

# Estimation of Cellulolytic Bacteria from Cowdung in Awka Central Abattoir, Anambra State, Nigeria.

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## ABSTRACT

Bacteria obtained from cow-dungs from selected slaughtering points in Awka central abattoir were assessed for cellulose degradation. The qualitative screening of the isolates was determined by culturing the isolates on a cellulose selective medium. The isolates were obtained and identified as *Serratia marcescens*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis* and *Lactobacillus* spp. Three of the isolates with the highest cellulolytic activity are *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Streptococcus mutans*. *Pseudomonas aeruginosa* gave the highest diameter clear zone value of  $12\text{mm} \pm 0.25\text{mm}$  *Streptococcus* spp. follows with clear zone of  $11\text{mm} \pm 1.02\text{mm}$  and *Bacillus subtilis* had the least diameter clear zone of  $10\text{mm} \pm 1.04\text{mm}$ . The three isolates were selected for optimization because they had a clear zone up to 10mm and above. These isolates were subjected to qualitative screening by culturing the isolates in a liquid medium with different incubation periods (12, 24, 48, 60, 72, 84 and 96hrs) at shack and static conditions. Potential activities of Fpase and CMC-ase were found in *Pseudomonas aeruginosa* (0.94u/m), *Bacillus subtilis* (0.88u/m) and *Streptococcus mutans* (0.6u/m) all at 48hrs incubation respectively. Microbes in cow dung have a potential to be Cellulolytic as the degradation of cellulose is an environmental friendly approach that can reduce the accumulation of agricultural wastes.

**Key Words:** Abattoir; Cellulolytic Bacteria; Cowdung; Hydrolysis Qualitative and Quantitative screening.

## INTRODUCTION

Cellulose is considered the most abundant biological polymer on aquatic and terrestrial ecosystems. It is also the main component of plant biomass [1]. It is the dominant waste material generated from agricultural industry in the form of stems, stalks, and husks. Lots of interests have been generated concerning the utilization of cellulose as an energy resource and feed [2]. Cellulose can be defined as a polymer made up of D-glucose units linked together to form linear chain through  $\beta$ -1, 4-glycosidic linkages. It is the substance that makes up most of the plant's cell wall. Due to the molecular linkages of cellulose, it is insoluble or does not dissolve easily in water. These molecules are formed into a criss-cross mesh which gives the strength and shape to the cell wall. Cellulose is commonly degraded by cellulolytic enzymes known as cellulases. Cellulases have a huge potential for application in biotechnology and other industries; such applications include malting and brewing, starch processing, clarification of juice, alcoholic beverage, pulp bleaching, animal feed production and textile processing [3]. Cellulases are the group of enzymes involved in the conversion of cellulosic substrates to fermentable sugars. Main members of this group include endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91) and  $\beta$ -glucosidase (EC 3.2.1.21). The endoglucanase hydrolyzes  $\beta$ -1,4 bonds in cellulose molecule, whereas exoglucanase cleaves the ends to release cellobiose and  $\beta$ -glucosidase converts cellobiose to glucose. However, the enzymes capable of degrading cellulose, known as cellulases are mainly produced by Bacteria, Fungi and Protozoan that hydrolyzes cellulose. Cellulolytic microbes have important role in environment for degradation of

cellulose and convert it into useful products. Cellulases are mainly classified into 5 types on the basis of types of reaction catalyzed- [4].

Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness. Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals, insect repellent, thermal insulator, oil and grease removal and biofuel [5].

Screening for microbial cellulose activity is typically performed as pure cultures of bacterial isolates were individually transferred in Carboxy-methylcellulose Agar (CMC) agar plates. After incubation for 48 hrs, CMC agar plates is flooded with 1 % congo red and allowed to stand for 15 mins at room temperature. One molar NaCl is thoroughly used for counterstaining the plates. Clear zones appeared around growing bacterial colonies indicating cellulose hydrolysis ability and cellulase production.

Cow dung is a waste product from bovine animals, which includes cattle, buffalo etc. Cow dung is the undigested residue of plant matter which is passed through the animal's gut. Being a mixture of feces and urine in the ratio of 3:1, it mainly consists of lignin, cellulose and hemicelluloses. Fresh Cow dung is greenish black in colour, but when exposed to air turned dark greenish brown. It also contains different minerals like nitrogen, potassium, along with trace amount of sulphur, iron, magnesium, copper, cobalt and manganese. Indigenous cow also contain higher amount of calcium, phosphorus, zinc and copper than the cross-breed cow [6]:[7]. Cow dung harbours a rich microbial diversity, containing different species of bacteria (*Bacillus* spp., *Corynebacterium* spp. and *Lactobacillus* spp.), protozoa and yeast (*Saccharomyces* and *Candida*) [7]. [8] have isolated many different bacterial genera such as *Citrobacter koseri*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Kluyvera* spp., *Morganella morganii*, *Pasteurella* spp., *Providencia alcaligenes*, *Providencia stuartii* and *Pseudomonas* spp. from cow dung.

Microbes in cow dung have a potential to be Cellulolytic as the degradation of cellulose in biomass wastes is an environmental friendly approach that can reduce the accumulation of agricultural waste and reduce the emission of greenhouse gases. This is considered suitable to improve environmental sanitation

## MATERIALS AND METHODS

**Study Area:** The study was carried out in Awka metropolis. Awka is the capital of Anambra state. The city has an estimated population of 301,667 as of the 2006 Nigerian census, and over 2.5 million as at 2018 estimate. Awka is located at 199.1 kilometers by road, directly north of port Harcourt in the center of the densely populated Igbo heartland in South East Nigeria. Awka central abattoir is located 3 kilometers away from Appeal court, along court road in Awka south Local Government Area with a population of 189,049 people in the area [9].

**Sample Collection:** The cow dung used in this study was collected from the Awka central Abattoir and Amansea Abattoir located in Awka metropolis of Anambra State. Seven (7) Cow dung samples were collected from seven slaughtering points in the two abattoirs used for the study. The samples were hygienically placed in an ice box at 4°C and transferred to Applied Microbiology laboratory, Nnamdi Azikiwe University-Awka in a sterilized sample bottle within 4hr for bacteriological analysis.

**Isolation and Identification of Bacteria:** One gram (1g) of the samples was dissolved into 10/ml distilled water in a clean, sterile 25/ml bottle. This was mixed thoroughly and serially diluted out with sterile distilled

water. One milliliter (1ml) each was introduced into 9/ml of water (diluent) in 10 folds sterile test tubes and each tube were mixed thoroughly. Then, test tubes with 1:1,000, 1:10000 and 1:100000 dilution factors were pour-plated on Nutrient agar (NA), MacConkey agar (MAC), and de Man Rogosa Sharpe agar (MRS) for bacterial load counts. All plates were incubated at 37°C for 48 hours. MRS plates were incubated anaerobically in a closed jar with a candle, which created microaerophilic condition at 35°C for 48 hours. Mannitol salt Agar (MSA) plates were also incubated at 37°C for 48 hours. The colonies observed after the incubation periods were counted and recorded [10].

**Molecular Characterization of Isolates:** The Isolates screened were taken to a special laboratory located at Queen Elizabeth road, Ibadan, for DNA sequencing using molecular tools. Two (2/mLs) of bacterial cells broth was added to a ZR Bashing TM Lysis Tube. Then 750ul Lysis Solution was added to the tube. It was secured in a bead fitted with 2 ml tube holder assembly and process at maximum speed for > 5 minutes. The ZR BashingBeadTM Lysis Tube was centrifuged in a microcentrifuge at > 10,000 x g for 1 minute. Up to 400 ul supernatant was transferred to a Zymo-SpinTM IV Spin Filter (orange top) in a collection tube and centrifuge at 7,000 x g for 1 minute. Then add 1,200 ul of Bacterial DNA Binding Buffer to the filtrate in the Collection Tube from Step 4. Eight hundred (800 ul) of the mixture was transferred from Step 5 to a Zymo-SpinTM IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute. The flow was discarded through from the Collection Tube and repeat Step 6. Then add 200 ul DNA Pre-Wash Buffer to the Zymo-Spin TM IIC column in new collection Tube and centrifuged at 10,000 x g for 1 minute. 500 ul Bacterial DNA Wash Buffer was to the Zymo-SpinTM IIC column and centrifuge at 10,000 x g for 1 minute. The Zymo-SpinTM IIC column was transferred to a clean 1.5 ml microcentrifuge tube and 100ul (35 ul minimum) DNA Elution Buffer was added directly to the column matrix. Then centrifuged at 10,000 x g for 30 seconds to elute the DNA [11].

**Initial screening of cellulase-producing bacteria using cellulose differential medium:** The screening of cellulase-producing bacteria was done using a method by [10]. The isolated bacterial strains were screened for cellulase enzyme production in submerged fermentation process. The production medium was prepared using 1% potato waste (as cellulose substrate), 0.2 %  $K_2HPO_4$ , 0.03 %  $MgSO_4$ , 1 % peptone, 0.25 %  $(NH_4)_2SO_4$  and autoclaved at 121°C for 15min. After sterilization, the medium was allowed to cool at room temperature. The medium was inoculated with 1 ml of selected bacterial isolates and incubated in a shaker (Eyela NTS- 331) at 35°C for 24hrs with an agitation speed of 140 rpm. After termination of the fermentation period, the fermented broth was centrifuged at 140 rpm for 10 min at 4°C to remove the unwanted material. The clear supernatant thus obtained after centrifugation served as crude enzyme source.

**Qualitative Screening for Cellulose Production:** Pure cultures of bacterial isolates were individually transferred in Carboxy-methylcellulose Agar (CMC) agar plates. After incubation for 48 hrs, CMC agar plates was flooded with 1 % Congo red and allowed to stand for 15 mins at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. Clear zones appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system [10].

**Assay of Cellulolytic Enzymes:** Cellulase activity was measured using the method by [12]. A reaction mixture which consisted of 0.2/mL of the crude enzyme solution and 1.8mL of 0.5% carboxymethyl cellulose (CMC) in 50mm sodium phosphate buffer (pH7) was incubated at 37°C in a water bath for 30min. Afterwards, 3mL of Dinitro-benzouic acid reagent was added to terminate the reaction. The resulting colour was developed by boiling the mixture for 5mins. Optical Density of the samples was measured at 595nm against a blank containing all the reagents with the exception of the crude enzyme as done by [13].

**Quantitative screening of Cellulolytic Bacteria (Fpase):** Pure cultures of selected bacterial isolates were individually maintained on CMC supplemented agar slants at 4°C, until used. The bacterial isolates were inoculated in broth medium containing 0.03 %  $MgSO_4$ , 0.2 %  $K_2HPO_4$ , 1 % glucose, 0.25%  $(NH_4)_2SO_4$

and 1 % peptone at pH 7 for 24h of fermentation period. After 24h of fermentation period these were used as inoculum source. Each pure bacterial isolates gotten from the inoculum was grown on sterilized cellulose mineral salt medium containing Sterilized wood pulp of 50gm (Whatman Filter paper) as the only source of carbon and energy. 0.2 %  $K_2HPO_4$ , 0.03 %  $MgSO_4$ , 1 % peptone, 0.25 %  $(NH_4)_2SO_4$  and autoclaved at 121°C for 15min. The cultures were incubated for 37°C for 96h. Zone of clearance on the cellulose agar plates was observed after flooding with Congo red for qualitative estimation. For quantitative estimation, CMC agar medium containing 1.0 % peptone, 0.2 %  $K_2HPO_4$ , 1 % agar, 0.03 %  $MgSO_4 \cdot 7H_2O$ , 0.25%  $(NH_4)_2SO_4$  was prepared and used for the cellulolytic activity time study as described by [10]. Fifty milliliter of the medium was poured into three flasks containing sterilized filter paper, which was initially soaked in 1.0 mL and 10 mM sodium phosphate buffer pH 7.0 at 37°C. This acts as the carbon source (Cellulose) for the bacteria. Five milliliter of each of the bacterial cell culture adjusted to 0.5  $OD_{500}$  nm was pipetted in to the flasks. The reaction was stopped by adding 1.0mL 3,5-dinitro salicylic acid (DNS) reagent at each time to determine the optical density at 546nm. Growth, change in the medium and cellulose utilization potential of the bacteria isolates were determined quantitatively by time course study of enzyme activity and also monitoring the bacterial biomass at time intervals of 12h, 24h, 48h, 72 and 96h as described by [14].

**2.8. Statistical Analysis:** One sample t-test was used to analyze the mean values of the bacterial isolates under study using SPSS version 17.

## RESULTS

### Isolation and Identification of Bacteria

**Table 1.** Shows the morphology of cow dung samples collected from different slaughter points in the study area

Sample collection site	Colour	Odor	Form
CA <sub>1</sub>	Greenish brown	Rotten egg smell	Slurry
CA <sub>2</sub>	Dark brown	„	Slurry
CA <sub>3</sub>	Greenish black	„	Watery
CA <sub>4</sub>	Greenish brown	„	Watery
AA <sub>1</sub>	Dark brown	„	Solid
AA <sub>2</sub>	Greenish brown	„	Slurry
AA <sub>3</sub>	Dark brown	„	Slurry

Key: CA<sub>1</sub> = Sample collected from 1<sup>st</sup> slaughter point in Central abattoir

CA<sub>2</sub> = Sample collected from 2<sup>nd</sup> slaughter point in Central abattoir

CA<sub>3</sub> = Sample collected from the 3<sup>rd</sup> slaughter point in Central abattoir

CA<sub>4</sub> = Sample collected from the 4<sup>th</sup> slaughter point in Central abattoir

AA<sub>1</sub> = Sample collected from the 1<sup>st</sup> slaughter point in Amansea abattoir

AA<sub>2</sub> = Sample collected from the 2<sup>nd</sup> slaughter point in Amansea abattoir

AA<sub>3</sub> = Sample collected from the 3<sup>rd</sup> slaughter point in Amansea abattoir

### Colonial Examination of the Bacteria Isolates

Table 2 shows the Colonial examination of the bacterial isolates. They were identified as *Streptococcus mutans*, *Pseudomonas aeruginosa*, *staphylococcus aureus*, *Serratia spp*, *Esherichia coli*, *bacillus subtilis* and *Lactobacillus spp*.

**Table 2:** Colonial examination of the bacteria isolates

Isolates	Colonial characteristics	Probable organisms
1	Colonies are white to grey in colour with round, heaped and raised elevation. Presence of an entire margin	<i>Streptococcus mutans</i>
2	Greenish-blue in colour, grape-like smell. Smooth mucoid slime layer seen on a translucent-opaque low convex elevation	<i>Pseudomonas aeruginosa</i>
3	Opaque, pink-red coloured bacteria. Presence of a fishy to urinary odor. Umbonated elevation and entire margin	<i>Serratia marcescenes</i>
4	Opaque colourless colonies with black on the center. Low convex elevation with smooth elevation.	<i>Salmonella typhi</i>
5	Translucent donut shaped pink coloured shiny texture. Convex with an entire margin, creating concentric growth ring in the colony.	<i>Esherichia coli</i>
6	Presence of a rough, opaque, fuzzy slightly yellow edged bacterium with an irregular margin.	<i>Bacillus subtilis</i>
7	Presence of small, irregular and round shape with shinny whitish cream. Convex, entire, opaque without pigment.	<i>Lactobacillus spp.</i>

### Qualitative Screening for cellulose production

Qualitative estimation of cellulose degradation by observing the hydrolysis Capacity (zone of clearance) on cellulose agar plates after flooding with Congo red and incubating for 96h at about 37°C. Table 3 shows the zone of clearance in diameter and zone of colony of the isolates on cellulose agar plate after flooding and incubation. *P. aeruginosa* had the wildest zone of clearance of about 48mm, diameter of 13mm and zone of colony of 3.7mm, which was slightly wider than *Streptococcus mutans* that had zone of clearance of 46mm, diameter of 12mm and zone of colony of 2.3mm. *B. Subtilis* had a moderate zone of clearance of 40mm, diameter 12mm and zone of colony of 1.3mm. ), during the screening process as incubated in carboxy-methyl cellulose agar (CMC) for 48h. The supernatant was found to bear cellulolytic enzymes, supporting other previous studies that indicated the ability by known members of the *Bacillus* genus to secrete proteins extracellularly [15]. Three bacterial isolates were found to have the highest potential to produce cellulase, an enzyme that have the potential of degrading cellulose in cow dung, to ascertain the degradation potentials of these isolates in a biomass material.

**Table 3.** Screening test values for cellulose degradation by bacterial isolates

Isolates	Zone of inhibition(mm)
1	6
2	10
3	12
4	4
5	3



6	11
7	5

**Table 4.** The three selected isolates with the widest zone of inhibition

Isolates	Zone of clearance (mm)	Diameter (mm)	Zone of colony (mm)
3	48	13	3.7
6	40	12	1.3
2	46	20	2.3

**Key:**

Isolates 1= *Serratia marcescense*

Isolates 2= *Streptococcus mutans*

Isolates 3= *Pseudomonas aeruginosa*

Isolates 4= *Esherichia coli*

Isolates 5= *Salmonella typhi*

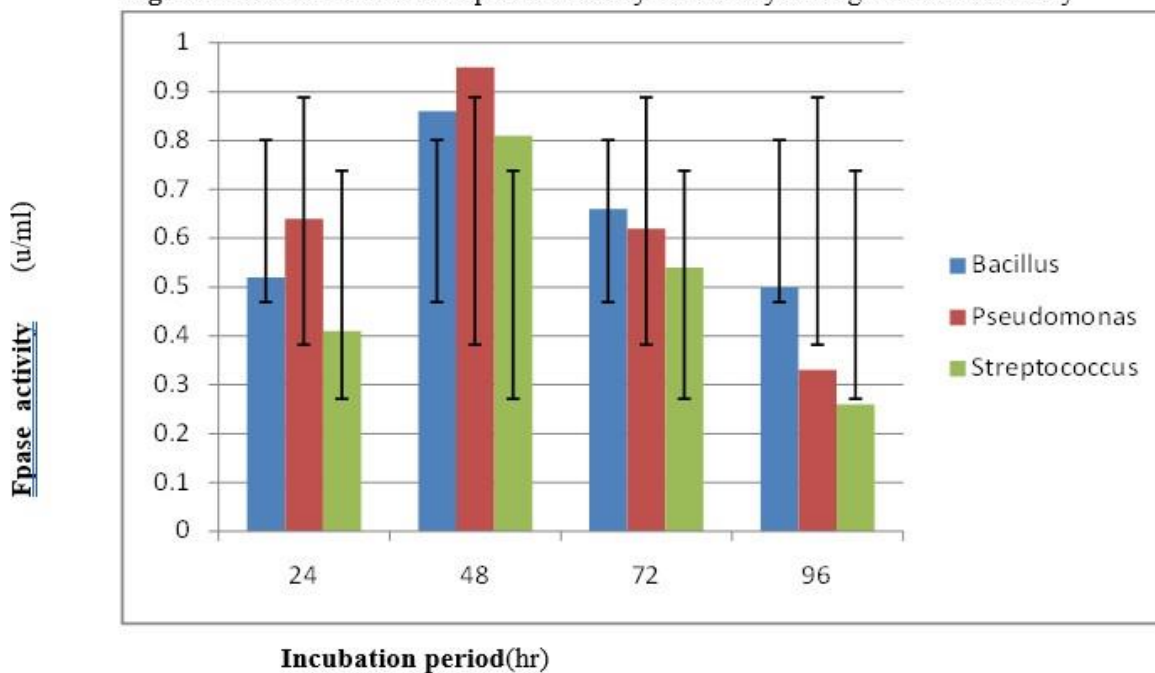
Isolate 6 = *Bacillus subtilis*

Isolates 7= *Lactobacillus spp.*

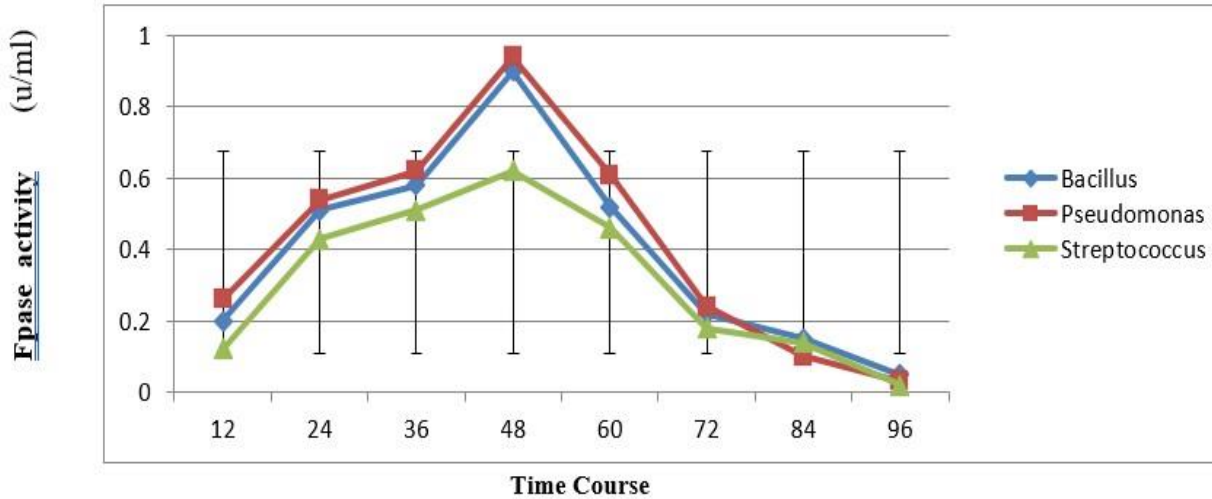
**Effect of incubation period on enzyme activity during time course study**

The time course of the production of cellulase by *P. aeruginosa* at 48h followed an exponential growth of the organism with maximum enzyme production of 0.95u/ml. The second highest enzyme production was seen in *Bacillus subtilis* which yielded a enzyme production of 0.94u/ml at 48h. The least was recorded by *Streptococcus mutans* with a mild cellulase production of 0.62u/ml at 48h. The increase level of cellulolytic activity during the incubation period can be attributed to the efficient utilization of the carbon and nitrogen sources in the biomass material, thus indicating a maximum production of cellulase as shown in figure 1. This report is similar with the report by [16] that cellulose degraders in the cow dung could be mesophilic or slightly thermophilic temperatures of incubation to be able to optimize a significant cellulase production.

**Figure 1.**Effect of incubation period on enzyme activity during time course study



**Figure 2.** Quantitative determination of Fpase in the medium



## DISCUSSION

The morphology of the cow dung samples observed were shown in table 1. The observation agrees with the observation obtained by [6] in their study on the current status of cow dung as a bioresources for sustainable growth. Table 1 also shows that cow dung occurs in watery, slurry and solid form which could easily have a direct or indirect contact with water causing an environmental pollution of water bodies as was described by [20]. This result also agreed with the studies carried out by [21], [22] and [23] which showed the presence of bacteria in the cow dung. The colonial characteristics of the selected isolates are shown in Table 2. These characteristics agree with the bacterial characteristics guidelines published by [18]. The work done by [19] confirms the identification of the bacteria from the cow dung used in the present study that animal excreta are major source of intestinal tract pathogens which could be shed in feces. These pathogens may be persist for a period of days, weeks or months depending on the type of pathogen and the environmental condition in which they could have the potential to increase the risk of occurrence of infections and diseases that pose huge threat to human health.

The isolated bacteria strains were screened for cellulose enzyme production in a cellulose differential medium in a submerged process according to the process described by [10]. Table 3 reported seven bacteria and their zone of inhibition as *Bacillus subtilis*(11mm), *E.coli* (2mm), *Streptococcus mutans* (10mm), *Serratia spp*(6mm),*Salmonda tyflu*(2mm), *Pseudomonas aeroginsa*(12mm), during the screening process as incubated in carboxy-methyl cellulose agar (CMC) for 48h. The supernatant was found to bear cellulolytic enzymes, supporting other previous studies that indicated the ability by known members of the *Bacillus* genus to secrete proteins extracellularly [15]. Three bacterial isolates (*Pseudomonas aeroginsa* 12mm), *Bacillus subtilis* 11mm) and *Streptococcus mutans* (10mm), were found to have the highest potential to produce cellulase, an enzyme that have the potential of degrading cellulose in cow dung, to ascertain the degradation potentials of these isolates in a biomass material. Table 4. shows the zone of clearance, diameter and the zone of colonies of the three bacteria isolates that will be optimized after flooding the medium with congo red. *Pseudomonas aeroginosais* the highest bacteria with a zone of clearance of 48mm, Diameter of 13mm and zone of colony of 3.7mm, followed by *Streptococcus mutans* with zone of clearance of 46mm, diameter of 20mm and zone of colonies of 2.3mm while *Bacillus subtilis* had the least zone of clearance of 40mm, diameter of 12mm and 1.3mm of zone of colonies. Thus, the result revealed certain bacteria have high potential to degrade cellulose. This result agrees with the work done by Umaru, (2013) who identified *Bacillus spp*, *Pseudomonas spp*, *Serratia spp* and some alcaligenes as suitable microorganisms for cellulose degradation of animal excreta. The result is also in line with the work done by [24], who reported that out of 368 isolates obtained from cow dung which he studied the degradation abilities of some bacteria isolated from cow dung and goat droppings, *Bacillus stearothermophilus* was predominant with a wide range of 70mm, thus contrary to the present study that observed that *Pseudomonas aeroginosa* was the predominant

cellulose degrader. [25] reported that *P. aeruginosa*, *Bacillus* spp and *serratia* spp have the ability to secrete Cellulolytic enzyme like Endo-1,4-  $\beta$  -glucanases, Exo-1,4-  $\beta$  -glucanases that cleave the internal bonds in the cellulose chain there by reducing them to simple sugars.

The increase level of cellulolytic activity during the incubation period can be attributed to the efficient utilization of the carbon and nitrogen sources in the biomass material, thus indicating a maximum production of cellulase as shown in figure 1, where at 48hr *P.aeruginosa* yielded a cellulase of 0.95u/ml, followed by *B. subtilis* which yielded 0.85u/ml and *S. mutans* had a relatively close yield of 0.8u/ml. This report is similar with the report by [16] that cellulose degraders in the cow dung could be mesophilic or slightly thermophilic temperatures of incubation to be able to optimize a significant cellulase production.

Fpase was determined in the medium for cellulolytic activity and monitoring through a time course study as shown in figure 2. Using a line graph, the rate of Cellulolytic activity, showed that at 48h, *P. aeruginosa* yielded 0.94u/ml which is the climax of enzymatic production, followed by a slightly lower production seen in *B. Subtilis* at 48h *Streptococcus mutans* showed a sharp drop in the cellulolytic activity with a range of 0.62u/ml at 48h, which can be attributed to some certain enzymatic reaction. There was significant degradation of biomass material (wood pulp) by the bacterial isolates from cow dung in the study conducted by [17] on the wood pulp degradation for paper industries. Using a cellulose medium incubated in a shaking water bath, He found out that *Pseudomonas aeruginosa* was observed to be the most effective bacterial isolate (highest degradation potential) followed by *Lactobacillus* and *Serratia* spp, thus conforms to the present study. [15] noted that Proper knowledge of bacterial degradation will help to eliminate or reduce environmental pollution from biomass material such as cow dung.

## CONCLUSION

The result of estimation of the bacterial isolates from cow dung reveals that it can be effective when applied to any biomass material as a bioremediation technique or conversion of toxic or hazardous material to a friendlier and less life threatening raw material for industrial purposes. Application of cow dung in proper and sustainable way can enhance productivity of yield in farming system, thereby minimizing the chances of bacterial and fungal pathogenic disease. Based on this research, it can be concluded that cow dung can be used as a source for isolation of cellulase producing bacteria

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