BIODEGRADATION OF CORNHUSK BY *BACILLUS CEREUS* ISOLATED FROM SOIL

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ABSTRACT

The isolation and identification of cellulolytic bacteria from soil for the biodegradation of cornhusk was carried out in this study. Cornhusk and soil samples were collected from a dump site in Minna, Nigeria. Carboxymethylcellulose (CMC) medium was inoculated with 1 ml of diluted soil by pour-plate method; the isolate with highest hydrolytic zone was selected and identified using standard cultural, biochemical and molecular tests. Cornhusk was sterilized in an autoclave prior to degradation tests using the isolate. The degradation potential of cornhusk by the isolate was measured using percentage cellulose loss. The effect of temperature (4^{o}C, 28^{o}C and 40^{o}C) on the degradation of the cornhusk was determined for 28 days. The highest degradation (80.55±0.77 %) was observed at 40^{o}C followed by 68.53±0.74 % at 4^{o}C while the least was 37.90±0.14 % at 28^{o}C. The control (un-inoculated cornhusk) had percentage degradation of 2%, 5% and 8% at 4^{o}C, 28^{o}C, and 40^{o}C respectively. The organism with the highest degradation potential was identified as *Bacillus cereus* R5-339. The pH during cornhusk degradation at 28^{o}C ranged from 4.67±0.09 to 7.43±0.16, while the pH at 4^{o}C and 40^{o}C ranged from 4.27±0.06 to 7.45±0.06 and 7.50±0.01 to 4.22±0.06 respectively. The test organism demonstrated the ability to degrade cellulose. Therefore, more research should be carried out if it could give better results when in consortium with other organisms.

Keywords: Biodegradation, carboxymethylcellulose, cornhusk, hydrolytic zone, *Bacillus cereus*
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INTRODUCTION

Cellulolytic bacteria degrade cellulolytic materials under normal environmental conditions; therefore many strains of these species of bacteria modify and solubilize lignocellulolytic material comprehensively, although their capacity to completely degrade lignin is incomplete [1].According to Tartoura and Youssef [2], above fifty percent (50%) of *Bacillus* spp. produce extracellular cellulases. *B. subtilis B. polymyxa, B. licheniformis,B. pumilus, B. brevis, B. firmus, B. circulans, B.megaterium* and *B. cereus* are known to be cellulose and hemicellulose degraders [1].

Prasad *et al*. [3] pointed out that the complete degradation of cellulose is made by a cellulolytic enzyme produced by microorganisms that are present in soil, marine and lake sediments, water and animal guts. It has been established that there are three main types of enzymes found in the cellulose system that degrade cellulose: exo- 1, 4-glucanase, endo- 1, 4-glucanase and glucosidase, which are produced both under aerobic and anaerobic conditions. Increase in waste generated and its management play an important role in our environment. Environmentalists are confronted with ways of managing these wastes with eco-friendly approach by the application of microorganisms and this is in many ways better than other available technologies. Bacteria utilize organic waste as source of nutrients, which does not produce pollution, unpleasant odour and sludge [4].

Cellulolytic bacteria possess some other characteristics that are responsible for the degradation of organic matter, beyond free bacterial cellulases, is the opportunity for whole cells in bacterial co-culture and strains with multiple exploitable characteristics to reduce the time and cost of current bio-conversion processes. So, isolation and characterization of cellulase-producing bacteria will continue to be an important aspect of research in biodegradation and bioremediation of cellulose containing wastes [5]. Therefore, the aim of this research was to isolate cellulolytic bacterial species from soil for the biodegradation of cornhusk.

MATERIALS AND METHODS

Sample collection (soil and cornhusk):
Soil**:** Soil sample at a depth of 12 cm was collected from Kpakungu dumping site located in Minna, Nigeria and transferred into a sterile Erlenmeyer flask

with the aid of sterile spatula. Cornhusk: Cornhusk (500 g) was obtained from dumping site into sterile polyethylene bags and taken to Microbiology laboratory, Federal University of Technology, Minna, Nigeria.

Isolation of cellulolytic bacteria

Soil sample was serially diluted until a dilution of 10^6 was obtained and then an aliquot of 1 ml of the diluent was inoculated on the surface of a media containing 1.0 % peptone, 1.0 % carboxymethyl-cellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and 0.2 % gelatin at pH 7 using the pour plate method in triplicates, and incubated for 48 h at 37°C [6].

Screening of cellulolytic bacteria

Isolated colonies on the plates were flooded with 1 % Congo red for 15 min at room temperature (28ºC±2) and counterstained with 1 M NaCl [7]. The colony having greater hydrolytic zone was subcultured on nutrient agar and incubated at 37°C for 24 h [8].

Biochemical and molecular characterization of bacterial isolates

Bacteria isolated were subjected to biochemical characterization using conventional methods described by Cheesbrough [9]; they were subsequently identified by comparing their characteristics with those of known taxa. Molecular characterisation of the isolate was carried out by DNA extraction [10] and amplification of the 16S rRNA gene by Polymerase chain reaction (PCR) reaction.

Polymerase chain reaction (PCR) reaction cocktail used consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System. Thermalcycler (Applied Biosystem Inc., USA) was used in PCR initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 seconds, 50°C for 60 sec and 72°C for 1 min 30 sec ; and a final extension at 72°C for 10 min and froze at 4°C [11].

The integrity of the amplified about 1.5 Mb gene fragment was checked on a 1 % agarose gel to confirm amplification. This was done by mixing 8 µl of amplified product to 4 µl of gel loading dye and ran on the solidified agarose gel at 110V for 1 h, and the image was captured under UV light. Also the amplified product was checked on a nanodrop (Model 2000 Thermo Scientific, USA) to quantify the concentration of the amplified product.

Purification of amplified product

After gel integrity, the amplified fragments were purified using ethanol in order to remove the PCR reagents. Briefly, 7.6 µl of 3 Molar of Na acetate and 240 µl of 95 % ethanol were added to 40 µl of amplified product in a sterile 1.5 µl Eppendorf tube, mixed thoroughly by vortexing and stored at -20°C for at least 30 min. Centrifugation was done at a temperature of 4°C for 10 min at 13,000 g followed by the removal of supernatant after which the pellets were washed by adding 150 µl of 70 % ethanol, mixed and then centrifuged for 15 min at 7,500 g and 4°C. Again all supernatants were removed, the tube was inverted on paper tissue and allowed to dry in the fume hood at room temperature (28ºC±2) for 10-15 minutes. The fragments were then resuspended with 20 µl of sterile distilled water and stored at -20°C prior to sequencing. The purified fragment was checked on a 1.5 % agarose gel ran on a voltage of 110 V for 1 h as previous, to confirm the presence of the purified product [11].

Sequencing of DNA fragment

The amplified fragments were sequenced using a Genetic Analyzer (3130xl sequencer of Applied Biosystems, USA) using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and Molecular Evolutionary Genetic Analysis (MEGA6) were both used for all alignment and phylogenetic analysis so as to ascertain the results obtained from each of them [12].

Biodegradation of cornhusk

Twenty (20) g of cornhusk was weighed, bore-milled and transferred into five (5) Erlenmeyer flasks, and distilled water (60 ml) was measured into the flasks, and they were sterilized at 121ºC for 15 min 18.5 psi. After cooling down, the flasks were then inoculated with 5ml of a suspension of the isolate McFarland's standard.The same conditions were repeated to another flask but without inoculation with the isolate, this served as the control. The set of flasks were incubated on a rotary shaker at ambient temperature (28ºC±2) for 28 days [13].

Determination of effect of temperature on biodegradation of cornhusk

Twenty (20) g of cornhusk was weighed, bore-milled and transferred into ten conical flasks (5 conical flasks for 4°C and 40°C, respectively). The first set of 5 conical flasks were incubated at 4°C, and the last set at 40°C for 7, 14, 21 and 28 days respectively and then compared with the treatments at ambient temperature [13].

Determination of degradation rate

After 28 days of incubation, the cornhusk was rinsed with sterile water, dried in an oven at 60°C and reweighed. The weight loss due to degradation was calculated using the following formula:

$$Weight\ loss\ (\%) = \frac{W - W1}{W} \times 100$$

Where, W=Initial weight, W_1=Final weight after 28 days of incubation [14].

Analysis of cellulose content in cornhusk

One gram of the degraded cornhusk samples were weighed and treated with a mixture of nitric acid and acetic acid (1:8 proportion) by boiling at 100°C in an apparatus that contains a Liebig's condenser. It was allowed to cool, filtered through Whatman's filter paper in a funnel, and then the filter paper containing an insoluble residue was dried in oven at 60°C and reweighed. The cellulose content was determined by subtracting the initial weight of cornhusk from final weight after drying [15].

Isolation of cellulolytic bacteria

Thirteen isolates were obtained from the three cultured plates, during cellulase production screening, the isolates with wider hydrolytic zone was the target and was used for the research

Screened cellulolytic bacteria

The hydrolytic zone of colonies ranged between 0.4 cm and1.8 cm. The isolate with the widest hydrolytic zone was further subcultured onto fresh nutrient agar and used for biodegradation of cornhusk. The photograph of PCR amplicons after agarose gel electrophoresis is shown in Plate I. The approximate size of the amplicon is 1500 Kb. The bacterial isolate used was 99% identical to *Bacillus cereus* strain (Plate I).

Percentage weight loss and cellulose reduction of cornhusk at ambient temperature

Percentage weight loss and cellulose reduction of cornhusk after 28 days of incubation at ambient temperature (28°C ± 2) are presented in Table 1. The least percentage weight loss of 0.00±0.00 % was obtained at zero (0) day of degradation and for the control because their weights did not reduce while the highest weight loss occurred at 28th day of degradation with 11.73±0.0%. Highest percentage cellulose reduction (37.90±0.14 %) was also observed at 28th day of degradation and least (1.1±0.14%) at zero (0) day of degradation

RESULTS

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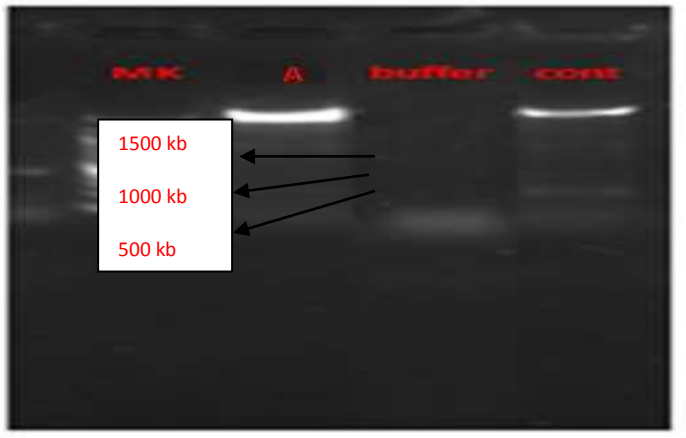

Plate I: PCR amplicons of 16S rRNA gene from *Bacillus cereus*.
Key: MK= DNA Ladder 1500kp, 1000 kb and 500kb A= Isolate under investigation (*Bacillus* sp.), Buffer: Negative control, Cont = Control

Table 1: Percentage weight loss and cellulose loss of cornhusk at ambient temperature

Days	W (g)	Control (W)	W_1 (g)	Control (W)	W_2 %	Control (W_2%)	W_3 (%)	Control (W_3%)
0	20	20	20.00	20	0.00±0.00^{f}	0.00	1.1±0.14^{f}	0.30±0.00^{e}
7	20	20	19.09	20	4.51±0.50^{d}	0.00	24.40±0.57^{c}	1.24±0.01^{d}
14	20	20	18.56	20	6.95±0.07^{c}	0.00	17.20±0.28^{d}	2.50±0.00^{c}
21	20	20	18.03	20	9.82±0.04^{b}	0.00	27.99±0.01^{b}	3.74±0.00^{b}
28	20	20	17.66	20	11.73±0.0^{a}	0.00	37.90±0.14^{a}	5.00±0.00^{a}

Key: W = Initial weight, W_1 = Weight after degradation, W_2 = Percentage weight loss, W_3 = Cellulose loss after degradation, Control = Cornhusk without *Bacillus cereus*

Values are means of duplicate measurement. Means with the same superscript were not significantly different at $P > 0.05$ and means with different superscript were significantly different at $P < 0.05$.

Partial sequence of the isolate

The data obtained from the Genbank database showed the identity of the isolate. Sequencing and phylogenetic analysis carried out on the isolate after it was subcultured into new sterile nutrient agar revealed that it was 99% identical to *Bacillus cereus* strain R5-339 16S ribosomal RNA gene. Blasts results from the molecular characterization of the isolate are presented in Table 2::

Table 2: Results of partial sequence of the isolate

Description	Maximum score	Total score	Query cover	E-value	% Identity	Accession
***Bacillus cereus* strain R5-339 16S ribosomal RNA gene, partial sequence.**	2676	2676	99%	0.0	99%	JQ659737.1

Percentage weight loss and cellulose reduction of corn husk at 4^{o}C

Percentage weight loss and cellulose reduction of cornhusk at 4^{o}C are shown in Table 2. The percentage weight loss ranged from 0.06±0.02% obtained at zero (0) day and 16.82±0.03% while 68.53±0.74% cellulose loss was obtained at 21st day degradation, control had 2.00±0.00% and 1.01±0.01% was obtained at zero (0) day of degradation at 4^{o}C (Table 3).

Table 3: Percentage weight loss and cellulose loss of cornhusk at 4^{o}C

Day	W (g)	Control (W)	W_1 (g)	Control (W_1)	W_2 %	Control (W_2 %)	W_3 (%)	Control (W_3%)
0	20	20	19.99	20.00	0.06±0.02^{d}	0.00±0.00^{e}	1.01±0.01^{f}	0.26±0.00^{c}
7	20	20	19.20	19.97	4.15±0.21^{c}	0.03±0.00^{d}	45.80±0.28^{b}	0.40±0.00^{c}
14	20	20	18.80	19.92	6.5±0.71^{b}	0.08±0.00^{c}	38.03±0.04^{c}	0.98±0.00^{b}
21	20	20	19.92	19.89	0.42±0.02^{d}	0.11±0.01^{b}	68.53±0.74^{a}	1.50±0.02^{a}
28	20	20	16.60	19.97	16.82±0.03^{a}	0.15±0.00^{a}	3.01±0.02^{d}	2.00±0.00^{a}

key: w = initial weight, w_1 = weight after degradation, w_2 = percentage weight loss, w_3 = cellulose loss after degradation, control = cornhusk without *Bacillus cereus.*

Values are means of duplicate measurement. Means with the same superscript were not significantly different at $P > 0.05$ and means with different superscript were significantly different at $P < 0.05$.

Percentage weight loss and cellulose reduction of cornhusk at 40°C

Percentage weight and cellulose reduction at 40°C ranges from 0.00±0.00 % on zero (0) day and 14.95±0.07 %, 1.09±0.01 % and 80.55±0.77% after 28 days of degradation. Highest weight loss was observed at 28th day of degradation and least at 0 (zero) day of degradation (Table 4).

Table 4: Percentage weight loss and cellulose reduction of cornhusk at 40°C

Day	W(g)	Control (W)	W_1 (g)	Control (W_1)	W_2 (%)	Control (W_2 %)	W_3 (%)	Control (W_3 %)
0	20	20	20.00	20.00	0.00±0.00^{f}	0.00±0.00^{c}	1.09±0.01^{f}	0.00±0.00^{e}
7	20	20	19.41	19.99	2.46±0.09^{d}	0.15±0.00^{b}	33.54±0.76^{d}	0.80±0.00^{d}
14	20	20	19.38	19.97	3.05±0.08^{b}	0.15±0.00^{b}	48.49±0.72^{c}	1.80±0.00^{c}
21	20	20	19.86	19.96	0.69±0.02^{c}	0.20±0.00^{a}	65.00±0.57^{b}	3.00±0.00^{a}
28	20	20	17.02	19.95	14.95±0.07^{a}	0.21±0.00^{a}	80.55±0.77^{a}	8.00±0.00^{a}

Key: W = Initial weight, W_1 = Weight after degradation, W_2 = Percentage weight loss, W_3 = Cellulose loss after degradation, C = Control (Cornhusk without *Bacillus cereus*)

DISCUSSION

Soil sample from a refuse dump site soil in Minna, Nigeria was serially diluted and plated on carboxylmethylcellulose (CMC) medium in this study. The highest hydrolytic zone obtained during screening was 1.8 cm and the least was 0.5 cm. The isolate with the highest hydrolytic zone used for the study was identified as *Bacillus cereus* as established by the results from morphological and biochemical tests carried out. Phylogenetic analysis also revealed that the isolate was *Bacillus cereus.* Bacteria from the soil where agricultural wastes are dumped have been proven to be the best producer of cellulase with the enzymes responsible for the hydrolysis of cellulose [16]. Biodegradation of cornhusk was carried out for twenty eight days at different temperatures (4°C, 28°C and 40°C). The maximum weight loss (11.73±0.0 %) was obtained at 28th day of degradation at 40°C. This was significantly higher ($P < 0.05$) than those obtained at 7, 14, and 21 days with 4.51±0.50 %, 6.95±0.07% and 9.82±0.04% degradation respectively. The highest weight loss obtained at 28th day of degradation could be as a result of longer time given for degradation compared to those with fewer days. The longer time period may have contributed to the increased microbial load and more cellulase secretion that hydrolyzed cellulose component of the cornhusk. However, the lack of degradation at day zero (0) and control may be because the enzyme had not been secreted by the bacterial isolate. Similar results were obtained from the report of Sameen *et al.* [17] where *Bacillus megaterium* was used for the degradation of cellulose. They reported no degradation in their control samples because *Bacillus megaterium* was not inoculated.

The highest percentage cellulase reduction of cornhusk obtained after 28 days of degradation (37.90±0.14 %) may be as a result of the rate of cellulase production in the substrate. However, there was also significant rate of degradation with 24.40±0.57%, 17.20±0.28% and 27.99±0.01% on 7th, 14th, and 21st days of degradation respectively. Highest degradation activities that occurred on the 28th day could be as a result of abundant enzyme synthesized by *Bacillus cereus* R5-339. This is in agreement with the findings of Arusha *et al.* [18] that reported a maximum cellulase production by *Bacillus* strains used in the degradation of agricultural wastes after 28 days.

The percentage weight loss of cornhusk at 40°C presented in Table 4 showed that the weight loss at 28th day of degradation (14.95±0.07 %) was significantly higher ($P < 0.05$) than the percentage weight loss obtained from other days and the control. These are similar to the findings of Nema *et al.* [19], Mukesh *et al.* [20] and Arusha *et al.*[18] where they reported the increase in the cellulase activities till 30th day of their degradation. The percentage cellulose reduction of cornhusk at 40°C ranges from 80.55±0.77

% at 28th day of degradation to 1.09±0.01 % on zero (0) day of degradation. On 7th day of degradation, 33.54±0.76 % was lost, while 48.49±0.72 % and 65.00±0.57 % of cellulose in cornhusk was lost on 14th, 21st day of degradation and 8.00±0.00 % cellulose was lost in control sample. These findings are in agreement with results reported by Nguyen and Vuong [21] that the cellulase enzymes perform maximally at temperatures ranging from 35°C to 45°C, although it is at variance with the finding of Arusha *et al.* [18] where *Bacillus* strains used produced maximum cellulase at 30°C. These results were similar to that of Ray *et al.* [22] who reported that minimum cellulase yield was observed when fermentation was carried out at 45°C, while maximum yield was obtained at 40°C by *B. subtilis* and *Bacillus circulans* this could be as a result of ability of the organisms to survive at high temperature.

The weight loss and cellulose reduction occurred in control samples, which may be due to the pre-treatment undergone by these samples. This agrees with the argument of Lin and Gi-Hyung [23] that reduction of particle size, crystalline structure and thermal pretreatments such as sterilization at 121°C in an autoclave were responsible for the slight reduction in weight and cellulose content of cornhusk.

The pH value showed that there was a significant reduction in the pH from 7.43±0.16 at day 0 of degradation to 4.67±0.09 on the day 28 of degradation. The reduction in pH could be due to the production of acids during the degradation process [24]. This is similar to the findings of Arusha *et al.* [18] who reported that the crude cellulase produced by *Pseudomonas* sp. and *Bacillus* sp was found to be active at pH 5. But, the result is not in agreement with the findings of Joseph *et al.* [25] that reported the optimum pH for crude cellulase obtained from three *Bacillus s*trains after 48 h, 72 h and 96 h to be pH 7.0. The deviation from their findings could be as a result of increased incubation period (28 days) adopted in this study.These results are also in agreement with the findings of Olanbiwoninu and Fasiku [26] that used *Bacillus pumilus*and *Bacillus subtilis* for the production of amylases and cellulases using sweet potato peels. Optimum pH for the production of cellulase by all the organisms in their research ranged from 5 - 7. These results obtained during the research were also in agreement with the findings of Goya and Soni [27], Azzeddine *et al.* [28] and Trinh *et al.* [29] who reported pH 5, 6 and 7 respectively as the optimum pH for production of cellulase from *Bacillus* species.

CONCLUSION

Cellulolytic bacteria are undoubtedly abundant in the soil in large numbers cohabiting each other. The isolate with the highest hydrolytic activity when screened was *Bacillus cereus.* The highest cellulose and weight loss was achieved at 40°C with 80.55±0.77 and 14.95±0.07 respectively after 28th day of treatments. The bacterium had the great capacity to hydrolyze cellulose from plant source**.**

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