{2+}, Hg^{2+}, Na^{2+}, and $MgCl_2$ were shown to be potent inducers while Zn^{2+} and Fe^{2+} were potent inhibitors.

In conclusions, (i) *B. clausii, Bacillus* sp. NER and *Bacillus* sp. CSS-8 strain with broad substrate spectrum, high activities at large temperatures, and pH ranges can form a good consortium for biodegradation of solid organic waste, (ii) All the studied bacterial isolates showed significant production of extracellular enzymes, (iii) There was a significant difference between the times taken for maximum enzymeproduction by the individual isolates, (iv) Enzymes produced by different isolates had broad range specificity on the substrates, (v) Temperature changes influenced the activities of cellobioses. At the same time, cellulases and xylanases act over a wide temperature range, (vi) pH changes influenced the activities of cellulases and cellobioses but not on xylanases, and (vii) Cellulase and cellobioses were stimulated by Ca^{2+} and Mg^{2+}. At the same time, Cu^{2+} and Ag^{+} inhibited the enzymes. However, xylanases were inhibited by the metal ions.

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