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PRODUCTION OF CELLULASE ENZYMES USING *Bacillus* species ISOLATED FROM CASSAVA WASTE

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Abstract

Cellulase enzymes play important roles in the degradation of cellulose. Species from the genus *Bacillus* that are known for their robust enzyme production, have also been identified as efficient cellulase producers. The aim of this research was to investigate the production of cellulase enzymes using *Bacillus* species isolated from cassava wastewater. Cassava waste samples were collected aseptically and *Bacillus* species were isolated using serial dilution and the pour plate method. Characterization and identification were carried out through standard microbiological techniques. Screening for cellulase production was conducted using carboxymethyl cellulose (CMC) agar. Production of cellulase was by submerged fermentation. Enzyme activity was determined using the dinitrosalicylic acid (DNS) assay. The highest and lowest values of the bacteria count recorded across the various wastewater samples were 1.84 ± 0.259 x 107 CFU/mL and 3.7 ± 0.125 x 105 CFU/ml respectively. The isolated *Bacillus* spp. exhibited significant cellulolytic activity, with clear hydrolysis zones observed on CMC agar. The highest cellulase activity was recorded at 205.71 U/ml under optimized conditions of pH 6.5–7.5 and a temperature of 37°C. This study confirmed the ability of *Bacillus* spp. to use cassava waste as a cost-effective substrate for enzyme production, highlighting its potential for large-
Scale industrial applications.

INTRODUCTION

Enzymes are proteins that function as biological catalysts, accelerating chemical reactions. The molecules that enzymes operate upon are called substrates, and enzymes convert these substrates into various compounds

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called products (Horowitz, 1999). Cellulases are a group of enzymes that catalyze the breakdown of cellulose, which is the most abundant organic polymer on Earth, into simpler sugars. This capability render cellulases invaluable in various sectors, including biofuels, textiles, food, and paper industries. This enzyme also plays a key role in hydrolyzing β-1, 4-glycosidic linkage in cellulose, a dominant component in plant cell wall (Horowitz, 1999). Cellulase which contributes to a large proportion of industrial enzymes in the global market, indicate their status as an important enzyme class in the market. In fact, cellulase is the third largest industrial enzymes by dollar volume and accounts for approximately 20 % of the total enzyme market in the world. The strong demand for cellulase is attributed to its major applications in the pulp and paper, textile, food and beverage, detergent, animal and feed industries (Behera and Ray, 2016).

Cassava, *Manihot esculenta*, is a tropical root crop widely cultivated in Africa, Asia, and Latin America. The crop is highly drought-resistant and also thrives in poor soil environment. In addition to its nutritional value, its nutritional value, cassava is also essential in industries producing starch, ethanol, and animal feed. For thousands of years, people have grown cassava, mostly in tropical and subtropical climates. It is an essential crop for food security because of its hardiness and capacity to flourish on unfavorable soils (Sadhu and Maiti, 2013). Microorganisms, including bacteria, fungi, and actinomycetes, are prolific producers of cellulase enzymes. Fungi, particularly species such as *Trichoderma* and *Aspergillus*, are renowned for their high cellulase yields. Microbial cellulose production is preferred over chemical synthesis due to its efficiency, sustainability, and lower environmental impact. The production process is influenced by various factors, such as the type of microorganism, substrate composition, pH, temperature, and aeration (Arotupin, 2007).

Among the microorganisms capable of producing cellulase, *Bacillus* species are of particular interest due to their adaptability and efficient enzyme production. *Bacillus* species are Gram-positive, rod-shaped bacteria known for their ability to secrete several extracellular enzymes, including cellulases. These bacteria are found in diverse environments and are especially prolific at degrading plant material. Their ability to thrive under various conditions makes them an ideal candidate for cellulase production (Singh and Kumar, 2011). *Bacillus* species such as *B. subtilis* and *B. licheniformis* are recognized for producing thermostable and alkaline-tolerant cellulases, which are essential for industrial processes that often require high temperatures and specific pH ranges. The efficiency of *Bacillus* spp. in converting cellulose into simple sugars makes them valuable for the biofuel industry, where lignocellulosic biomass is converted into fermentable sugars for ethanol production.

Using cassava waste as a substrate for cellulase production presents a dual benefit: it addresses waste disposal issues and provides a low-cost, renewable resource for enzyme production. Previous studies have demonstrated the feasibility of using agricultural wastes, such as corn stover and wheat straw for cellulase production, highlighting the potential of such substrates in reducing production costs and enhancing sustainability. Cassava waste, which is rich in cellulose, is an ideal candidate for this purpose. Cassava peels and other residues are rich in organic matter, leading to rapid decomposition and the release of greenhouse gases if not properly managed. Additionally, the leaching of nutrients and organic compounds can contaminate soil and water bodies, affecting local ecosystems. Economically, improper waste management can incur significant costs for cassava processing industries and local governments. Converting cassava waste into valuable products like cellulase enzymes, can alleviate these environmental issues and provide an additional revenue stream; hence, this study was carried out to explore the production of cellulase enzymes using microbial isolates from cassava waste water.

MATERIALS AND METHODS

Collection of Samples:

Cassava wastewater was obtained from a cassava processing factory located at Idokpa town under Ikpoba-Okha

Local Government Area, Edo State. The samples were collected using a clean container and transported to the Microbiology laboratory of the University of Benin for analysis. Stock samples were maintained at 4 ^{o}C in the refrigerator. The chemicals used were of analytical grade, whereas distilled water was used.

Media Preparation / Isolation of Microorganisms:

Nutrient agar and Carboxymethyl Cellulose (CMC) agar were prepared according to the manufacturer's instructions. The samples were autoclaved for 15 min at 121 °C. The associated microorganisms in the samples were isolated using serial dilutions and the pour plate method. In total, 25 ml of cassava wastewater was diluted with 225 ml of sterile distilled water. The sample was serially diluted. 1 mL of dilution factor, 10^{-5} of the samples was inoculated on Nutrient agar, using the pour plate method. The inoculated media were incubated at 37 °C for 24 – 48 hrs for bacterial growth. Discrete colonies that developed on the plates were counted and recorded as colony forming unit per milliliter (CFU/ml) (Dimowo and Omonigho 2017)

Enumeration of Microorganisms:

The enumeration of microorganisms was carried out using the colony counter. After incubation, colony-forming units (CFU) were counted, and microbial load was calculated using the formula below;

$$\frac{CFU}{mL} = \frac{\text{No. of colonies}}{\text{Volume of inoculum plated}} \times \frac{1}{\text{Dilution Factor}}$$ (Dimowo and Omonigho, 2017)

Isolation of Pure Cultures:

The streak plate method was used for the isolating pure cultures. The newly prepared media was poured into petri-dishes and allowed to solidify. Bacteria with different characteristics were sub-cultured by transferring 1 x 2 cm² of bacterial tips-containing agar onto the center of fresh nutrient agar media using a sterile wired loop. This was done to avoid picking two different colonies at a time. The process was carried out very close to the flame to ensure proper sterility (Asikhia and Dimowo, 2023).

Characterization of Isolates:

Bergey's Manual of Determinative Bacteriology was used as a reference guide for identifying the isolated bacteria. This manual classifies bacteria based on a range of characteristics, including morphological characteristics such as colony appearance and cell shape, Gram staining results to differentiate Gram-positive and Gram-negative bacteria and biochemical reactions based on enzymatic activities.

Screening of Cellulase-Producing Bacteria:

For primary screening, isolated bacteria were grown on 1 % carboxymethylcellulose (CMC) media and incubated at 28 ± 2 °C for 15 min. The Petri dishes were then flooded with Iodine solution (0.1 %). The reagent solution was discarded and the plates were washed with 1M NaCl solution for 15–20 mins. A clear zone around the colony indicates the presence of cellulase enzyme (Andrade and Pastore, 2013). Bacteria isolates capable of decomposing CMC were identified by the emergence of a clear zone surrounding the colony after testing with Congo red (Silwal and Chhetri, 2022).

Production of Cellulase

The selected bacterial cultures were individually maintained on CMC agar slant at 4 °C. The selected bacterial culture was inoculated in broth medium containing 1.0 gm peptone, 0.3 gm meat extract, 0.5 gm NaCl and 100 ml of distilled water. After the incubation period, the bacterial cells were used as inoculum.

The selected bacterial isolates were studied for cellulase enzyme production in the submerged fermentation process. 250 ml Erlenmeyer flask containing 50 ml of the production medium containing 3.0 gm Carboxymethylcellulose, 1.0 gm peptone, 0.3 gm meat extract, 0.5 gm NaCl, and 100 ml distilled water was used. The medium was autoclaved at 121 °C for 15 min. After autoclave, the medium was inoculated with 3.0

ml of bacterial isolates and inoculated in a rotary shaker at 37±2 °C for 9 days of fermentation period with agitation speed of 100 rpm. The enzyme activity was checked at an interval of 24 hrs, after centrifugation, of the crude enzyme source.

Estimation of Cellulase Activity

The estimation of cellulase enzyme activity was assayed using 3, 5 - dinitrosalicylic acid (DNSA) reagent (Asikhia and Dimowo, 2023) by estimating the amount of reducing sugars released from CMC. The crude enzyme was added to 2.0 ml of 1 % CMC solution to the tube as the substrate. The solution was incubated in a test tube at 55 °C for 30 min. 1 ml DNSA solution to stop the enzyme activity. The solution was kept in a water bath for 15 min and then 6 ml of distilled water was added. Absorbance was measured at 540 nm using the spectrophotometer (Dimowo *et al.,* 2021). Cellulase production was estimated using glucose calibration curve. One unit (U) of enzyme activity is expressed as the quantity of enzyme, required to release 1 µmol of glucose per minute under standard assay conditions (Jabeen and Qazi, 2014).

RESULTS

Table 1; depicts the total heterotrophic bacteria count isolated from cassava wastewater using nutrient agar. The highest count was obtained from sample B, which had a count of $2.08 \pm 0.08 \times 10^7$cfu/ml and the lowest count, was obtained from sample D, which had a count of $3.7 \pm 0.125 \times 10^5$ cfu/ml

TABLE 1: Total heterotrophic bacterial count isolated from cassava waste (cfu/ml)

Sample	Total bacterial count (cfu/ml)
A	$1.84 \pm 0.259 \times 10^7$
B	$2.08 \pm 0.08 \times 10^7$
C	$2.4 \pm 0.259 \times 10^6$
D	$3.7 \pm 0.125 \times 10^5$

Table 2: Cultural and morphological characteristics of isolates

Sample	**Shape**	**Size**	**Elevation**	**Margin**	**Surface**	**Color**	**Gram Reaction**	**Shape and arrangement**
A	Circular	Small	Convex	Entire	Dull	Grey	-	Rod-shaped
B	Irregular	Small	Convex	Undulate (wavy)	Dull	Pinkish-grey	-	Rod-shaped
C	Circular	Large	Convex	Entire	Smooth	Pale yellow	+	Rod-shaped, single celled
D	Circular	Medium	Convex	Undulate	Dull	Grey	+	Rod-shaped, single celled

Based on visual observation and after performing Gram's staining procedure, cultural and morphological characteristics of the colonies on the agar plate were recorded as follows.

Table 3: Biochemical characterization

Biochemical Test	Result	Interpretation
Urease Test	Negative (-)	No production of urease enzyme; urea not hydrolyzed.
Motility Test	Positive (+)	*Bacillus* spp. exhibits motility.
Catalase Test	Positive (+)	Presence of catalase enzyme; bubbles observed.

Biochemical tests such as the urease, motility catalase tests were performed. Biochemical tests such as the urease, motility catalase tests were performed.

Figure 1: Rapid plate screening for Cellulase Production

Figure 1; explains the result of the preliminary screening of the *Bacillus* spp. isolate for cellulase activity, utilizing starch as a sole source of carbon using a culture plate method showed the presence of a halo ring on the culture plate. This indicates that the organism (*Bacillus* species) uses starch as its sole source of carbon. Carboxymethyl cellulose (CMC) agar was used.

Figure 2, illustrates the cellulase activity of *Bacillus* spp. as a function of the number of days. The enzyme activity values of *Bacillus* species isolated from cassava wastewater samples during cellulase production on day 3 was 138.29 U/ml and on day 7 it increased to 182.86 U/ml. The highest enzyme activity was 205.71 U/ml on day 9 of production. The lowest enzyme activity value of 126.29 U/ml was obtained from *Bacillus* species on day 1.

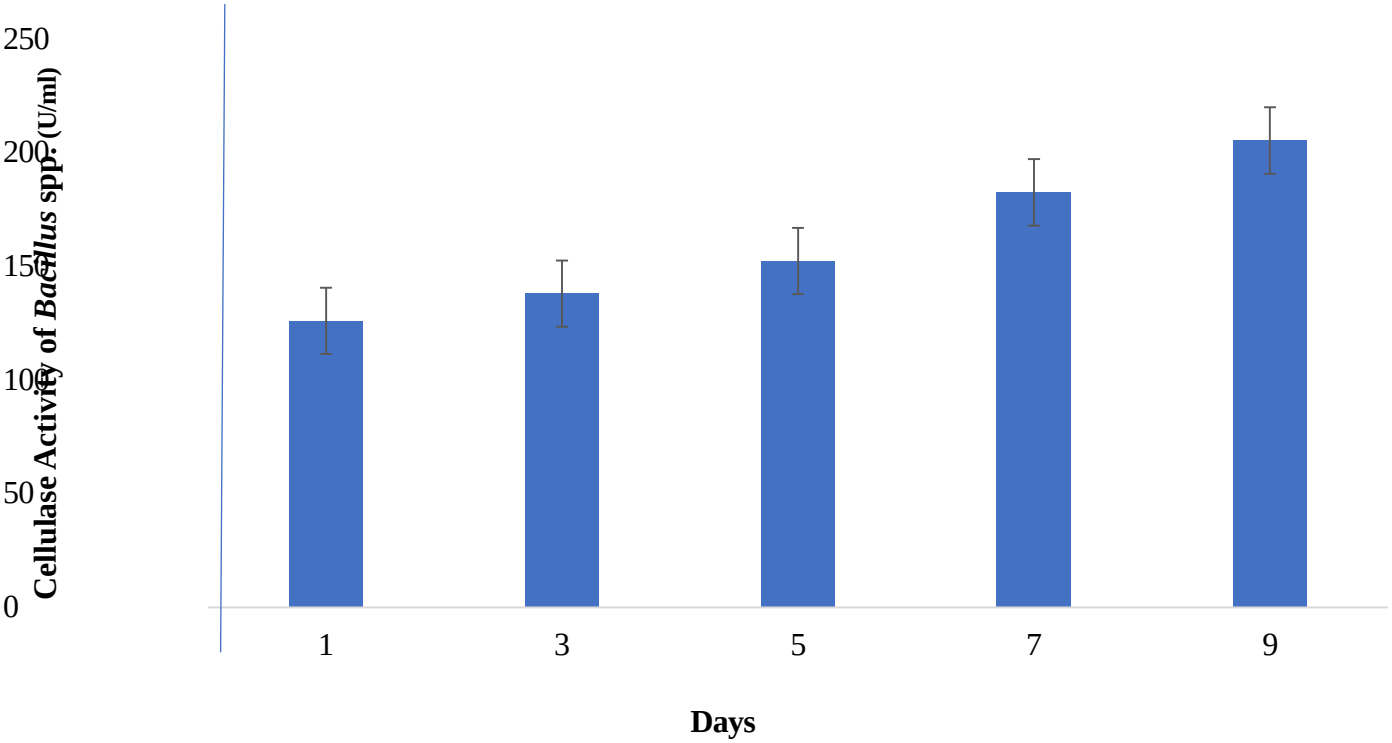

Figure 2: Cellulase activity of *Bacillus* species during production.

Discussion

According to Arotupin (2007), the potential of microorganisms obtained from cassava wastewater was evaluated for the production of amylase and cellulase. This study's findings also highlight the potential of *Bacillus* species that were isolated from cassava waste to function as efficient cellulase enzyme producers. A sustainable approach to waste management

was demonstrated when cassava waste – which is a major environmental pollutant due to its high organic content was effectively used as a substrate for isolating bacteria that produce cellulase. Therefore, our findings demonstrate the potential of cassava waste as a cost-effective substrate for cellulase production, addressing both waste management issues and the demand for industrial enzymes. The presence of *Bacillus* species as well as other bacterial species was confirmed by the microbial isolates varied cultural, morphological, and biochemical traits. Most of the isolates had rod-shaped Gram-positive bacteria, according to the Gram staining performed, which is in line with earlier research on *Bacillus* species isolated from cellulose- rich environments (Singh and Kumar, 2011). The Congo red assay was used to verify the capacity of these isolates to produce cellulase enzymes; distinct zones of clearance indicated cellulolytic activity. Their ability to form clear zones on CMC agar plates suggests robust cellulase production, confirming previous research done by Behera and Ray (2016), which highlighted the cellulolytic potential of *Bacillus* spp. in lignocellulosic environments.

Submerged fermentation, the method which was used in this study to produce cellulase, showed high enzyme activity during its use. Based on previous reports on cellulase-producing *Bacillus* species, this study verified that *Bacillus* spp. flourish at temperatures between 35°C and 45°C and pH ranges between 6 and 8. Enzyme activity increased exponentially and the highest cellulase enzyme activity was recorded on the ninth day at 205.71 U/ml. These results highlight how *Bacillus* species can adapt to varoius environmental conditions, making them viable options for industrial enzyme production.

Also, sources of carbon and nitrogen had a significant impact on cellulase production. For maximum cellulase yield, carbon sources like starch and nitrogen sources such as peptone and ammonium nitrate were essential in the production process. High cellulose content and accessibility in cassava wastewater make it a promising substrate for cellulase synthesis.

This study also showed that *Bacillus* species-produced cellulases have potential uses in waste management, textile processing, and biofuel production. For example, the importance of cellulases in the biofuel sector is demonstrated by their ability to hydrolyze cellulose into fermentable sugars. Employing cassava waste as a substrate offers a commercially feasible raw material for the synthesis of cellulase while simultaneously lowering environmental pollution. The results of this study also highlight the necessity for scalable and economical cellulase production techniques. Solid-state fermentation (SSF) is a technique that can increase enzyme yield while using less energy and water, making the process more environmentally friendly for industrial use.

Although, the study achieved significant outcomes, several challenges were noted. The heterogeneity of cassava waste results in difficulties in maintaining uniformity during fermentation. This variability in substrate composition may have affected cellulase activity and yield. Future studies should focus on optimizing pretreatment methods to enhance cellulose accessibility while minimizing inhibitors like lignin. Another limitation was the scale of fermentation. Although laboratory-scale submerged fermentation provided valuable insights, industrial-scale production requires additional considerations such as cost, equipment design, and process integration. Exploring genetic engineering to develop *Bacillus* strains with enhanced cellulase activity and stability could address some of these challenges.

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