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Partial Purification and Characterisation of Cellulase from Sugarcane as affected by postharvest storage of Sugarcane (*Saccharum officinarum* L) stem

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ABSTRACT

This study was aimed at evaluating the effect of storing sugar cane stem at room temperature on the activity of its cellulase enzyme. Cellulase was partially purified and characterised from freshly harvested sugarcane (FHS) and stored sugarcane (SS) (*Saccharum officinarum* L) using 80% ammonium sulphate precipitation and dialysed; the FHS had 136.52 units/mg protein while the SS 184.53 units/mg proteins. The K_m value of cellulase of SS was 0.09 mg/ml while that of FHS was 0.540 mg/ml. The substrate specificity on different cellulose materials (orange peel, banana peel, maize starch, sugarcane bagasse, maize cob and apple pomace) showed varying results for the two enzyme sources. The enzyme from FHS showed 100% activity with banana peel and sugar cane bagasse while the enzyme from SS showed 100% activity with the peels of orange and banana and sugar cane bagasse. Maize cob and apple pomace as carbon source showed very little cellulase activities, 17.4% for FHS and 26.6% for SS. The optimum pH value of partially purified cellulase of FHS was 4.0 while that of SS was 7.0 and the enzyme was optimally active at 40°C for both sources. At the concentrations of 1.0 mM and 10.0 mM, Ca^{2+} and Na^{2+} caused the enzyme activity to increase by 100% residual activity in both FHS and SS. Cellulase of stored sugarcane have increased activity compared with cellulase of freshly harvested sugarcane since it exhibited a very low K_m.

Keywords: Cellulase, purification, pH, sugarcane, specific activity, temperature

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INTRODUCTION

Cellulase is important and essential in the conversion of cellulose into fermentable sugar (Li et al., 2009). This is a complex enzyme consisting of endo-*β*-glucanase (EC 3.2.1.4), exo-*β*-glucanase (EC 3.2.1.91) and *β*-glucosidase (EC 3.2.1.21) the combined action of these three enzymes components is necessary when cellulose is to be hydrolysed (Gielkens et al., 1999; Lin et al., 2009; Balasaravanan et al., 2013).

Cellulase enzymes are important in industrial applications, especially in alcoholic fermentation, malting and brewing, in the textile industry for softening of cotton and denim finishing, in the laundry industry for the production of detergents, fibre modification, in the pulp and paper industry and extraction of fruit and vegetable juices (Hanif et al., 2004; Zhou et al., 2008).

Cellulose is considered the most abundant biomass on the earth; it is made up of long polymers of glucose units with β 1-4, linkage (Shallom & Shoham, 2003; Venkata et al., 2013). Cellulose accounts for half of the dry weight of plant biomass and secondary sources of agricultural wastes (Haruta et al., 2003). There are huge quantities of agricultural and industrial cellulosic waste which has attracted global attention for its use as a renewable resource for conversion to bio-based products and bioenergy (Li et al., 2009). Cellulose degradation by microorganisms converts cellulose through acid or enzymatic hydrolysis into soluble sugars. This shows microbial cellulose utilisation is very important in the largest material flows of the biosphere. However, there are still large quantities of cellulosic materials that are yet to be exploited (Lynd et al., 2002; Sethi et al., 2013).

The sugarcane bagasse is one of the most abundant agricultural wastes and a possible energy source when considering the production of second-generation ethanol since sugarcane bagasse is rich in hydrolysable polysaccharides (UNICA, 2012). Sugarcane bagasse contains 45–55% cellulose, 20–25% hemicellulose, 18–24% lignin, 1–4% ash and less than 1% wax (Thomas, 2009). The cellulosic and lignocellulosic residue is an attractive feedstock for ethanol production as it is available in large amounts and at a low cost. The conversion of waste materials to fuels and chemicals has an economic value while reducing environmental impacts (Pereira et al., 2008).

To the best of the present authors' knowledge, this is the first study to examine the effect of post-harvest storage of sugar cane stem on the cellulase activity of sugar cane. This study was designed to evaluate the cellulase activity of sugar cane affected by postharvest storage of its stem at room temperature.

MATERIALS AND METHODS

Material

Mature sugarcanes *Saccharum officinarum* with no blemish were freshly harvested from a local farm in Ile-Ife, Osun state, Nigeria.

Sample Preparation

The freshly harvested sugar cane stem was transported to the laboratory in an ice container where they were washed in cold saline and divided into two lots, one was stored at room temperature 28±2°C for five days termed stored sample (SS) while the other was used immediately and termed freshly harvested (FHS).

Preparation of crude enzyme

The sugar canes were scrapped gently and then cut into bits. One hundred grams (100 g) of sugar cane was homogenised in a Warring Blender using three volumes of 0.05 M citrate buffer of pH 4.8. The homogenate was filtered using a double layer cheese cloth then centrifuged using Centurion cold centrifuge (R-1880) at 4000 rpm for 30 minutes at room temperature. The aliquot of the supernatant was assayed for its cellulase activity and to determine its protein concentration. The supernatant was then precipitated with 80% ammonium sulphate.

Enzyme Assay

Cellulase activity was measured based on Zhang et al. (2006). One millilitre (1.0 ml) of enzyme extract, 1.5 ml of 1 % viscous carboxymethyl cellulose were mixed in 0.05 M citrate buffer of pH 4.8. The mixture was incubated in a water bath at 50°C for 1 hour. The experimental and control tubes underwent incubation at the same temperature and time. 2 ml of 3,5- dinitrosalicylic acid (DNSA) reagent was added to terminate the reaction. The mixture was boiled for 30 minutes, cooled and optical density was measured at 540 nm. One unit of cellulase activity is the amount of enzyme that released a reducing sugar equivalent to 1 µmol glucose per minute under the specified assay conditions. A standard calibration curve of glucose was made and used for the estimation.

Determination of Protein Concentration

The protein concentration was measured Bradford (1976) and Bovine Serum Albumin (BSA) was used as the standard.

Ammonium Sulphate Precipitation

Ammonium sulphate $(NH_4)_2SO_4$ salt was added to the crude enzyme, stirred gently until the whole salt had completely dissolved in the supernatant; this brought the supernatant to 80% $(NH_4)_2SO_4$ saturation (560 g/L). The mixture was kept in the fridge overnight at 4°C; it was then centrifuged at 4,000 x g for 30 minutes at room temperature. The precipitate was collected and re-suspended in 0.1 M phosphate buffer of pH 7.2. The ammonium sulphate precipitate was then desalted using sephadex G 25 to remove the ammonium sulphate salt.

Determining Kinetic Parameters

The desalted fraction from $(NH_4)_2SO_4$ precipitation was then used for the kinetic studies. The kinetic parameters (K_m and V_{max}) of the enzyme were determined by

varying concentrations of 1% carboxymethyl cellulose and measuring the initial reaction velocities (µmol of glucose/min) at 50°C for 1 hour. The reaction mixture contained varying concentration between 0.1 ml and 1.0 ml of CMC solution and cellulase activity was determined as earlier described. Plots of (1/V) versus 1/[S] were made according to Lineweaver and Burk (1934).

Effect on some Cellulosic Substrates

The substrate specificity of the enzyme was determined using different compounds that include: orange peel, banana peel, maize starch, sugarcane bagasse, maize cob and apple pomace in a typical cellulase assay mixture. The percentage activity of the enzyme was measured using CMC as the control.

Effect of pH on the Enzyme Activity

The effect of pH on the enzyme activity was measured using the method proposed by Agboola and Okonji (2004). Fifty mili molar (50 mM) of citrate buffer with pH 3-5; 50 mM of phosphate buffer with pH 6-8 and 50 mM of borate buffer with pH 9-10 were used. The cellulase activity was assayed as described in the enzyme assay section.

Effect of Temperature

The varying temperatures between 40°C and 100°C were used to investigate the effect of temperature on the enzyme activity and to measure its optimum temperature. The assay mixture was initially incubated at the temperature above for 10 minutes; the reaction was then initiated with the addition of an aliquot of the enzyme which had been equilibrated at the above temperature. The cellulase activity was assayed as previously described (Zhang et al., 2006).

Effects of Cations on the Enzyme Activity

The effects of various cations on the activity of cellulase were carried out. The cations tested were Ca^{2+}, Na^{2+}, Ba^{2+}, Mn^{2+} and K^{+} at 1.0 mM and 10 mM in a typical cellulase assay mixture. The chlorides of the metals were dissolved in distilled water. The control has no cations and it has 100% activity.

RESULTS

Tables 1 and 2 show the results of partial purification of cellulase from both freshly harvested sugarcane (FHS) and stored sugarcane (SS) respectively. The specific activity of enzyme after partial purification using ammonium sulphate precipitation and dialysis were found to be 136.52U/mg and 184.53U/mg for FHS and SS respectively.

Table 1
Purification table for cellulase of freshly harvested sugarcane FHS

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification fold
Crude extract	36.1	4160	115.24	100	1
80% $(NH_4)_2SO_4$ precipitation	10.76	1469	136.52	35	1.19

Table 2
Purification table for cellulase of stored sugarcane SS

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification fold
Crude extract	46.8	6689	142.9	100	1
80% $(NH_4)_2SO_4$ precipitation	17.9	3303	184.53	49.4	1.19

Kinetic Parameters

The Lineweaver-Burk plot to determine the kinetic parameters (K_m and V_{max}) of cellulase from freshly harvested and stored sugarcane for carboxymethyl cellulose (CMC) are presented in Figures 1 and 2 while the values obtained from the figures are presented in Table 3. It showed that FHS has Km of 0.540 and V_{max} of 38.02 while SS has Km of 0.09 and V_{max} of 40.98

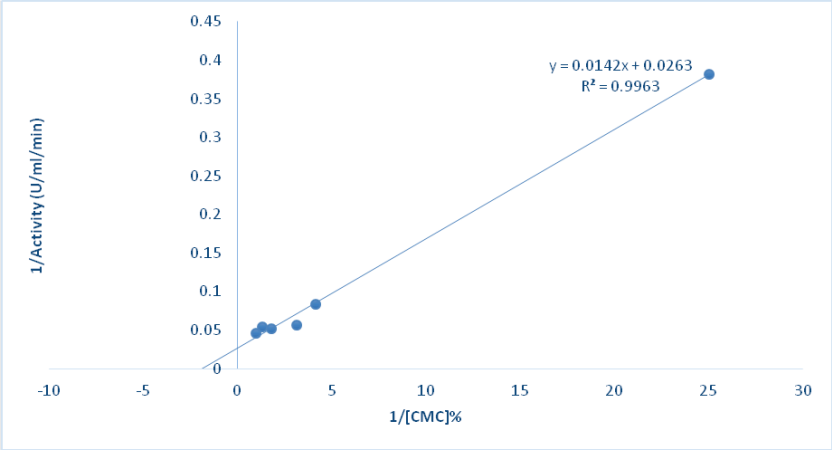

Figure 1. Lineweaver-Burk plot of 1/V against 1/[S] at varying concentration of CMC for FHS

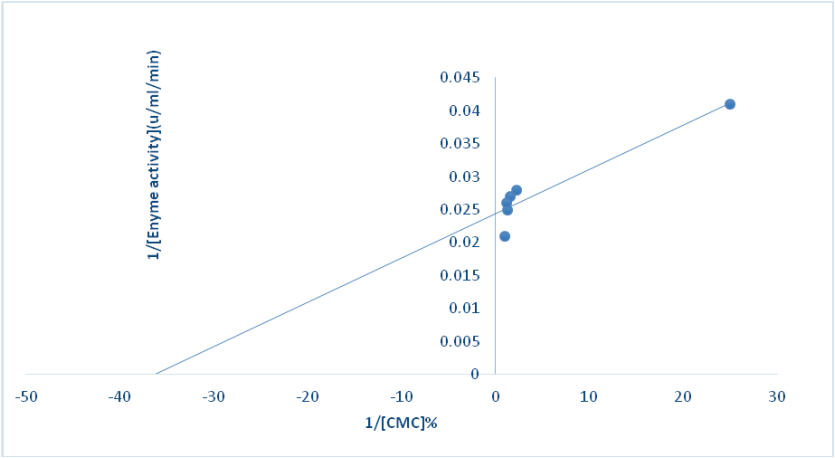

Figure 2. Lineweaver-Burk plot of 1/V against 1/[S] at varying concentration of CMC for SS

Table 3
Summary for the kinetic parameter of cellulase for FHS and SS

Substrates	Km	Vmax
CMC(FHS)	0.540	38.02
CMC (SS)	0.09	40.98

Effect on other Cellulosic Substrates

The results of enzyme specificity are shown in Figure 3 and Figure 4 for FHS and SS respectively. The enzyme from FHS showed 100% activity with banana peel and sugar cane bagasse while the enzyme from SS showed 100% activity with orange peel, banana peel and sugar cane bagasse.

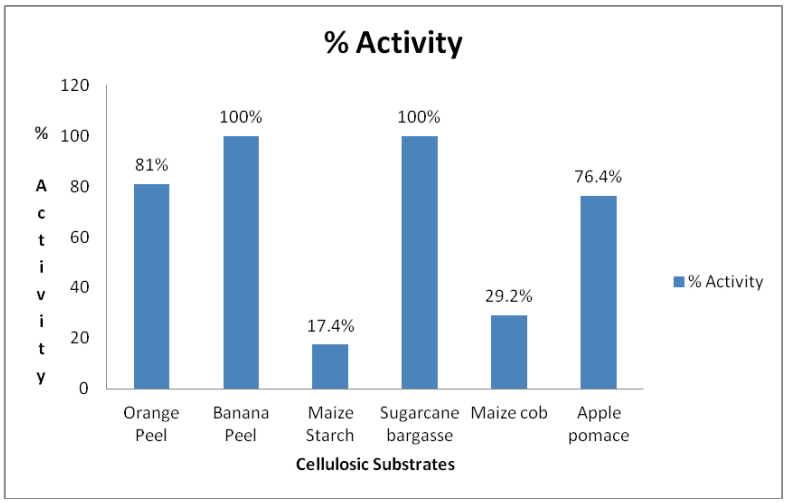

Figure 3. % substrate specificity of cellulase of freshly harvested sugarcane FHS

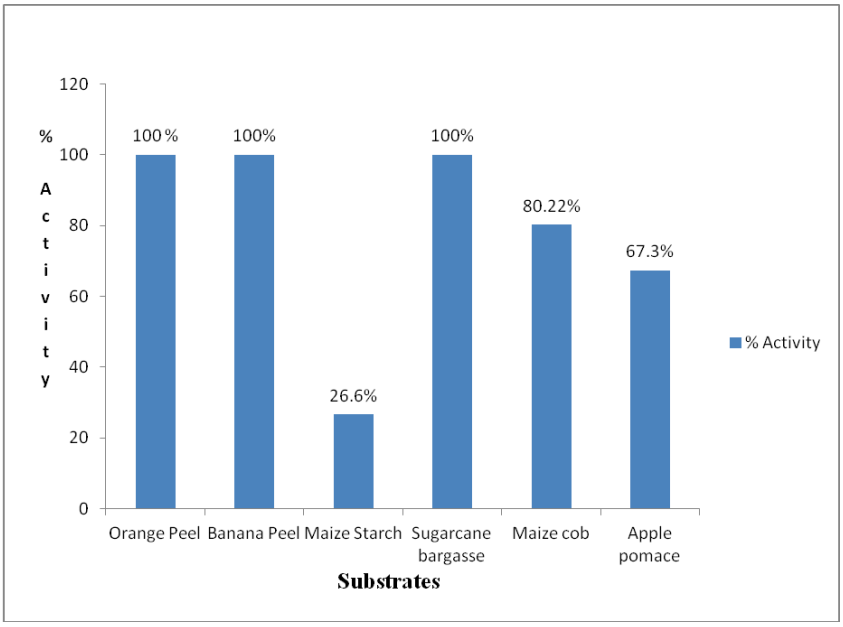

Figure 4. % substrate specificity of cellulase of stored sugarcane SS

Effect of Temperature

Partially purified cellulase of both freshly harvested FHS and stored sugarcane SS exhibited a maximum activity at 40°C as shown in Figures 5 and 6.

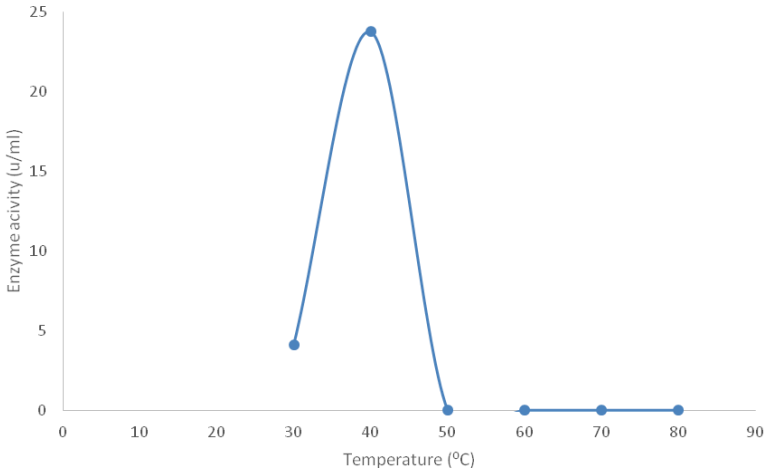

Figure 5. Effect of temperature on freshly harvested sugarcane FHS cellulase

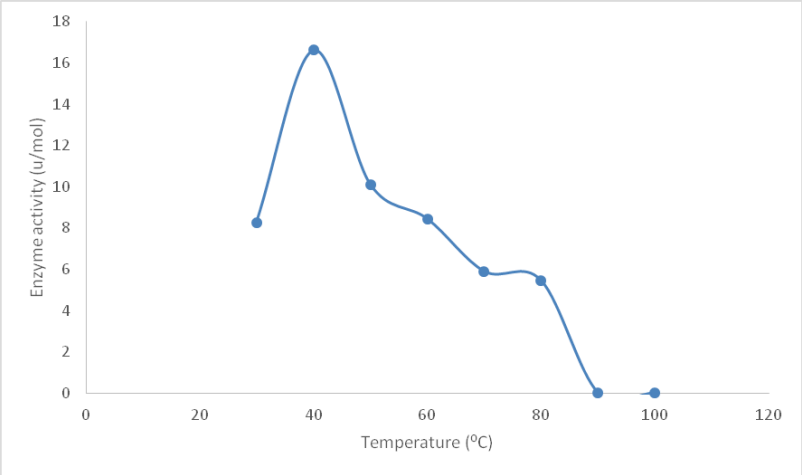

Figure 6. Effect of temperature on stored sugarcane SS cellulase

Effect of pH

The cellulase activity of freshly harvested sugarcane (FHS) exhibited an optimum pH of 4.0 while that stored sugarcane (SS) showed an optimum pH of 7.0 as seen in Figures 7 and 8 respectively.

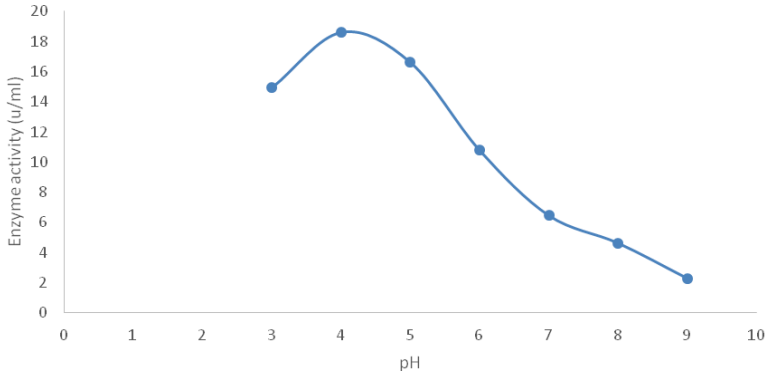

Figure 7. Effect of pH on cellulase of freshly harvested sugarcane FHS
Enzyme assay at different buffers and pH: 50 mM citrate buffer (pH 3-5); 50 mM phosphate buffer (pH 6-8) and 50 mM borate buffer (pH 9-10)

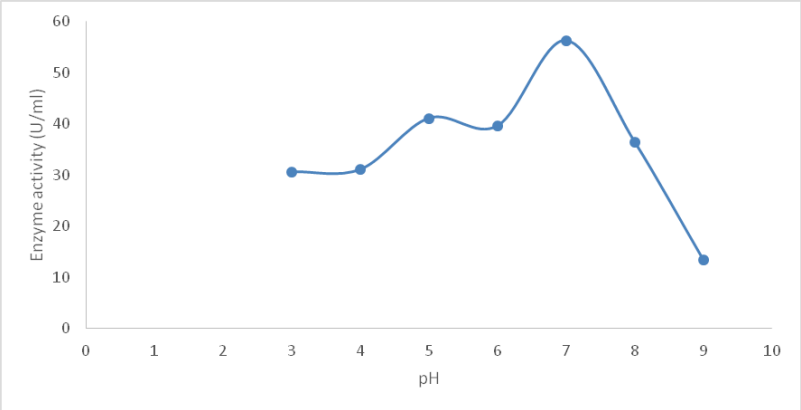

Figure 8. Effect of pH on cellulase of stored sugarcane SS
Enzyme assay at different buffers and pH: 50 mM citrate buffer (pH 3-5); 50 mM phosphate buffer (pH 6-8) and 50 mM borate buffer (pH 9-10)

Effects of Metallic Salts on the Enzyme Activity

The result of the effect of chloride salts shown in Table 4, indicated the activity of cellulase from FHS and SS were not inhibited by chloride salts.

Table 4
Effect of chloride salts on activity of cellulase of freshly harvested sugarcane FHS and stored sugarcane SS

Chloride salts	FHS % Residual activity		SS % Residual activity	
	1mM	10mM	1mM	10mM
$CaCl_2$	100	100	100	100
NaCl	98.79	100	100	100
$BaCl_2$	67.67	78.43	70.54	76.32
$MnCl_2$	62.45	76.04	69.45	74.86
KCl	76.76	85.43	76.32	83.96

DISCUSSION

In postharvest, enzymes can be active and their activity can positively or negatively influence organoleptic characteristics of fruits. It is imperative to understand the different reactions the enzymes catalyse in plant tissues, so as to exploit their advantages and avoid their undesirable effects (Tomás-Barberan & Espin, 2001). Sugarcane suffers postharvest losses in recoverable sugar as a result of deterioration of stale cane (Solomon, 2009).

The specific activity of partially purified cellulase using 80% ammonium sulphate precipitate obtained from FHS and SS samples were 136.52 units/mg protein and 184.53 units/mg protein respectively. The SS had a higher specific activity of cellulase

than the FHS. Some enzyme activities have been reported to increase when fruits are stored at ambient temperature. Barrelt and Gonzalez (1994) reported increase in Polygalacturonase activity in stored cherry, also acid invertase activity has been reported to have 1.5 - 7.0-fold increase in SS (Solomon et al., 1990; Batta & Singh 1991; Saxena et al., 2010). The increase in the activity of cellulase enzyme in SS may be due to physiological activities that continue in fruit after harvesting, which is the breakdown of cellulose to glucose for the sustenance of the sugarcane and this required cellulase enzyme in the conversion of cellulose to glucose. Physiological activities continue in all plant crops following harvesting (Rhodes, 1980). The increase in cellulase activity can also be attributed to stalling and senescence, also exogenous cellulase of bacteria origin as a result of infections of the sugarcane stem by microorganism that breakdown cellulose with the help of cellulase enzyme to supply their carbon source.

The K_m of the partially purified cellulase from freshly harvested sugarcane was 0.540 mg/ml which showed that the cellulase from sugarcane has affinity for carboxymethyl cellulose (CMC) as substrate. The K_m values of cellulase from different sources using CMC as substrate have been reported, Enokibara et al., (1991) 0.28% for cellulase from *Favouls arcularicas*, Busto et al., (1996) 1.32% from T. reesei, Begum & Absar (2009) 0.83% from *Aspergillus oryzae* and Bakare et al., (2005) 3.1mg/ml from *Pseudomonas fluorescens*.

The difference in K_m value of cellulase enzyme may be due to the different sources of its isolation. The K_m value of cellulase in this work for SS was found to be 0.09 mg/ml which showed that the cellulase from SS has a higher affinity for carboxymethylcellulose (CMC); this is expected as the cellulase enzymes in SS are more active metabolically in breaking down cellulose than in FHS. Since the cellulase of the SS exhibited the highest enzyme activity.

The optimum temperature for the partially purified cellulase of FHS and SS was at 40°C. The optimum temperature for the activity of cellulase enzyme varies; Bakare et al., (2005) reported 35°C for cellulase from *Pseudomonas fluorescens*. Fagbohunka et al. (2012) reported 30°C for cellulase from the haemolymph of giant African snail (*archachatina marginata*) while 60°C was the optimum temperature of cellulase of the peel and corm of *Amorphophallus paeoniifolius* (Singh et al., 2014, 2015). The temperature at which enzymes performed optimally depends on the temperature of the environment at which the source of the enzyme thrives best. When the temperature is higher than the optimum temperature, the enzyme gets denatured and the activity decreases (Bakare et al., 2005).

Partially purified cellulase from freshly harvested sugarcane exhibits an optimum pH of 4 while cellulase from SS has an optimum pH of 7. The enzyme functions within the pH range of 3 to 8. The optimum pH of 4 obtained for cellulase of FHS may be due to the physiological state of a fresh sugar cane. The optimum pH of 7 for

cellulase of stored sugarcane may be due to the physiological activities in SS which has resulted in deterioration of the cane. Lionnet (1986) observed that during deterioration of sugarcane, sucrose is lost, lactic acid and ethanol production increases - which could make the pH tends towards neutral.

The substrate specificity of the partially purified cellulase enzyme from FHS and SS was tested using various carbon sources such as orange peel, banana peel, maize starch, sugarcane bagasse, maize cob and apple pomace. In this study, it was found out that when maize starch was used as a carbon source, very little cellulase activities were detected, 17.4% for FHS and 26.6% for SS, whereas higher activities i.e. 100% were detected when orange peel, banana peel and sugarcane bagasse were used as a carbon source in both FHS and SS. Cellulase production is a big factor in the degradation of cellulosic material and it is essential to ensure cellulase production economically viable. The cost of the substrate is very important in the economics of an enzyme production; therefore, different substrates can be utilised for cellulase production for comparison (Ahmed et al., 2009). The differences in the activities of cellulase in these cellulosic materials may be due to the physicochemical constituents of these carbon sources. Physicochemical composition of agro-residues such as cellulose, hemicellulose, lignin, nitrogen, and minerals could influence enzyme activities, so also is the presence of an activator or an inhibitor in the agro-residues and the diffusion of the catabolite (Patagundi et al., 2014)

Chloride salts of calcium, sodium, barium, manganese and potassium improved the residual activity of the enzyme. This improvement in the residual activity of cellulase has been reported by Fagbounka et al. (2012) on the cellulase from the haemolymph of giant African snail (*Archachatina marginata*).

CONCLUSION

In summary, this study showed the presence of cellulase activity in FHS and SS. Cellulase of SS showed an increased activity compared with cellulase of FHS), this shows that storing sugarcane at room temperature 28±2°C causes increase in the activity of the cellulase enzyme. The present study thus shows that cellulase from SS is suitable for commercial applications.

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