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Adansonia digitata (Baobab) fruit pulp as substrate for *Bacillus* Endoglucanase production

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In recent years the cost of carbon source for microbial enzymes production has necessitated a drive towards cheaper and sustainable sources. In this study, *Adansonia digitata* (Baobab) fruit pulp was utilized as substrate for *Bacillus* endoglucanase production and the effects of varying temperature, pH, incubation period, inoculum size and substrate concentration on endoglucanase production were investigated. The cellulolytic activity of the isolates was screened base on halo of clearance on Carboxymethyl cellulose agar, while the endoglucanase activity of the selected *Bacillus* species was monitored using 3, 5 - dinitrosalicylic acid (DNS) method. Out of 4 *Bacillus* species screened, only *Bacillus amyloliquefaciens* and *Bacillus subtitis* showed cellulolytic activity with *B. amyloliquefaciens* having the highest activity of 20.0 mm. Therefore *B. amyloliquefaciens* was selected for studies on the effect of fermentation conditions on endoglucanase activity. The maximum yield of endoglucanase was produced at temperature of 55 °C, pH 2, 1% inoculum size and 1% substrate concentration for 4 days incubation period. The results of this study suggest that *Adansonia digitata* fruit pulp can be harnessed at low concentration for large scale endoglucanase production by *Bacillus*, and the endoglucanase produced by *B. amyloliquefaciens* could be a thermostable enzyme with novel characteristics suitable for application in biofuel and textile industry.

Keywords: *Adansonia digitata*, *Bacillus amyloliquefaciens,* cellulase, Endoglucanase. fermentation

In recent years the high cost of carbon sources for industrial production of microbial enzymes has necessitated a shift to other cheaper sources of carbon (Ajayi and Fagade, 2003). Plants are the most common source of renewable carbon and energy on the earth with annual cellulose production estimates of about 4 x 10^9 tons (Coughlan and Mayer, 1990). Cellulose is world's most abundant organic substance (Ruttloff, 1987) and comprises a major storage form of photosynthesized glucose. It is the major component of biomass energy (Scott *et al.*, 1987). Because a large proportion of vegetation added to soil is cellulose therefore, decomposition of cellulose has a special significance in the biological cycle of carbon (Lederberg, 1992).

Cellulase is a multienzyme system composed of several enzymes with numerous isozymes, which act in synergy (Grassin and Fauquembergue, 1996). The basic enzymatic

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process for the depolymerization of cellulose requires three types of enzymes: Endoglucanase (EG or C_X), hydrolyses internal β-1,4 glucan chain of cellulose at random, primarily within amorphous regions and displays low hydrolytic activity towards crystalline cellulose (Walsh, 2002; Grassin and Fauquembergue, 1996); Exoglucanase i.e., exoacting cellobiohydrolases (CBH), removes cellobiose from the non-reducing end of cello-oligosaccharide and of crystalline, amorphous and acid or alkali treated cellulose; alpha-Cellobiase or β-glucosidase (BGL), hydrolyses cellobiose to yield two molecules of glucose which completes the depolymerization of cellulose (Himmel *et al.*, 1994).

Cellulases have enormous potential in industrial applications. Glucose produced from cellulosic substrate are further used as substrate for subsequent fermentation or other processes which could yield valuable end products such as ethanol, butanol, methane, amino acid, single-cell protein etc., (Walsh, 2002). Cellulases have been used for several years in food processing, feed preparation, waste-water treatment, detergent formulation, textile production and in other areas. Additional potential applications include the production of wine, beer and fruit juice. Nevertheless, all these uses are of rather small magnitude compared with cellulase requirements for bioconversion of lignocellulosic biomass to fuel ethanol (Philippidis, 1994).

Cellulases were initially investigated several decades back for the bioconversion of biomass. With the shortage of fossil fuels and the arising need to find alternative sources of renewable energy and fuels, there is a renewed interest in the bioconversion of lignocellulosic biomass using cellulases and other enzymes. Many attempts have been made to produce low-cost cellulases which lead to several works on the use of substrates such as corn cobs, wheat straw, sugar cane bagasse, aspen wood, and waste from newspaper industry (Liming and Xueliang, 2003). Lignocellulosic materials are being highlighted as the next substrates in endoglucanse production (Thangaswamy *et al*., 2011). *Adansonia digitata* (Baobab) fruit pulp is among the most common but underutilized fruits in Africa. Considering the abundance of *Adansonia digitata* in Northern Nigeria and the importance of cellulases, this study was aimed at screening indigenous *Bacillus* isolates from fadama soil for cellulolytic ability and providing a better understanding of conditions for the production and activity of cellulases by *Bacillus amyloliquefaciens* using *Adansonia digitata* (Boabab) fruit pulp as substrate.

Materials and Methods

Sample collection

One hundred grams of *Adansonia digitata* fruit pulp was purchased in Mabera area within Sokoto metropolis, Nigeria.

The *Bacillus* species were previously isolated from fadama soil within Usmanu Danfodiyo University Permanent site using standard procedures as described by Oyeleke and Manga. (2008) and identified following series of biochemical test as described by Holt *et al*. (1994).

Preparation of Inoculum

The inoculum was prepared as described by Ibrahim *et al*. (2011b). It was prepared by suspending the young active colonies from the culture into sterile distilled water, by serial dilution and plating, the number of viable colonies in the inoculum was found to be 2.2×10^6 CFU/ml was used as inoculum for the subsequent studies.

Screening for cellulase production

Carboxyl methyl Cellulose (CMC) agar plates were prepared by enriching nutrient agar with 1% CMC using the method of Maki *et al.* (2011). The isolate was spread on the solidified CMC agar and was incubated for 48 hrs to express cellulose depolymerization through cellulase production into its surrounding medium. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 M NaCl (Maki *et al.*, 2011). To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on CMC agar were measured.

Production of cellulase enzymes

The two bacteria species were separately grown and tested for production of cellulase in submerged culture in a salt medium, containing 0.01% $MgSO_4$, 0.1% $(NH4)_2SO_4$, 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.05% Na-citrate, supplemented with 0.1 % *Adansonia digitata* fruit pulp as carbon source. The cultures were grown at 37^oC for 24 h. Culture broth was sampled at different times during growth to determine cellulase production by measurement of absorbance at 540 nm.

Effect of fermentation conditions

The effects of fermentation conditions such as pH, temperature, inoculum size, incubation period and substrate concentration were studied as described by (Bertrand *et al.* 2004). Initial pH of the medium was set at 2.0, 3.0, 4.0, 5.0, and 6.0 using 1 N HCl or 1 N NaOH (at constant inoculum size of 1%, temperature 55 ^oC, incubation period of 3 days and 1% substrate concentration). Based on the results of this experiment a pH of 2.0 was adopted in subsequent experiments. The inoculum size of 1, 2, 3, 4 and 5%; temperatures 35 ^oC, 45 ^oC, 55 ^oC, and 65 ^oC, incubation periods of 1, 2, 3, 4 and 5 days and substrate concentration (*Adansonia digitata* fruit pulp) of 1, 2, 3, 4 and 5% were studied using the same procedures. At certain intervals the fermentation medium was agitated. Each assay was carried out in duplicate and the mean of the duplicate analysis was reported in each figure.

Endoglucanase assay:

Endoglucanase activity was determined by the method of Mandels *et al.*, (1976). Endoglucanase activity was measured by monitoring the amount of reducing sugar liberated from carboxyl methylcellulose (CMC). Endoglucanase activity was assayed by adding 1ml of enzyme (fermented broth supernatant) to 0.5 ml of 1% (w/v) CMC as a substrate and incubated for 30 min at 37 ^oC in a water bath. The reaction was stopped by adding 1 ml of 3, 5 dinitrosalicylic acid, followed by boiling for 10 min. The final volume was made to 5 ml with distilled water and the absorbance due to the produced 3-amino, 5- nitrosalicyclic acid was measured at 540 nm with a spectrophotometer (Jenway 6100). One unit of enzyme activity was expressed as the amount of enzyme required to release one micromole of reducing sugar / mL under the standard assay condition.

Results

The cellolulytic activity of the *Bacillus* species based on the zone of clearance on carboxymethylcellulose (CMC) agar was measured and presented in Table 1. *Bacillus amyloliquefaciens* had the highest cellolulytic activity of 20.0 mm, followed by *Bacillus subtilis* with zone of 18.0 mm while *Bacillus brevis* and *Bacillus firmus* had no zone of clearance.

Bacillus amyloliquefaciens which showed the highest zone of clearance was selected for further study on the effect of temperature on the activity of endoglucanase produced the result presented in Figure 1. The result shows that the cellulase yield was maximum at 55ºC with a reducing sugar concentration of 0.45 mg/ml and showed slight reduction in the activity at 65ºC with reducing sugar concentration of 0.41mg.

The effect of pH on the endoglucanase activity of *Bacillus amyloliquefaciens* was examined at various pH values ranging from 2 to 6 as shown in Figure 2. The maximum activity was displayed at pH 2 with a reducing sugar concentration of 0.20 mg/ml and the least activity was displaced at the pH of 5 with a reducing sugar activity of 0.14mg/ml.

Figure 3 shows a gradual increase in endoglucanase activity through 24, 48, 72 and maximum at 96 hours with reducing sugar concentration of 0.43mg/ml.

The endo-glucanase activity showed a gradual decrease on further extension of incubation period beyond 4 days. There was no increase in the endoglucanase activity when the size of the inoculum was increased from 1 - 5% (Figure 4). The maximum cellulase activity was 0.43 mg/ml when 1% of inoculums were used. After that, there was a gradual decrease in endoglucanase activity to 0.39 mg/ml when inoculums size was increased to 5%.

The effect of substrate concentration was shown in Figure 5. From the figure substrate concentration of 1% gave the highest endoglucanase activity with reducing sugar value of 0.44 mg/ml while the substrate concentration of 5% gave the lowest endoglucanase enzyme of 0.24 mg/ml yield from *Bacillus amyloliquefaciens*.

Table 1: Result of cellulase activity of the identified *Bacillus* species

Bacterial species	Diameter of zones of clearance (mm)
B. amyloliquefaciens	20.0
B. subtilis	18.0
B. brevis	-
B. firmus	-

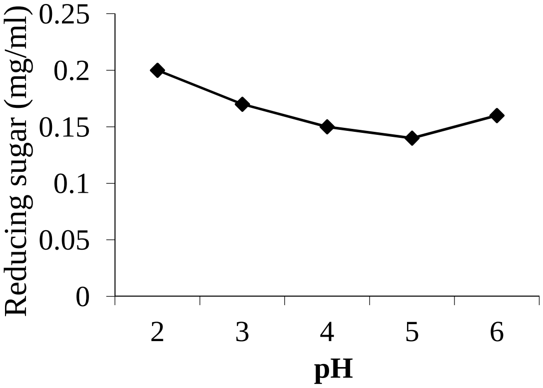

Figure 2: Effect of pH on endoglucanase produced by *B. amyloliquefaciens*

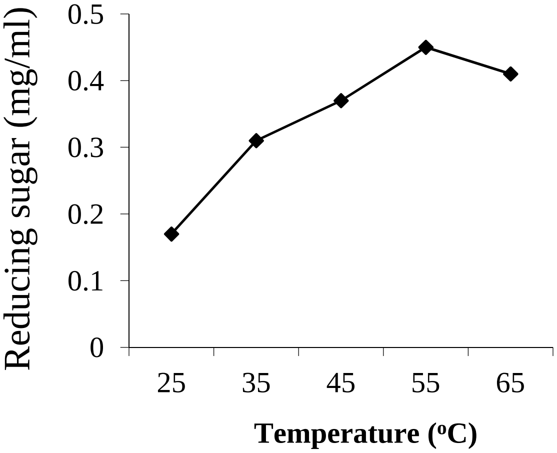

Figure 1: Effect of temperature on endoglucanase enzyme produced by *B.amyloliquefaciens*

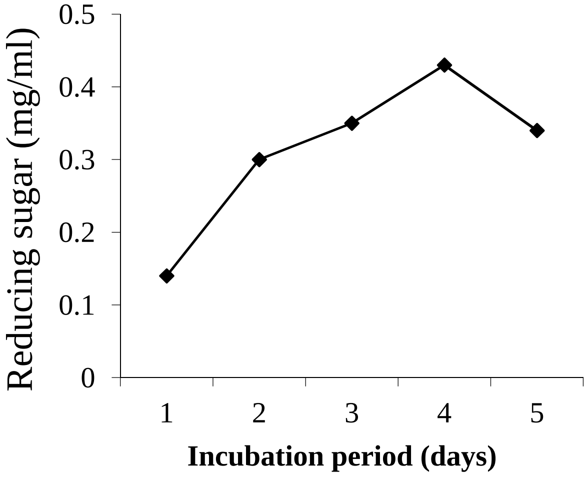

Figure 3: Effect of incubation period on endoglucanase produced by *B. amyloliquefaciens*

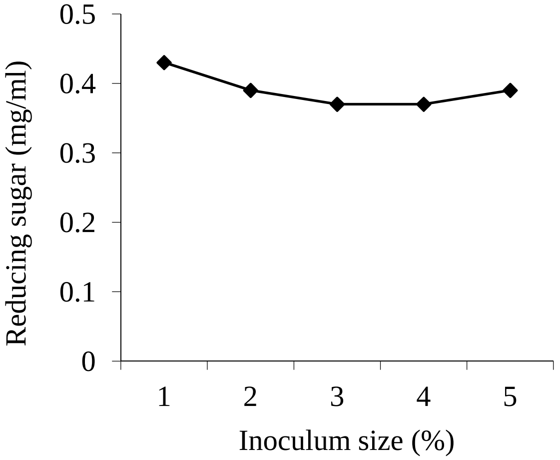

Figure 4: Effect of inoculum size on endoglucanase produced by *B. amyloliquefaciens*

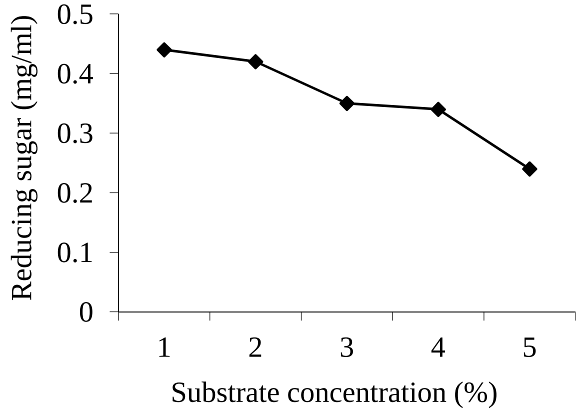

Figure 5: Effect of substrate concentration on endoglucanase produced by *B. amyloliquefaciens*

Discussion

The cellolulytic activity of *B. amyloliquefaciens* in this study is somewhat greater than *Bacillus subtilis.* This variation in cellolulytic activity produced by these species may be due to differences in their genetic makeup. Ibrahim *et al*. (2011a) observed similar result on *Bacillus* spp screened for amylase production. *B. amyloliquefaciens* has been reported to produce endoglucanase (Thangaswamy *et al.*, 2011). This *B. amyloliquefaciens* with high cellolulytic activity could be a potential candidate for large scale endoglucanase production.

The *B. amyloliquefaciens* in this study showed maximum activity within reported temperature range (55 ^{o}C). The reported alkaline cellulases from *Bacillus* sp. present an optimum activity from 40 to 70°C (Hakamada *et al.*, 1997; Ito, 1997; Christakopoulos *et al.*, 1999; Mawadza *et al.*, 2000; Kim *et al.*, 2005). This shows that the endoglucanase produced by *B. amyloliquefaciens* is a thermophilic endoglucanase with promising potentials for exploitation by industries or processes that operate at this temperature.

Enzymes have pH range within which they function best with their activity being maxima at the optimum pH and decreased at higher or lower pH values (Lehninger, 1993). The endoglucanase in this study showed an optimum pH which was found to be acidic (pH 2). A slightly near neutral pH (pH 4.8) has been reported (Vries and Visser, 2001; Thangaswamy *et al.*, 2011). This implies that endoglucanase from *B. amyloliquefaciens* holds commercial value for industries that carry out their operations at acidic pH.

The effect of incubation period on endoglucanase production increased progressively and attained the peak activity at the fourth day of incubation and declined on the fifth day. The current result slightly differs from that of Thangaswamy *et al.* (2011) who reported peak activity at the first three days. The decline in endoglucanase activity on 5th day may be explained by the fact that at this stage the isolates have entered their late stationary phase. In other words, the isolates have a long lag and initial stationary phase. Production of enzymes is usually initiated during the log phase of the growth and reaches maximum levels during the initial stationary phase (Sudharhsan, *et al.*, 2007). Even though extra cellular enzymes are produced from log phase to initial stationary phase, within the phases the production may vary.

Decrease in endoglucanase activity with further increase in inoculum size might be due to clumping of cells which could have reduced sugar and oxygen uptake rate, and also enzyme release and may probably be due to limiting nutrients at higher inoculum size (Dhanya *et al*., 2006).This implies that one percent of this isolate is needed for maximum production of cellulase which makes inoculum development and viability much easier in an industrial process.

Further increase in *Adansonia digitata* fruit pulp beyond 1% did not result in proportionate increase in endoglucanase yield. Mandels and Reese (1959) also reported that maximum yield of cellulase were obtained on 1% substrate. This may be due to the fact that certain sugars are inhibitors of enzyme production while others stimulate enzyme production. Ibrahim *et al.* (2011a) reported that *Adansonia digitata* fruit pulp inhibited the production of amylase by *Bacillus licherniformis*.

Conclusion

The study shows that *Bacillus subtilis* and *Bacillus amyloliquefaciens* from indigenous fadama soil can produce cellulase when cultured on *A. digitata* fruit pulp as substrate and the optimum conditions for cellulase production are at a temperature 55 °C, pH 2, 1% inoculum size and substrate concentration for 4 days incubation period. The results of this study suggest that *Adansonia digitata* fruit pulp can be harnessed at low concentration for large scale bacillus cellulase production, and the cellulase produced by *B. amloliquefaciens* could be a thermostable enzyme with novel characteristics suitable for application in biofuel and textile industry.

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