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Optimization of Culture Condition in the Production of Bioenzymes by Bacteria Isolated from Poultry Waste in Sokoto State, Nigeria

[1]Hauwa, H., [2]S.B. Oyeleke and [3]Adamu, Shahidah Ahmed

[1]Faculty of Science, Department of Microbiology. Faculty of Science. Sokoto State University, Sokoto.

[2]Faculty of Science, Department of Microbiology. Federal University of Science And Technology Minna , Niger State.

[3]Department of Microbiology. Faculty of Science.Sokoto State University, Sokoto
(08069303535) h_hauwau@yahoo.com

Abstract

Poultry wastes obtained from a poultry farm in Sokoto metropolis were analyzed for cellulose producing bacteria. Bacillus megaterium, Bacillus laterosporus, and Bacillus amyloliquifeciens isolated were screened for their ability to produce cellulase enzyme. All the isolates showed cellulose activity by exhibiting a wide halo on caboxymethylcellulase medium (CMC).The fermentation process was optimized using the following parameters, inoculum size, pH, Substrate concentration, temperature, and incubation periods. Cellulase activity was determined using DNS method, Banana peels was used as a substrate for the production of the enzymes, this was analysed with atomic absorbtion spectrophotometer (AAS).Cellulase enzyme was produced at innoculum size 1, 2, 3, 4, and 5%. pH 3, 5, 7, 9, and 11. Substrate concentration 1g, 2g, 3g, 4g, and 5g. Temperature 35, 45, 55, 65, and 75, for 1, 2, 3, 3, and 5, days respectively. Bacillus laterosporus recorded the highest cellulase activity 0f 0.37mg/ml in 5% substrate concentration among all the isolates while Bacillus amyloliquefeciens recorded highest cellulose production at pH3 with 45mg/ml Bacillus laterosporus recorded highest activity of cellulose production with 0.71mg/ml Temperature was also studied in the cellulose production and Bacillus laterosporus showed highest activity at 75^{0}C with activity of 0.66mg/ml. This study showed that Bacillus laterosporus was the best cellulase producing bacteria among all the isolates.

Key words: *poultry wastes, cellulase, Banana peels, Bacteria, Enzymes.*

INTRODUCTION

Cellulose is the principal constituent of the cell wall of most terrestrial plants. The source of cellulose is in plants and it is found as micro-fibrils (2-20nm in diameter and 100 – 40,000nm long). These form the structurally strong frame work in the cell walls. Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulose-containing raw materials and waste products that are not exploited or which could be used more efficiently. Various biomass inducing residues including lignocellulosic material, paper waste, pulses, cereals straw and bagasses have been widely used as carbon sources for commercial cellulose fermentation Brijwani, and Vadlani, (2011), Wen, *et al.*, (2005) Belghith, *et al.*, (2001).

The problem in this respect is however to develop processes that are economically profitable. Cellulose-containing wastes may be agricultural, urban, or industrial in origin, sewage sludge might also be considered a source of cellulose since its cellulosic content provides the carbon needed for methane production in the anaerobic digestion of sludge. Agricultural wastes include crop residue, animal excreta and crop processing wastes slashing generated in logging, saw dust formed in timber production and wood products in forestry originated activities (Pranner, 1979).

The previous negative attitude in which wastes were viewed self consciously as valueless and even offensive and for disposal only has been replaced in large part by a positive view in which wastes are recognized as raw materials of potential value (Pranner, 1979). Currently, there are two major ways of converting cellulose to glucose: chemical versus enzymatic. Enzymatic hydrolysis of cellulose is an important reaction in nature for it marks the first step in the decay of

cellulose, the most abundantly occurring organic In the early 1970s, the oil crisis generated interest in using cellulose as a chemical and energy resource. One promising approach was to hydrolyze the cellulose to glucose with fungal enzymes and then to ferment the glucose to ethanol which could be used as a liquid fuel (Mandels *et al.*, 1974).

Sources of Cellulase

Cellulases are easily obtained from microbial and fungal sources, but vertebrates lack the ability to produce endogenous cellulases, and hence are reliant upon gastrointestinal microorganisms for cell wall degradation of ingested plants. The beneficial effects of microorganisms in the digestive processes of terrestrial animals are well established (Combe *et al.*, 1976; McBee, 1977; Goldin, 1986; Moriarty, 1990). Some investigations have also suggested that microorganisms have a beneficial effect in fish digestive processes e.g. microbial breakdown of chitin (Minami *et al.*, 1972; Goodric and Morita, 1977; Danulat and Kausch, 1984; Kono *et al.*, 1987), p-nitrophenyl-β-N-acetylgalactosamine and collagen (MacDonald *et al.*, 1986), cellulose (Stickney and Shumway, 1974; Trust *et al.*, 1979; Saha and Ray, 1998; Bairagi *et al.*, 2002 a, b, 2004; Saha *et al.*, 2006), and the vitamin B12 producing ability of the bacteria (Sugita *et al.*, 1991). However, the specific cellulolytic activity shown by the bacterial species is found to depend on the source (Saxena *et al.*, 1993).

Microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Enzyme production is closely controlled in micro organisms and therefore, to improve its productivity these controls can be exploited and modified. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size (carbon source and cellulose quality), pH value, temperature, presence of inducers, medium additives, aeration, growth time, etc. (Immanuel *et al.*, 2006). Therefore, attention has been focused on studying the cellulolytic activity and cellulase enzyme production by several micro organisms in various products as well as in various environments. To establish a successful fermentation process it is necessary to make the environmental and nutritional conditions favorable for the micro organism for over-production of the desired metabolite.

Thus an elaborate investigation is therefore, required to establish the optimum conditions to scale up enzyme production in an individual fermentation process. Although there are reports on the influence of various fermentation variables on cellulase production by different bacteria and fungi isolated from various natural environments (Garcia-Martinez *et al.*, 1980; Stewart and Parry, 1981; Coral *et al.*, 2002; Rajoka, 2004; Immanuel *et al.*, 2006), information on the optimum fermentation conditions for cellulase production by fish gut bacteria is lacking.

MATERIALS AND METHODS

The cellulolytic activity of the isolated organism was determined as described by Ghose, (1987). Loop full of each isolate was streaked aseptically on carboxymethyl cellulose medium. The plate was then incubated at 37 ^{o}C for 48 hours for bacterial isolates. After incubation period, the culture was flooded with 0.1% Congo red dye solution and later 40% Nacl solution and allowed to stand for 15-20 minutes. A zone of clearance formed around the bacterial colonies. The zone of clearance was measured using a meter rule. This represents the cellulolytic activity of the bacterial isolates.

Medium composition for cellulase production

The fermentation media used was Mary Mandels mineral salts solution and it was used along with different carbon and nitrogen sources. The medium (**M1**) contained the following (per L) Banana peels, 20g ; Peptone,1g ; $(NH_4)2SO_4$, 1.4g ; KH_2PO_4, 2g ; $CaCl_2$, 0.3g ; $MgSO_4.7H2_0$, 0.3g ; Urea, 0.3g ; trace metal solution (2.5g $FeSO_4$; 0.98g $MnSO4.H_2O$; 1.76g $ZnSO_4.H_2O$; 1.83g $COCl_{l2}.6H_2O$ dissolved in 1000ml of distilled water and heated to homogenized ; pH 6.8 (Jeffries, 1996).

Cellulase assay

Liquid media was used for the quantitative assay of cellulase production from the three bacterial strains. Cellulase activity was measured according to the method of Denison and Koehn (1977). The production of reducing sugar (glucose) from CMC substrate through cellulolytic activity was measured at 540 nm by the dinitrosalicyclic acid method using glucose as the standard.

One cellulase unit (U) was defined as the amount of enzyme per milliliter culture filtrate that released 1 microgram glucose per minute.

Optimization of Cellulase Production Process

Effect of optimum pH on cellulase production by Bacteria isolates

The reaction mixtures of 120 hrs containing enzymes from the different bacteria isolates were prepared; the optimum pH for enzyme cellulase activity was examined by running the assay activity between pH ranges of 3.0, 5.0, 7.0, 9.0, and 11.

The enzyme activity for each pH was determined by adding DNSA reagents and the absorbance read at 540 nm.

Effect of optimum temperature on cellulase production by Bacteria isolates

The optimal temperature for activity was determined by assaying the activity of the enzyme at different temperature ranges of 35 ^{0}C, 45 ^{0}C, 55 ^{o}C, 65 ^{o}C, and 75 ^{0}C.

Effect of incubation period on cellulase production by Bacteria isolates

The incubation was carried out for different periods in this study including days 1, 2, 3, 4, and 5 for the bacteria isolates after which an assay was determined by Dinitrosalicyclic method (Betrand *et a*l, 2004).

Effect of innoculum size on cellulase production by Bacteria isolates

This activity was determined by assaying the enzymes at different sizes of 1%, 2%, 3%, 4% and 5% and the enzyme solution was maintained with different sizes of the innoculum.

Effect of substrate concentration on cellulase production by Bacteria isolates

The effect of substrate concentration was determined by assaying the activity of the enzymes of each bacteria with different substrate concentration of 1, 2, 3, 4and 5%. DNSA reagent was added and the absorbance was taken on the spectrophotometer at 540nm as described by Betrand *et a.,l (*2004).

RESULTS

The effect of substrate concentration on cellulase activity produced by bacterial isolates is shown in Fig1. *Bacillus laterosporus* had maximum activity recorded in 2% substrate concentration with cellulase activity of 0.35 mg/ml and the least activity recorded in 5% substrate concentration with activity of 0.15 mg/ml *Bacillus megaterium* recorded maximum cellulase activity in 2% substrate conc with activity of 0.37 mg/ml, which was then followed by a decrease in activity as the substrate concentration increases. *Bacillus amyloliquifeciens* recorded its least activity in 5% substrate concentration with activity of 0.14 mg/ml and the highest recorded at 1% substrate concentration with activity of 0.36 mg/ml.

The effect of pH on the activities of cellulase enzyme produced by bacterial isolates is shown in figure 2. From the result the highest activity produced by *Bacillus laterosporus* was at pH of 5 with activity of 0.36 mg/ml, these agrees with the report of Lynd *et al*., 2002 and Gautam (2011) Who reported maximum yield at pH 5 and 6 by *A. niger* and *Trichoderma* sp while the least recorded at pH value of 9 with cellulase activity of 0.10 mg/ml. *Bacillus megaterium* had an activity of 0.40 mg/ml at pH of 5 this was followed by a gradual decrease as the pH increases. *Bacillus amyloliquifeciens* recorded maximum activity of 0.45 mg/ml at pH of 3.There was a slight decrease as the pH approaches 11 with cellulase activity of 0.19 mg/ml. This shows that *Bacillus amyloliquifeciens* recorded the highest cellulase activity.

The effect of innoculum size on the activity of cellulase produced by bacterial isolates. It can be seen in figure 3 that *Bacillus laterosporus* exhibited high cellulase activity of 0.94 mg/ml and 0.65 mg/ml in 5% and 4% respectively, minimum cellulase activity was recorded at 3% with 0.46 mg/ml. *Bacillus megaterium* and *Bacillus amyloliquifeciens* also recorded maximum activity 1n 3%. *Bacillus megaterium* had cellulase activity of 0.85 mg/ml while *Bacillus amyloliquifeciens* had 0.89 mg/ml from the result *Bacillus laterosporus* recorded the highest cellulase activity while *Bacillus megateruim* recorded the least activity. lower innoculum size require longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme, an increase in the number of cells in the innoculums would ensure a rapid proliferation and biomass synthesis. When innoculum size was increased from 1-5% there was increases in enzyme production but after that the activity was decreased (Fig 3) due to depletion of nutrients by the enhanced biomass, which resulted dwindle in metabolic activity (Kashyap *et al*., 2002). A balance between the increasing biomass and accessible nutrient would yield an optimal enzyme production (Ramachandran *et al*., 2004).

The effect of incubation period on the activity of cellulase producing isolates. From figure 4 it can be seen that *Bacillus laterosporus e*xhibited high cellulase activity of 0.71 mg/ml after 120 hrs (day 5) These agrees with the report of Gautam *et al* ., (2011) who reported optimum yield on the 5th day but contrary to his other report of highest cellulase activity by *A.niger* and *Trichoderma* sp on the 4th and 6th day which was suitable for commercial point of view Kang et al., (2004) , and it might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation on secretary machinery of the enzmes (Nochur *et a*l., 1993), the least activity recorded was at 48 hrs (day 2) with 0.4 mg/ml .*Bacillus megaterium* recorded its highest activity after 48 hrs (day 2) (0.56 mg/ml) this was followed by a decline in cellulase activity at 72 hrs (day 3) and 120 hrs (day 5).

The least activity recorded was at 96 hrs (day 4) (0.21 mg/ml) *Bacillus amyloliquifeciens* had its highest activity recorded at 24 hrs (day 1) this was followed by a slight decrease as the hours increases, to120 hrs (day 5) the activity drop to 0.18 mg/ml which was the least recorded.

The effect of temperature on cellulase production by the bacterial isolates is shown in figure 5 from the figure *Bacillus laterosporus* had cellulase activity of 0.04 mg/ml at 35 ºC, this was followed by a slight increase at 45 ºC (0.55 mg/ml) these disagrees with the report of Ray *et al* (2007) who reported that minimum cellulose yield was observed when fermentation was carried out at 45 ºC by *B. subtilis* and *B.circulans* it was then followed by a sharp decrease at 55ºC and 65ºC 0.23 mg/ml and 0.13 mg/ml but as the temperature increases to 75ºC the activity also increases to 0.66 mg/ml. *Bacillus megaterium* exhibited its high cellular activity at 55ºC with 0.58 mg/ml but the activities fluctuated by increasing and decreasing as temperature rises and fall the lowest recorded for *Bacillus megaterium* was at 35ºC with cellulase activity of 0.36 mg/ml . *Bacillus amyloliquifeciens* had its highest activity at 35ºC with 0.59 mg/ml, it was then followed by a slight decrease as the tempreture increases with the lowest activity at 75ºC (0.30 mg/ml). From the result *Bacillus laterosporus* recorded the highest cellulase activity. These agrees with the findings of Bakare *et al.*, (2005) who found that cellulose enzyme producded by *pseudomonas fluorescence* was activated at 30-35 ºC, also disagrees with the report of immanuel *et al.*, 2006 who reported maximum endogluconase activity in *cellulomonas Bacillus* and *Micrococcus* sp at 40 ºC .

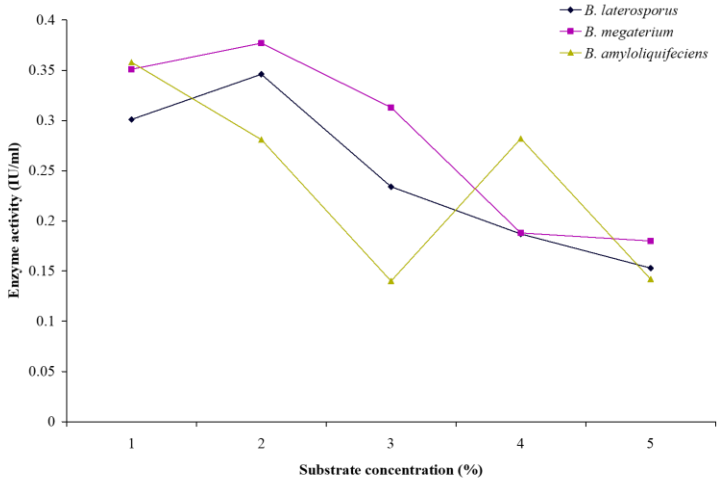

Figure 1; Effect of substrate concentration on cellulase activity produced by *B. laterosporus, B. megaterium, B. amyloliquifeciens*

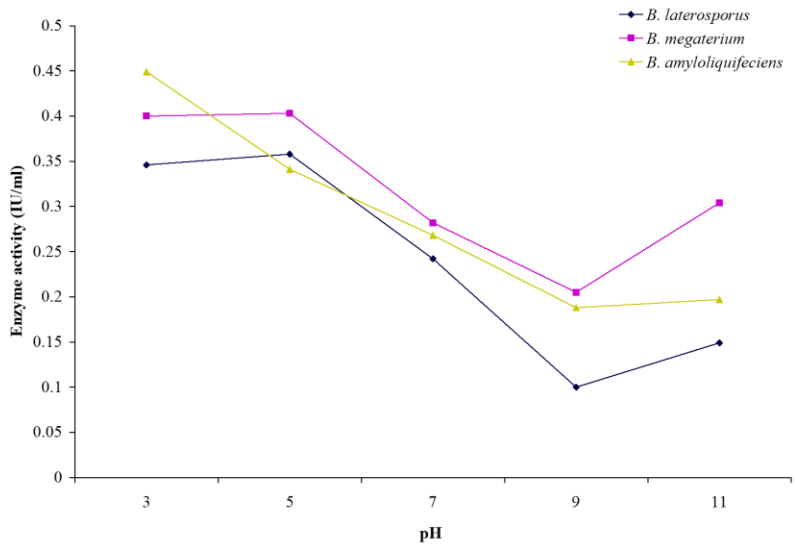

Figure2: Effect of pH on cellulase activity produced by *B. laterosporus, B.megaterium, B. amyloliquifeciens*

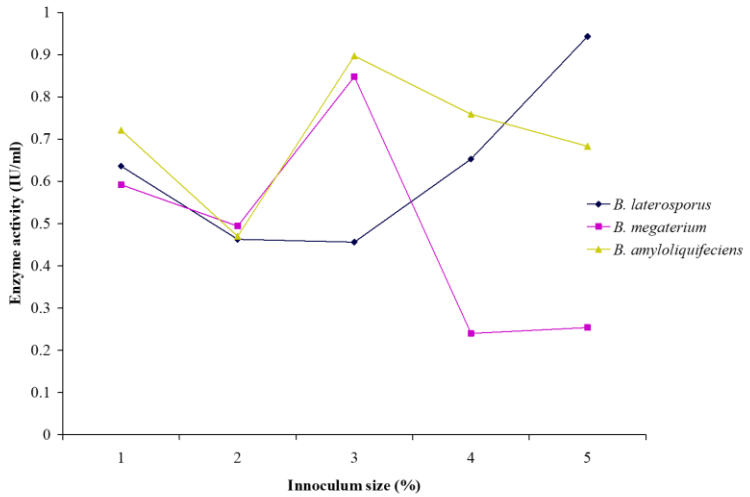

Figure 3: Effect of innoculum size on cellulase activity produced by *B. laterosporus, B.megaterium, B. amyloliquifeciens*

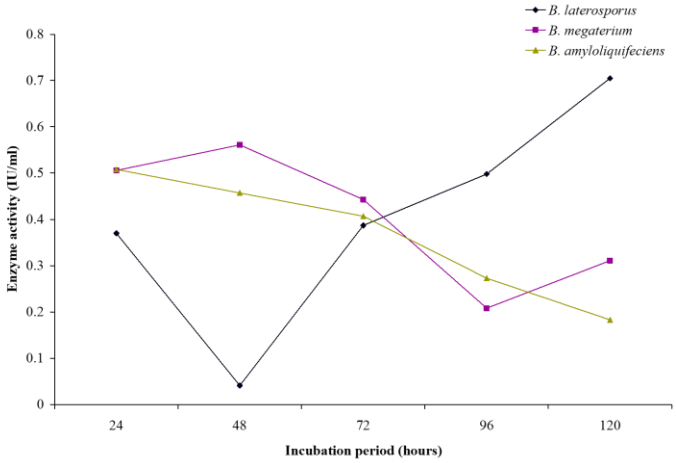

Figure 4: Effect of incubation periods on cellulase activity produced by *B. laterosporus, B.megaterium, B. amyloliquifeciens*

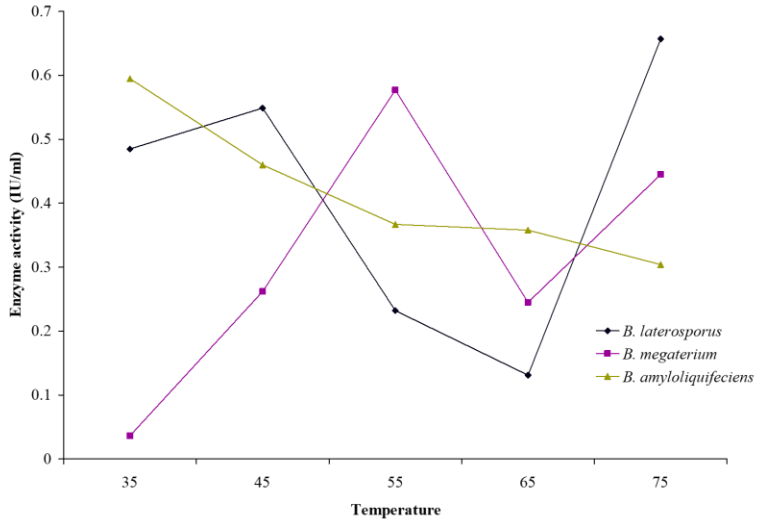

Figure 5: Effect of Temperature on cellulase activity produced by *B. laterosporus, B. megaterium, B. amyloliquifeciens*

DISCUSSION

Three species of *Bacillus* were characterized and identified on the basis of macroscopy and microscopy. The macroscopic and microscopic characterization revealed the presence of *Bacillus laterosporus, Bacillus megaterium, and Bacillus amyloliquifeciens*. All the bacterial isolates were hydrolizers of carboxymethyl cellulose meduium, based on cellulolytic activity of the isolates following their zones of clearing on CMC medium. Temperature and pH are the most important factor that influence enzyme activity .*Bacillus laterosporus* had an enzyme activity of 0.44 mg/ml at 35^{o}C this was followed by an increase in enzyme activity as the temperature increase, with maximum activity of 0.56 mg/ml. cellulase enzymes when hydrolyzed by cellulolytic microorganisms instead of being left alone for natural degradation, these enzymes can be utilized effectively under optimum conditions, to produce cellulase as realized as the temperature increased beyond 45^{o}C the enzyme activity decreased ,The minimum enzyme activity for *Bacillus laterosporus* was recorded at 75^{o}C with enzyme activity of 0.17 mg/ml while *Bacillus magaterium* had an enzyme activity of 0.56 mg/ml at 35^{o}C and the temperature increased to 45^{o}C enzyme activity also increases to 0.66 mg/ml this was followed by a sharp increase in enzyme activity of 0.82 mg/ml at 55^{o}C. Hence, further increase in temperature lead to a decrease in enzyme activity of 0.32 mg/ml at 75^{o}C .This is in line with the report of Kelly *et al* (1997) that during isomerization, temperature is preferably maintained within the range of 20-90^{o}C and the best activity is obtained at 50-75^{0}C.

Further increase in cellulose concentration beyond the level that gave optimum cellulase enzyme production will result in proportionate decrease in yield. This is at variance with Haapela *et al*., (1995) and Jeffries, (1996) who reported that maximum enzyme activity was recorded with the medium concentration of cellulose at 10g/l. Lynd *et al*., 2002), reported that the production of cellulase for the utilization of cellulose is induced only in the presence of specific substrate but suppressed when easily utilizable sugars such as glucose are available.

CONCLUSION

This study revealed that poultry wastes and Banana peel, are of domestic and industrial agro-wastes, that can be use to produce large amounts of cellulase enzymes when hydrolyzed by cellulolytic microorganisms instead of being left alone for natural degradation, these enzymes can be utilized effectively under optimum conditions, to produce cellulose.

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