

Cultural Conditions Necessary for Optimal Cellulase Yield by Cellulolytic Bacterial Organisms as They Relate to Residual Sugars Released in Broth Medium

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Abstract

A total of 115 samples made up of 42 (36.5%) rumen fluid, 36 (31.3%) cowdung and 37 (32.2%) soil samples were collected with the aid of sterile swab sticks except for rumen fluid samples which were collected by use of stomach tubes inserted into mouths of cows and by suction, liquor was collected into prewarmed thermo flasks under continuous flushing with carbon dioxide. Soil samples were collected into sterile universal containers. All samples were obtained from abattoirs situated at three locations in Benin City, Nigeria. Samples were investigated for cellulolytic bacteria by Filter Paper Yeast Mineral broth method. Cellulase production was assayed by Carboxymethyl cellulose submerged broth culture while residual sugar yield and other cellulolytic activities were determined by 3, 5 – Dinitrosalicylic acid, Filter Paper, Microcrystalline and Viscometric methods. Cellulolytic bacterial organisms isolated from both soil and rumen fluids were *Bacillus subtilis*, *Clostridium cellobioparum* and *Clostridium thermocellum*. *Pseudomonas aeruginosa* was isolated from both soil and cowdung samples while *Erwinia* spp was obtained from both rumen fluid and cowdung samples. *Bacillus circulans* and *Serratia* spp were obtained from soil samples only. *Clostridium thermocellum* and *Erwinia* spp produced the highest and lowest cellulase yields respectively. All isolates at 40°C and pH 6, recorded optimal sugar yields in culture broth of which *Clostridium thermocellum* recorded the highest. Lowest yields were recorded at 30°C and pH 3 although there was significant difference in individual yields ($P < 0.05$). *Clostridium thermocellum* recorded optimal cellulolytic activities at 50°C and pH 6. All isolates attained optimal cellulolytic activities at $32.6 \pm 6.2^\circ\text{C}$ and $\text{pH } 6.29 \pm 0.9$ with other broth cultural conditions kept constant. Implications of these findings are discussed.

Keywords: Cultural, Conditions, Optimal, Cellulase, Bacteria, Sugars, Broth

1. Introduction

Cellulase is an inducible enzyme complex involving synergistic action of endoglucanase (C_x), exoglucanase (C_1) and cellobiase (C_B) (Zaldivar *et al.*, 2001). The high cost of cellulase production owing to use of pure chemicals in production coupled with low enzyme activities limits its industrial use (Kalra and Banta, 2008). Major impediments to exploiting the commercial potential of cellulases are the yield stability and the cost of cellulase production. Research should therefore be aimed at taking advantage of the commercial potential of existing and new cellulases in nature (Coral *et al.*, 2002).

Both fungi and bacteria have been heavily exploited for their abilities to produce a wide variety of cellulases and hemicellulases. A lot of emphasis has been placed on the use of fungi because of their ability to produce copious amounts of cellulases and hemicellulases which are secreted into broth medium for easy extraction and purification. Besides, fungal enzymes are often less complex than bacterial hydrolases and can therefore be more readily cloned and produced via recombination in a rapidly growing bacterial host such as *Escherichia coli* (Maki *et al.*, 2009). However, the isolation and characterization of novel glycoside hydrolases from bacteria are now becoming widely exploited mainly because bacteria have a higher growth rate than fungi a property that

allows for higher recombinant production of enzymes. Bacterial hydrolases are often more complex and are often expressed in multi-enzyme complexes providing increased function and synergy.

Most importantly, thermophilic, psychrophilic, alkaliphilic, acidophilic and halophilic bacteria inhabit a wide variety of environmental and industrial niches that are extremely resistant to environmental stress (Maki *et al.*, 2009). Bacteria also present an attractive potential for the exploitation of cellulases and hemicellulases due to their rapid growth rate, enzyme complexity and extreme habitat variability (Maki *et al.*, 2009).

Beyond free bacterial cellulases, is the opportunity for whole cells in bacterial co-culture and strains with multiple exploitable characteristics to reduce time and cost of current bio-conversion processes (Miranda *et al.*, 2009). Not only can these strains survive the harsh conditions found in the bio-conversion processes, they often produce enzymes that are stable under such extreme conditions. This may increase rates of enzymatic hydrolysis, fermentation and product recovery. As a result, isolation and characterization of cellulase producing bacteria will continue to be an important aspect of biofuel research.

Cellulase of *Paenibacillus* spp was found to have maximum CMC activity at 60°C, pH 6.5. Due to the promising thermostability and slight acidic tolerance of this enzyme, it has good potential for industrial use in the hydrolysis of soluble cellulose as well as activity on microcrystalline sources of cellulose (Wang *et al.*, 2008). *Paenibacillus campinasensis* BLII is a thermophilic spore-forming bacterium which was found to grow between 25 and 60°C over a wide range of pH. Optimal growth is around neutral pH at 55°C. The physiological properties of this strain and the vast number of free glycosyl hydrolases produced, give this strain potential for use in bio-refining industry. (Ko *et al.*, 2007).

Bacillus subtilis offers a potentially more valuable thermostable enzyme for the bio-refining industry due to extreme heat tolerance. Cultivation of thermophiles offers several advantages such as reducing the risk of contamination and viscosity thus making mixing easier and this leads to a high degree of substrate solubility while reducing the cost of cooling (Li *et al.*, 2008). *Bacillus agaradhaerens* was shown to have increased optimal thermostability from 50 – 60°C and optimal pH range of 7 – 9.4 (Hirasawa *et al.*, 2006). *Cellulomonas flavigena* enzyme was found to have optimum cellulase and xylanase activities at pH 6 with an optimum temperature at 50°C (Perez-Avalos *et al.*, 2008).

Zaldivar *et al.* (2001) observed that cellulase production by *Trichoderma aureoviridae* is best if pH doesn't fall below 3.5 and at an optimum temperature of 28°C. Pei-Jun *et al.* (2004) reported a pH of 6.5 for optimum cellulase production by *Trichoderma koningii*. Kalra and Banta (2008) reported pH and temperature of 5 and 30°C respectively as optimal for cellulase yield. Cellulolytic endoglucanase activity of *Cellulomonas* spp, *Bacillus* spp and *Micrococcus* spp on coir fibre was optimal at pH 7 and temperature 40°C (Immanuel *et al.*, 2006). Some previous studies reported that the agricultural wastes of lignocellulosics are used as carbohydrate source to produce commercially important products such as ethanol, glucose and single cell protein (Solomon *et al.*, 1999).

Carbon source, cellulose quality, pH value, temperature, presence of inducers, medium additives, aeration and growth time have been reported to be important parameters in optimizing cellulase production (Immanuel *et al.*, 2006) and among these, pH is of major interest (Judasz *et al.*, 2004).

As a result of the high cost of cellulase production with its low enzyme activities owing to the use of chemicals in its industrial production, efforts are needed to economize cellulase production through media (cultural) optimization and use of supplements and/or additives (Kalra and Banta, 2008). Researches have been carried out and are still ongoing all aimed at isolating potential cellulase producing microorganisms from diverse habitats (Ray *et al.*, 2007).

Cellulases have uses as/in chemicals, fuels, food, brewery, wine, animal feeds, textile, laundry, pulp/paper and agriculture (Bhat 2000; Sun and Cheng, 2002). It is on record that most African countries such as Nigeria spend foreign exchange to import cellulases. Cellulases can be locally sourced invitro by first screening for cellulolytic bacteria and optimizing their cultural conditions for maximal yield. The technology can then be scaled up by our industries and invariably, government can be helped to save scarce foreign exchange.

Cellulosic wastes will yield free cellulose after delignification. Bacterial cellulases can convert cellulose to reducing sugars which on fermentation, is converted to ethanol – a raw material for methane and biogas production. Ethanol can be dehydrated to obtain ethylene gas. This study is therefore aimed at investigating the cultural conditions necessary for optimal cellulase yield by cellulolytic bacterial organisms as they relate to residual sugars released into broth medium with the following objectives: (1) isolate and screen for bacterial cellulase producers peculiar to the study environment (2) determine sample site distribution of cellulolytic

organisms (3) determine cellulase production capacity of cellulolytic isolates (4) determine residual sugar production levels of isolates at varying temperature and pH conditions.

2. Materials and Methods

A total of 115 samples made up of 42(36.5%) rumen fluid, 36(31.3%) cow dung and 37(32.2%) soil samples were collected from abattoirs situated at three locations in Benin City, Nigeria. Cowdung samples were collected with sterile swab sticks while soil samples were obtained from the immediate environment of the abattoir littered with cowdung in sterile universal containers. Soil was dug about 20cm deep (Okoth *et al.*, 2007) before samples were collected. Rumen samples (taken from recently killed cows) were obtained by use of stomach tubes inserted into the mouths of cows and by suction, liquor was collected into prewarmed thermo flasks under continuous flushing with carbondioxide. All collected and well labeled samples were taken to the laboratory for immediate processing.

Cow dung samples were processed by dipping the swab end of each stick into 5ml sterile distilled water (after breaking off the stick). This set up was shaken and using sterile pasteur pipettes (with intermittent rinsing in sterile water), two drops (0.04ml) of resulting suspension were streaked on carboxymethyl cellulose agar medium described by Bassey (2002).

Soil samples were collected into 20ml sterile plastic universal containers from which soil suspensions were made. Twenty grams (20g) of each soil sample was weighed out and dissolved in 50ml of sterile water in sterile screw capped 100ml bottles. This was properly mixed and using sterile glass pipettes, two drops (0.04ml) of resulting suspension of each sample were streaked on sterile carboxymethyl cellulose agar medium as described by Bassey (2002).

The composition of the medium described by Bassey (2002) per litre (1000ml sterile water) is: Cellulose powder (8.0g), Agar – agar (30.0g), K_2HPO_4 (1.65g), $(NH_4)_2SO_4$ (1.60g), Yeast extract (1.0g), Cysteine (0.5g), $CaCl_2$ (0.096g), $MgSO_4$ (0.096g), 0.1% Resazurin (0.1ml), pH 7.2. The pH 7.2 was maintained by dropwise addition of 5M NaOH.

Agar – agar powder together with the cellulose powder (carboxymethyl cellulose) was gradually dissolved in 200ml distilled water first and made up to 500ml. A paste suspension was obtained (A). The other constituents were first dissolved in 200ml distilled water and made up to 500ml (B). A and B were then mixed together and mixture was distributed into 2(500ml) screw capped bottles and autoclaved at $121^{\circ}C$ for 15minutes. Molten medium was allowed to cool to $45^{\circ}C$ and 2 tablets of griseofulvin were dissolved into the molten agar aseptically i.e. 1 tablet (500mg) dissolved in 500ml molten agar. This was to prevent fungal contamination of medium. The preparation was dispensed into sterile glass petri dishes (in 2ml volumes) on sterilized laboratory bench. Surface of poured plates were bunsen flamed to remove air bubbles and plates were left to set.

The above medium (before pouring into plates) was modified by addition of thioglycollate powder (Biotec, UK) for isolation of strict anaerobes. The original medium was used to isolate facultative and strict aerobes. The original media (liquid and solid) were carboxymethyl cellulose mineral nutrient medium (CMCM nutrient medium) and carboxymethyl cellulose mineral agar medium (CMCMA medium). Whereas the former did not contain agar, the latter did. The modified media (liquid and solid) were: Thioglycollate carboxymethyl cellulose mineral liquid medium (TCMCM nutrient medium) and Thioglycollate carboxymethyl cellulose mineral agar medium (TCMCMA medium)

Processed rumen fluid, cow dung and soils samples were inoculated into sterile TCMCM and CMCM Liquid media screw capped bottles and incubated at $37^{\circ}C$ for 24hours. Whereas TCMCM was used to grow strict (obligate) anaerobes, CMCM medium was used to grow strict aerobes, facultative aerobes and anaerobes. Bottles that showed turbidity (cloudiness) were subcultured onto their corresponding solid media agar plates. Positive CMCM bottles were subcultured on CMCMA plates and incubated at $37^{\circ}C$ for 24hours while positive TCMCM plates were subcultured on TCMCMA plates and incubated inside anaerobic jar (Mackintosh & Fildes jar) at $37^{\circ}C$ for 24hours.

Preservation of Isolates/Bacterial Suspension Preparation

Pure cultures of bacterial isolates were obtained and preserved on CMCA slants by subculturing from CMCMA plates onto the slants aseptically and incubating at $37^{\circ}C$ overnight. The resulting stock cultures were kept in the refrigerator at $4^{\circ}C$ for further use. Anaerobic isolates on TCMCMA plates were immediately scrapped into TCMC broth containing heated iron fillings to obtain bacterial suspension. Bacterial suspensions of aerobic isolates were also prepared by scrapping surface of CMCA stock (slant) cultures into sterile CMC broth submerged culture in sterile 100ml screw capped bottles and bottles were shaken to mix.

Screening for Cellulolytic Bacterial

The Filter Paper Yeast Mineral Salts (FPYM) broth method by Dhawan and Gupta (1997) was used. This assay was used to separate cellulase producers from non- cellulase producing isolates.

One thousand milliliters (1000ml) of the prepared sterile FPYM broth medium was distributed in 50ml volumes into 500ml flasks and 15cm diameter whatman filter paper No. 1 was folded into a cone and placed inside each flask with the apex up. All flasks were properly stoppered with cotton wool and sealed with aluminum foil paper and autoclaved at 121°C for 15minutes. All flasks were allowed to cool and labeled appropriately. One milliliter (1ml) of the bacterial suspension of each isolate was aseptically dispensed into each flask. All inoculated and labeled flasks were incubated at 37°C for 24hours. Positive flasks (i.e. those with yellow and brown mottling/disintegration of filter paper were noted. Positive organisms which were cellulase producers were then identified and characterized.

Identification of Cellulolytic Bacterial Organisms

The identification and characterization of the cellulolytic bacterial organisms were carried out according to the methods of Cullimore (2000) and Cowan and Steel (1993). The characteristics considered included cultural, morphological, biochemical tests as well as sugar fermentation tests. The cultural characteristics observed were: colour, optical density, pigmentation, margin, elevation and forms of colonies. The morphological features revealed by gram stain included cell shapes and sizes. The following biochemical tests: motility, indole production, citrate utilization, urease, oxidase, methyl red, voges proskauer, H₂S production, beta galactosidase, phenylalanine deaminase and fermentation tests were carried out and the following cellulase producing bacterial organisms were identified accordingly:

Large, irregular spreading colonies on TCMCMA plates, gram positive rod shaped cells with swelling endospores occurring in singles, motile, lactose/sucrose fermenting (with acid and gas), cellulose fermenting (with acid) colonies which grew weakly on nutrient Agar - characteristic of *Clostridium cellobioparum*, (2) Large, irregular, spreading colonies on TCMCMA plates, gram positive bacilli with round central or terminal endospores, motile, lactose fermenting (with acid and gas), sucrose negative, cellulose fermenting (with acid and gas) colonies – characteristic of *Clostridium thermocellum* (3) Orange, bead like colonies with convex elevation, gram negative short rods occurring in chains with hollow centre, motile, voges proskauer positive, beta galactosidase positive, gelatin liquefaction positive, lactose, mannitol and cellulose fermenting (with acid only) colonies which only grow on media containing cellulose – characteristics of *Serratia* spp.

The others were: (4) Light brown, flat colonies with irregular edges and spreading rapidly on the surface of agar medium, gram positive bacilli in singles with spores centrally positioned, motile, oxidase positive, glucose fermenting (with acetoin and gas), cellulose fermenting (with acid and gas) and non sucrose fermenting – characteristic of *Bacillus subtilis*. (5) Light brown, flat colonies with irregular edges and spreading rapidly on the surface of agar medium, gram positive bacilli in singles with spores terminally positioned, non-motile, oxidase negative, non lactose fermenting, non mannitol fermenting, glucose fermenting (with acid and gas), cellulose fermenting (with acid only) and fructose fermenting (with acid and gas) colonies – characteristic of *Pseudomonas aeruginosa*. (7) Smooth, raised motile colonies, gram negative short bacilli in singles and short chains, voges proskauer positive beta galactosidase positive, lactose, glucose, mannitol fermenting (with acid gas) and cellulose fermenting (with acid only) – characteristics of *Erwinia* spp.

Cellulase Enzyme Production

For enzyme studies, CMCM and TCMCM broths were used to carry out cellulase production by both cellulolytic aerobes and anaerobes so identified. Broths were prepared by dissolving the mineral salts (as previously described) in sterile 1000ml distilled water and mixture was dispensed in 50ml volumes into sterile 100ml screw capped bottles (the number of bottles equivalent to the number of isolates). Following that, 0.5g of carboxymethyl cellulose powder (Biotec, UK) was added to the content of each bottle and shaken properly, screwed leaving little space. All bottles were autoclaved at 121°C for 15mins and allowed to cool after bottles were tightly screwed. All bottles were properly labeled. Five milliliters (5ml) of bacterial suspension of both aerobes and anaerobes as previously described, was inoculated aseptically into each broth as labeled. A sterile CMCM broth (without any inoculation) and a sterile TCMCM broth (without any inoculation) were set up as controls for aerobes and anaerobes respectively.

All inoculated bottles were incubated at 37°C for 24hours. At the end of incubation, 10ml of submerged broth culture of each isolate was collected into sterile centrifuge tubes and spun for 15minutes. Same treatment was done on controls and labeled as such. The supernatant containing the crude enzyme solution) was then partially purified according to the Acetone method of Okolo (2004).The partially purified enzyme solutions were then characterized by assaying for reducing sugars by DNS method of Miller (1972), C₁ (exoglucanase) activity by

filter paper method of Somogyi (1977), Cx (endoglucanase) activity by Mackenzie (1987), extracellular protein content by Lowry *et al.* (1951) and percentage loss of viscosity by Norkrans & Hammarstron (1963). Controls were treated as tests and all results recorded.

Reducing Sugars Assays by DNS Method

The amount of reducing sugars released by the cellulolytic bacterial organisms was determined by the Dinitrosalicylic Acid (DNS) method of Miller (1972). The 3, 5 dinitrosalicylic acid in hot alkaline solution (when boiled for 5 minutes) is reduced to 3 amino, 5 – nitrosalicylic acid which possesses a deep orange colour and determined spectrophotometrically. The DNS reagent solution was prepared by first dissolving 10g NaOH and 200g of sodium potassium tartrate (Rochelle's salt) in one litre of distilled water before the addition of 2g phenol and 10g DNS reagent in that order. The DNS solution was warmed for complete dissolution. Glucose (0.07% w/v) and sodium sulphite (5% w/v) solution were prepared separately and 1ml of each was added to 100ml of the DNS solution before use. The DNS solution was stored in a brown bottle and kept at 4°C in a refrigerator.

To prepare a standard Glucose Calibration (of absorbance versus glucose concentrations), 1g of D – glucose (sigma) was dissolved in 1000ml of distilled water and mixed properly. The mixture was serially diluted to give 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1mg/ml of distilled water. Each standard glucose dilution was then treated by making a further half dilution with sodium citrate/citric acid buffer prepared according to the method described by Srinivasan (1997). One milliliter (1ml) of the buffer was added to 1ml of each glucose standard dilution as prepared earlier. Three milliliters (3ml) of DNS solution was added and mixture was immersed in boiling water for 5 minutes, cooled and absorbance read at 540nm using the spectronic – N21 spectrophotometer.

A standard glucose curve of optimal density (absorbance) against glucose concentrations was plotted to obtain a standard calibration curve from which the sugar concentrations of unknown samples (test samples) were extrapolated using their respective absorbances (obtained by treating test samples as the diluted D – glucose concentrations).

Effect of pH Changes on Enzyme Yield in Submerged Culture

The components of the cellulose nutrient medium were weighed without the phosphate (i.e. K_2HPO_4 , KH_2PO_4) and dissolved in different citrated-phosphate buffer solutions of pH 3,4,5,6 and 7. The citrate-buffer solutions were prepared on the basis of the data compiled by Munro (1970). The first step was the preparation of stock solutions of 0.1M citric acid and 0.2M dibasic sodium phosphate. Citric acid was prepared by dissolving 19.21g of the acid in 1000ml of distilled water. The other was prepared by dissolving 71.7g $Na_2HPO_4 \cdot 12H_2O$ in 1000ml of distilled water.

All the broths were sterilized and allowed to cool. They were labeled and each bacterial suspension (as labeled) and prepared earlier was inoculated (in 5ml volume) into the broth. Anaerobiosis was maintained for *Clostridium cellobioparum* and *Clostridium thermocellum* by introducing heated iron fillings into the broths before inoculation. Broths were incubated at 37°C for 24 hours after which, residual sugars, protein content, C_1 , C_x and percentage loss of viscosity of carboxymethyl cellulose were determined and recorded.

Effect of Temperature Changes on Enzyme Yield in submerged culture

Identified cellulolytic bacterial organisms were incubated at 30°C, 40°C and 50°C to determine the temperature at which cellulase is optimally produced. The CMCM and TCMCM broths (as earlier prepared), were inoculated with 5ml of bacterial suspensions of aerobic and anaerobic organisms respectively. Heated iron fillings were introduced into the broths for the anaerobes. Five milliliters of bacterial suspension of each isolate was then inoculated into broth submerged cultures (as labeled) and incubated separately at 30°C, 40°C and 50°C for 24 hours.

Ten milliliters (10ml) of broth culture at each temperature was collected in centrifuge tubes and spun for 15mins. The supernatant (containing crude enzyme suspension) was then partially purified according to method described by Okolo (2004). The reducing sugars, protein content, C_1 , C_x activities and percentage loss of viscosity of carboxymethyl cellulose were determined at each temperature and recorded.

3. Results

Six (50.0%), 4 (33.3%) and 2 (16.7%) of the cellulolytic bacterial organisms were obtained from the soil, rumen fluid and cowdung respectively (Table 1) cellulolytic bacterial organisms isolated from both soil and rumen content were *Bacillus subtilis*, *Clostridium cellobioparum* and *Clostridium thermocellum*. *Pseudomonas aeruginosa* was isolated from both soil and cowdung samples while *Erwinia* spp was obtained from both rumen content and cowdung samples. *Serratia* spp, *Bacillus subtilis*, *Bacillus circulans*, *Clostridium cellobioparum*, *Clostridium thermocellum* and *Pseudomonas aeruginosa* were obtained from soil samples only (Table 1).

Cellulase producing abilities of isolated bacterial organisms are presented in Table 2. From the highest to the lowest cellulase producers, the organisms are *Clostridium thermocellum* (5.32 IU/ml), *Bacillus circulans* (4.80 IU/ml), *Bacillus subtilis* (4.64 IU/ml), *Pseudomonas aeruginosa* (3.61 IU/ml), *Clostridium cellobioparum* (3.17 IU/ml), *Serratia* spp (1.64 IU/ml) and *Erwinia* spp (1.23 IU/ml). Total mean exoglucanase activity (2.57 IU/ml)

was greater than total mean endoglucanase activity (0.91 IU/ml) as recorded for cellulolytic bacterial organisms. This was the reverse case for fungal organisms. Overall cellulase activity was 3.49 ± 0.13 IU/ml with no significant difference between activities of individual organisms ($P > 0.05$).

Presented in Table 3, are the reducing sugars released by cellulolytic isolates at 30°C, 37°C, 40°C and 50°C as well as at pH 3, 4, 5 and 6. All isolates at 40°C, recorded the highest reducing sugar of 5.11 ± 1.59 mg/ml of which *Clostridium thermocellum* recorded the highest 12.05 mg/ml. There was however, no significant difference ($P > 0.05$) among sugar yields of all isolates at 40°C. Although the least sugar yield (1.84 ± 0.27 mg/ml) was recorded at 30°C by all isolates, there was significant difference in the sugar yields obtained by the isolates at that temperature ($P < 0.05$). The next highest sugar yield (4.23 ± 1.66 mg/ml) as obtained for all isolates, was recorded at 50°C of which *Clostridium thermocellum* recorded the highest (11.13 mg/ml) yield. There was however, no significant difference in the recorded yields of sugar by the isolates ($P > 0.05$). At 50°C, *Serratia* spp, *Pseudomonas aeruginosa* and *Erwinia* spp recorded no sugar yield at all.

The sugar yields at varied pH values of 3, 4, 5 and 6 are shown also in Table 3. The bacterial isolates recorded the highest sugar yield (3.43 ± 0.88 mg/ml) at pH 6 followed closely by 2.94 ± 0.48 mg/ml recorded at pH 5. Also, *Clostridium thermocellum* at both pH values recorded the highest sugar yield. There was however no significant difference among the sugar yields as obtained by individual isolates ($P > 0.05$). At pH 3, the lowest sugar (0.83 ± 0.22 mg/ml) yield was recorded of which *Bacillus subtilis* and *Erwinia* spp recorded the lowest yields. *Pseudomonas aeruginosa* recorded no sugars at all at pH 3 even though there was a significant difference among the sugar yields at pH 3 ($P < 0.05$).

Presented in Table 4 are the optimal pH and temperatures at which the isolates recorded maximum cellulolytic activities in terms of cellulolytic parameters analyzed. At pH 7.0, *Serratia* spp and *Clostridium cellobioparum* optimally recorded yields of residual sugars, exoglucanase activity (C_1), endoglucanase activity (C_x), extracellular protein content and percentage loss of viscosity of carboxymethyl cellulose. *Bacillus subtilis* and *Erwinia* spp recorded the same cellulolytic activities (indices) at pH 6.5. *Bacillus circulans* and *Pseudomonas aeruginosa* at pH 5.5 obtained optimal yields while *Clostridium thermocellum* recorded maximum yields at pH 6.0. All isolates are likely to obtain maximum cellulolytic activities at pH 6.29 ± 0.9 of which there was no significant difference in the pH values ($P > 0.05$).

Temperature variation effect on the optimal cellulolytic activities of the isolates is also presented in Table 4. At 35°C, *Serratia* spp, *Bacillus subtilis* and *Bacillus circulans* obtained optimal exoglucanase activity, endoglucanase activity, residual sugar yield, extracellular protein content and percentage loss of viscosity of carboxymethyl cellulose. Whereas 40°C was optimal temperature for optimal cellulolytic activities of *Clostridium cellobioparum* and *Erwinia* spp, that of *Pseudomonas aeruginosa* was 33°C. *Clostridium thermocellum* recorded an optimum temperature of 50°C. All isolates are likely to obtain maximum cellulolytic activities at temperature 32.6 ± 6.19 °C of which there was no significant difference in the temperature values ($P > 0.05$).

4. Discussion

Bacteria present an attractive potential for the exploitation of cellulases due to their rapid growth rate, enzyme complexity and extreme habitat variability (Maki *et al.*, 2009). The high cost of cellulase production (due to use of pure chemical in production) coupled with low enzyme activities, limits its industrial use. Therefore, efforts are needed to economize cellulase production by media optimization (Kalra and Banta, 2008) and hence, isolation and characterization of cellulase producing bacteria will continue to be an important aspect of biofuel research (Hirasawa *et al.*, 2006).

Samples used in this study included rumen liquor, cowdung and soil samples obtained from the surroundings where cowdung littered. According to Doi (2008), cellulase producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material from forestry or agricultural wastes, the faeces of ruminants (cows etc). soil and organic matter.

Identified cellulase producing bacteria in this study included *Serratia* spp, *Bacillus subtilis*, *Bacillus circulans*, *Clostridium cellobioparum*, *Pseudomonas aeruginosa*, *Erwinia* spp and *Clostridium thermocellum*. In a similar study, Sangbrita *et al.* (2006) isolated *Bacillus* species from the intestine of tilapia and Chinese grass carp. Weng-Jing *et al.* (2005) isolated 15 mesophilic bacteria of which *Bacillus* species predominated.

Bacterial cellulase producers isolated in this study occurred in varying proportions in the sample sources examined (Table 1). Bacterial organisms isolated from cowdung were *Pseudomonas aeruginosa* and *Erwinia* spp. *Pseudomonas aeruginosa* (a strict aerobe) occurrence in the cowdung may be due to secondary bacterial proliferation. The inadequate and faulty observance of anaerobiosis and human error associated with media (culture media) constitution and incubation conditions may have been responsible for the rather small number of bacterial cellulase producers isolated compared to the sample size. In addition, most cellulolytic organisms are diet and location specific. Also, a very large proportion of rumen microorganisms are non-culturable rumen bacteria (Kamra, 2005).

The highest cellulase producing bacterial isolate from this study was *Clostridium thermocellum* (5.32 IU/ml) followed by *Bacillus circulans* (4.80 IU/ml) and *Bacillus subtilis* (4.64 IU/ml). This is low when compared with the report of Wen-Jing *et al.* (2005) who isolated *Bacillus cereus* with maximum cellulolytic activity of 7.9 – 28.0 IU/ml. Maximum cellulolytic activities of 67.02 IU/ml and 35.8IU/ml as reported by Sangbriba *et al.* (2006) for *Bacillus circulans* and *Bacillus megaterium* respectively is also far higher than those obtained in present study. A possible explanation for the low cellulase yield in this study could be due to inadequate adherence to strict anaerobic cultural conditions for *Clostridium thermocellum*. Also, the first three highest cellulase producers may be different strains from those known already since cellulolytic organisms are location specific. However, *Clostridium thermocellum* was the highest cellulase producer in this study. According to Johnson *et al.* (1981), *Cl thermocellum* is noted for producing a cellulase complex known as cellulosome which has a very high cellulolytic activity. Cellulosome eliminates the wasteful expenditure of microorganisms thus continuously producing copious amounts of free cellulases (Shoham, 1999).

A high accumulation of sugar is a clear manifestation of high enzyme production and activity (Ejechi, 1991). Glucose is one of the main products of cellulose breakdown (Bisaria and Ghose, 1981; Okolo, 2004). If cellulose containing wastes were to be used as substrates, the waste problem would be solved (Coombs, 1987). Glucose can be fermented to form ethanol which can be used as fuel. Ethanol can be dehydrated to produce ethylene – a major compound used in the chemical industries. Glucose (sugar) also has uses in the pharmaceutical, food and bioscience based industries (Coombs, 1987)

Although, statistically, there was no significant difference in residual sugar of all isolates (accumulated sugar in culture broth) as obtained by bacterial isolates at pH6 ($P>0.05$), the highest sugar yield ($3.43 \pm 0.88\text{mg/ml}$) was recorded at that pH. At 40°C , all isolates obtained the maximum sugar accumulation ($5.11 \pm 1.59\text{mg/ml}$) in broth culture. There was however no significant difference among individual yields ($P>0.05$). It appears therefore, that optimal temperature and pH for optimal residual sugar yield by cellulolytic bacteria in our environment are 40°C and 6 if all other submerged cultural conditions are maintained. At the other extreme, a temperature and pH of 30°C and 3 respectively may inhibit sugar accumulation in culture broth. This was shown by *Pseudomonas aeruginosa* which recorded total absence of sugar at 30°C and pH and 3. Also within these cultural conditions, *Bacillus subtilis* and *Erwinia* spp recorded less than 1.00mg/ml . The lowest accumulated broth sugar yields of ($1.83 \pm 0.27\text{mg/ml}$ and $0.83 \pm 0.22\text{mg/ml}$) were obtained at 30°C and pH 3 respectively.

Cellulolytic activities (indices) of all bacterial isolates peaked at $38.6 \pm 0.19^{\circ}\text{C}$ and pH 6.29 ± 0.94 although there was no significant difference among the individual values recorded ($P>0.05$). At pH 7.0, *Serratia* spp and *Clostridium cellobioparum* recorded optimal yields of residual sugars, exoglucanase activity, extracellular protein content and percentage loss of viscosity of carboxymethyl cellulose.

At 35°C , *Serratia* spp, *Bacillus subtilis* and *Bacillus circulans* recorded optimal cellulolytic activities. At pH 5 and 35°C , *Bacillus subtilis* recorded optimal yield of residual sugar, endoglucanase and exoglucanase activities. This differed from the finding of Immanuel *et al.* (2006) who recorded 40°C and pH 7 as optimal for endoglucanase activity of *Bacillus* spp. Also different, were the reports of Li *et al.* (2008) and Hiroswawa *et al.* (2006) who recorded 50°C and pH 7 as optimal for cellulase production by *Bacillus subtilis* and *Bacillus agaradherens* JAM-Ku-23.

Bacillus circulans at pH 4 and 35°C , recorded optimal residual sugar yield. At pH 6, maximum exoglucanase activity and percentage loss of viscosity of CM-cellulose were also recorded for *Bacillus circulans* at 35°C . Finding in this study differed from that of Sangbriba *et al.* (2006), who recorded maximum cellulolytic activities of *Bacillus circulans* and *B. megaterium* at 30°C and pH8. Ariffin *et al.* (2008) reported optimal endoglucanase activity of *Bacillus pumilus* EB3 at 37°C and pH 7 which is also different from findings in present study. *Clostridium thermocellum* however attained optimal cellulolytic activities at 50°C and pH 6. Perez-Avalos *et al.* (2008) worked with *Cellulomonas flavigena* and recorded 55°C and pH 6 as optimal for its cellulolytic activities. This finding is similar to that recorded for *Cl. thermocellum* in present study.

Findings in this study did not exactly agree with those of other authors as cited and the reason may not be far fetched. Most of the studies cited were carried out in other parts of the world especially in the temperate regions. Cellulolytic organisms by virtue of habitat or source, are location and diet specific. Hence findings are peculiar to our environment. This however, is not to say that findings are fool-proof owing to cultural and equipment efficiency challenges encountered in the course of prosecuting the research.

Though the total cellulase yield of $3.49 \pm 0.13\text{IU/ml}$ is much lower than commercial cellulase, it can be used in its crude and partially purified forms but at high ratios, to saccharify pretreated wastes first to sugars, then ethanol, ethylene, methane and biogas. Also, since all isolates attained maximum cellulolytic activities at $32.6 \pm 6.19^{\circ}\text{C}$ and pH 6.29 ± 0.94 , the problem of high cost of cellulase production owing to low enzyme activities of organisms used industrially for its production can be solved by meticulously observing the optimal cultural growth conditions reported in this study.

5. Conclusion

Cellulolytic bacterial organisms isolated included *Serratia* spp, *Bacillus subtilis*, *Bacillus circulans*, *Clostridium cellobioparum*, *Pseudomonas aeruginosa*, *Erwinia* spp and *Clostridium thermocellum* of which *Bacillus subtilis*, *Clostridium cellobioparum* and *Clostridium thermocellum* were isolated from soil and rumen fluid. Whereas *Pseudomonas aeruginosa* was isolated from both soil and cowdung samples, *Erwinia* spp was isolated from both rumen fluid and cowdung samples.

From highest to the lowest cellulase concentrations, *Clostridium thermocellum* produced the highest yield of cellulase in culture broth while *Erwinia* spp produced the least. All isolates at 40°C and pH 6, recorded optimal residual sugars in culture broth of which *Clostridium thermocellum* had the highest yield. There was however, no significant difference ($P > 0.05$) among sugar yields for all isolates at 40°C and pH 6. The lowest yields were recorded at 30°C and pH 3 although there was significant difference in individual yields ($P < 0.05$). At 50°C and pH 3, *Serratia* spp, *Pseudomonas aeruginosa* and *Erwinia* spp recorded no sugar yield in culture broth.

Serratia spp and *Clostridium cellobioparum* however at pH 7 and 40°C, recorded optimal cellulolytic activities in terms of residual sugars, exoglucanase activity, endoglucanase activity, extracellular protein content and percentage loss of viscosity of carboxymethyl cellulose. *Bacillus circulans* and *Pseudomonas aeruginosa* recorded similar cellulolytic activities at pH 5.5 but at varying temperatures of 35°C and 40°C respectively. *Clostridium thermocellum* recorded maximum cellulolytic activities at 50°C and pH 6.

All isolates are likely to attain optimal cellulolytic activities at $32.6 \pm 6.2^{\circ}\text{C}$ and $\text{pH } 6.3 \pm 0.9$ if other broth cultural conditions are kept constant. Though the total cellulase yield of 3.49 ± 0.1 IU/ml is much lower than commercial cellulase, it can be used in its crude or partially purified form but at higher ratios, to saccharify pretreated wastes first to sugars, then ethanol, ethylene, methane and biogas. Also, since all isolates attained maximum cellulolytic activities at $32.6 \pm 6.2^{\circ}\text{C}$ and $\text{pH } 6.29 \pm 0.9$, the problem of high cost of cellulase production and biogas (owing to low enzyme activities of organisms used industrially for their production) can be solved by meticulously observing the optimal cultural growth conditions reported in this study.

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Table 1. Sample Site Distribution of Cellulolytic Bacterial Organisms

Identified Bacterial Organisms	Soil	Rumen fluid	Cowdung
<i>Serratia spp</i>	+	-	-
<i>Bacillus subtilis</i>	+	+	-
<i>Bacillus circulans</i>	+	+	-
<i>Clostridium cellobioparum</i>	+	+	-
<i>Pseudomonas aeruginosa</i>	+	-	+
<i>Erwinia spp</i>	-	+	+
<i>Clostridium thermocellum</i>	+	+	-
Total: 12(100.0%)	6(50.0%)	4(33.3%)	2(16.7%)

Table 2. Cellulase Production by Cellulolytic Bacterial Organisms at 37°C, pH 7.2

Cellulolytic Bacterial Organisms	C ₁ (IU/ml)	C _X (IU/ml)	Total Cellulase Activity (IU/ml)
<i>Serratia spp</i>	0.89	0.75	1.64
<i>Bacillus subtilis</i>	4.06	0.58	4.64
<i>Bacillus circulans</i>	3.46	1.34	4.80
<i>Clostridium cellobioparum</i>	1.18	1.99	3.17
<i>Pseudomonas aeruginosa</i>	3.16	0.45	3.61
<i>Erwinia spp</i>	0.90	0.33	1.23
<i>Clostridium thermocellum</i>	4.36	0.90	5.32
Mean:	2.57	0.91	Mean ± S.E: 3.49 ± 0.13

(P > 0.05)

C₁ = Exoglucanase ActivityC_X = Endoglucanase Activity

Table 3. Residual Sugars Released by Cellulolytic Bacterial Organisms at 30°C, 37°C, 40°C, 50°C and pH 3, 4, 5, 6

Cellulolytic Bacterial Organisms	Temp: pH:	30°C	37°C	40°C	50°C
		3 (IU/ml)	4 (IU/ml)	5 (IU/ml)	6 (IU/ml)
<i>Serratia spp</i>	Temp	1.01	1.83	0.90	0.00
	pH	1.55	0.52	2.00	1.71
<i>Bacillus subtilis</i>	Temp	1.40	4.88	6.03	5.60
	pH	0.99	1.64	4.01	2.55
<i>Bacillus circulans</i>	Temp	1.46	4.58	5.60	5.10
	pH	1.08	2.91	4.11	1.34
<i>Clostridium cellobioparum</i>	Temp	2.52	4.03	8.13	7.77
	pH	1.04	1.11	3.21	4.50
<i>Pseudomonas aeruginosa</i>	Temp	1.62	3.53	3.05	0.00
	pH	0.00	2.60	1.05	3.03
<i>Erwinia spp</i>	Temp	1.80	1.66	0.04	0.00
	pH	0.08	0.93	2.01	2.65
<i>Clostridium thermocellum</i>	Temp	3.09	5.95	12.05	11.13
	pH	1.10	1.44	4.21	8.21
Mean ± S.E	Temp	1.84 ± 0.27	3.78 ± 1.03	5.11 ± 1.59	4.23 ± 1.66
Mean ± S.E	pH	0.83 ± 0.22	1.59 ± 0.33	2.94 ± 0.48	3.43 ± 0.88
P value	Temp	P < 0.05	P > 0.05	P > 0.05	P > 0.05
P value	pH	P < 0.05	P < 0.05	P > 0.05	P > 0.05

Table 4. Optimal Temperature and pH Conditions for Optimal Cellulolytic Activities by Bacterial Organisms

Cellulolytic Bacterial Organisms	Optimal pH & Cellulolytic Activities	Optimal pH Range (mean)	Optimal Temp. & Cellulolytic Activities	Optimal Temp. Range (mean)
<i>Serratia spp</i>	6 (Sug,C ₁ C _x) 8 (Prot, Visc)	6 - 8 (7.0)	30°C (Sug,Prot,C ₁ ,C _x) 40°C (Visc.)	30°C - 40°C (35°C)
<i>Bacillus subtilis</i>	5 (Prot,C _x) 8 (C ₁ ,Sug,Visc)	5 - 8 (6.5)	30°C(Sug,Prot,C _x) 37°C(C ₁),40°C(Visc)	30°C - 40°C (35°C)
<i>Bacillus circulans</i>	4(Sug),6(C ₁ ,Visc) 7(C _x ,Prot)	4 - 7 (5.5)	37°C(C ₁ ,Visc),30°C (Prot),40°C(Sug,C _x)	30°C - 40°C (35°C)
<i>Clostridium cellobioparum</i>	7(Prot,C _x),6 (Visc),8(Sug,C ₁)	6 - 8 (7.0)	30°C(Sug,Prot,C ₁) 40°C(Visc),50°C(C _x)	30°C - 50°C (40°C)
<i>Pseudomonas aeruginosa</i>	4(Sug,C ₁ ,C _x ,Prot) 7(Visc)	4 - 7 (5.5)	30°C(Sug,Prot,C _x) 37°C(Visc,C ₁)	30°C - 37°C (33°C)
<i>Erwinia spp</i>	6(C ₁ ,C _x ,Visc) 7(Sug,Prot)	6 - 7 (6.5)	30°C(Sug,Prot,C ₁) 40°C(Visc),50°C(C _x)	30°C - 37°C (40°C)
<i>Clostridium thermocellum</i>	6.5(Sug,Prot) 5(C ₁),7(C _x ,Visc)	5 - 7 (6.0)	60°C(Prot,C ₁ ,C _x) 50°C(Sug), 40°C(C _x)	40°C - 60°C (50°C)
Mean ± S.E:		6.29 ± 0.94		38.6 ± 0.19
		P > 0.05		P > 0.05

Sug – Residual sugar

C₁ – Exoglucanase activityC_x – Endoglucanase (Carboxymethyl cellulase) activity

Prot – Extracellular protein content

Visc – Percentage loss of viscosity of carboxyl methyl cellulose