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Isolation and Biochemical Characterization of Cellulase Produced by Bacterial Isolates from Sugarcane Waste Soil

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Cellulase is one of the most economically important enzyme, which aids in catalyzing cellulolysis, the decomposition of cellulose and other related polysaccharides. So the demand/importance of this enzyme in both domestic and commercial sectors cannot be over emphasized. In this research cellulase-producing bacteria were isolated from soil around sugarcane waste dumping area, which was identified to be *P. fulorescens* after numerous biochemical and microbiological analysis. The bacteria were then grown and used to ferment certain biomass, with the aim of using the organisms to produce the cellulase enzyme. The total protein/cellulase enzyme activity of the medium was ascertained. Optimization/characterization for maximum cellulase activity was done by varying the temperature, pH, enzyme concentration and substrate concentration, in which the optimum condition for cellulase production was ascertain to be at a temperature and pH of 40˚C and pH 7 respectively. SDS-PAGE electrophoresis was carried out to determine and reconfirm the presence and molecular weight of the isolated enzyme. The estimated extrapolated molecular weight of the enzyme was found to be 13.5KDa.

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1. INTRODUCTION

Cellulase is any of several enzymes produced chiefly by fungi, bacteria and protozoans, which catalyze cellulolysis (the decomposition of cellulose) and of some related polysaccharides. The name is also used for any naturally occurring mixture or complex of various enzymes, that act serially or synergistically to decompose cellulosic material [1]. In the first stage of cellulose decomposition, the polymer is broken down into disaccharide cellobiose in the presence of enzyme cellulase and then into glucose with the enzyme cellobiase. Cellulases break down the cellulose molecule into monosaccharides ("simple sugars") such as beta-glucose or shorter polysaccharides and oligosaccharides. Cellulose breakdown is of considerable economic importance, because it makes various major constituents of plants available for consumption and use in chemical reactions [2]. The specific reaction involved is the hydrolysis of the 1,4-beta-D-glycosidic linkages in cellulose, hemicellulose, lichenin, and cereal beta-D-glucans. Because cellulose molecules bind strongly to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides such as starch [3]. Most mammals have only very limited ability to digest dietary fibres such as cellulose by themselves. In many herbivorous animals, such as ruminants like cattle and sheep, hindgut fermenters like horses, cellulases are produced by symbiotic bacteria. Cellulases are produced by a few types of animals, such as some termites [4].

Several different kinds of cellulases are known, which differ structurally and mechanistically. Synonyms, derivatives and specific enzymes associated with the name "cellulase" include endo-1,4-beta-D-glucanase (beta-1,4-glucanase, beta-1,4-endoglucan hydrolase, endoglucanase D, 1,4-(1,3,1,4)-beta-D-glucan 4-glucanohydrolase), carboxymethylcellulase (CMCase), avicelase, celludextrinase, cellulase A, cellulosin AP, alkali cellulase, cellulase A 3, 9.5 cellulase, and pancellase SS [5]. Enzymes that cleave lignin have occasionally been called cellulases, but this old usage is deprecated; they are lignin-modifying enzymes. Cellulase are mostly used in commercial food processing, such as in coffee production and in the pulp and paper industry. During drying of beans, it performs the hydrolysis of cellulose [6]. Therefore, it is necessary for researchers to work on the various plants extract/materials that can be used for the production, extraction and isolation of cellulose, other than from food processing and hydrolysis of cellulose, which is economically not economical. This enzyme is however also vital in the fermentation of biomass, in biofuels production.

Sugarcane are several species of tall perennial true grasses of the genus *Saccharum*. It's an Andro-pogoneae, native to the warm temperate to tropical regions of South and Southeast Asia, Polynesia and Melanesia, which is normally used for sugar production [7]. It has stout, jointed and fibrous stalks that are rich in the sugar sucrose, which accumulates in the stalk internodes. The plant is two to six meter's (six to twenty feet) tall. All sugar cane species interbreed and the major commercial cultivars are complex hybrids [8]. Sugarcane belongs to the grass family Poaceae, an economically important seed plant family that includes maize, wheat, rice and sorghum and many forage crops. Sugarcane predominantly grows in the tropical and subtropical regions (sugar beets grow in colder temperate regions). Other than sugar, products derived from sugarcane include falernum, molasses, rum, cachaça (a traditional spirit from Brazil), bagasse and ethanol. In some regions, people use sugarcane reeds to make pens, mats, screens and thatch. Bagasse is the fibrous matter that remains after sugarcane or sorghum stalks are crushed to extract their juice. It is dry pulpy residue left after the extraction of juice from sugar cane. Some industries normally use bagasse as a biofuel and in the manufacture of pulp and building materials [9]. The vast demand of this enzyme in both domestic and commercial sectors is of paramount importance. This research work is aimed at the extraction of cellulase from sugarcane waste. By identifying the organisms present and using them for the production and optimization of cellulase enzyme.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil sample from sugarcane waste was collected from sugarcane waste stand at kofar Famfo Kano state, Nigeria in sterile bags and taken to the laboratory.

2.2 Carboxymethylcellulose Media Preparation

Carboxymethylcellulose Media was prepared using standard protocols [10] and the mixture was then autoclaved for 20 minutes at 121˚C.

2.3 Serial Dilution of Soil Sample

Serial dilution were done in sterile test tubes labeled $10^{\wedge-3}$, $10^{\wedge-4}$, $10^{\wedge-5}$ and $10^{\wedge-6}$ respectively, with a start-up volume of 100ml of distilled water, the tubes were capped and swirled gently to mix [11].

2.4 Screening and Isolation of Bacteria

Cellulose producing bacteria were isolated from soils by the dilution spread plate method using carboxymethycellulose agar media [12]. The gel was incubated at 45^0C for 24 hours. The plates were then flooded with 0.1% Congo red for 15 minutes and washed with 1M NaCl to visualize the hydrolysis zone. To indicate the cellulose activity of the organisms, diameter of the clear zone around colonies on the carboxymethylcellulose agar was measured [12].

2.5 Bacterial Identification

The bacterial isolates were identified by means of morphological examination and some biochemical characterization. The parameters investigated includes colonial morphology and gram reactions. The results were compared with Bergey‘s manual of determinative bacteria [10]. Standard biochemical identification analysis were carried out, which includes; Starch Hydrolysis Test [13], Gelatin Liquefaction [11], Fluorescent Pigment Test [14], Catalase Test [12], Oxidase Test [15].

2.6 Enzyme Production Medium

Production medium containing glucose 0.5gm, peptone 0.75gm, $FeSO_4$ 0.01gm, KH_2PO_4 0.5gm, and $MgSO_4$ 0.5gm. 10 milliliters of medium were taken into a 100ml conical flask. The flask was sterilized in an autoclave at 121^0C for 15 minutes and after cooling, the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37^0C in a shaker incubator for 10hours. At the end of the fermentation period, the culture was centrifuged at 5000g for 15 minutes to obtain the crude extract which serve as enzyme source [16].

2.7 Determination of Total Protein Content

Content of the protein in the clear supernatants was determined according to Bradford method using Serum Bovine Albumin (BSA) as standard and the concentration was expressed in milligram per milliliter (mg/ml) [17].

2.8 Enzyme Assay

Briefly a reaction mixture composed of 0.2ml of crude enzyme solution plus 1.8ml of 0.5% carboxymethylcellulose in 50mm sodium phosphate buffer (pH 7) was incubated at 37^0C in a shaking water bath for 30 minutes. The reaction was terminated by adding 3ml of dinitrosalicyclic acid reagent. The colour was then developed by boiling the mixture for 5 minutes. OD of the samples was measured against a blank containing all the reagents minus the crude enzymes [12].

2.9 SDS PAGE Electrophoresis

SDS page electrophoresis is normally done using a 12.5% polyacrylamide slab gel with a Tris-HCl buffer at pH 8.9. A 0.1% SDS-14% polyacrylamide slab gel is used, following the method of [18]. The gel was then stained with 0.1% coomassie Brilliant blue R-250 in 50% methanol 7% acetic acid and the background of the gel was de-stained with 7% acetic acid.

2.10 Effect of Change in pH Cellulase Activity

Flasks with broth containing the optimum concentration of substrate and carbon source are taken and the pH of the broth is adjusted to 7.0, 8.0, 9.0, 10.0 and 11.0 in different flasks using 1N-HCl and 1N-NaOH and sterilized. The cultures were then inoculated and incubated at particular temperature. At the end of incubation period, the cell-free culture filtrate is obtained and used as enzyme source [19].

2.11 Effect of Change in Temperature on Cellulase Activity

Production medium at pH 7 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different temperatures from 35, 40, 45, 50, 55, and 60^0C for 6 hours. At the end of incubation period, the cell-free culture filtrate is obtained and used as enzyme source [16].

2.12 Effect of Change in Enzyme Concentration on Cellulase Activity

The concentration of the enzyme used were increased from 0.2, 0.4, 0.6, 0.8, and 1.0mg/ml respectively, in different flasks. The cultures were inoculated and incubated at a particular temperature. At the end of incubation period, the cell free filtrate was used as enzyme source [19].

2.13 Effect of Substrate Concentration on Cellulase Activity

The concentration of glucose, which is the substrate that was used during the medium production was increased from 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0mg/ml respectively, in different tubes. The cultures were inoculated and incubated at a particular temperature. At the end of incubation period, the cell free filtrate was used as enzyme source [20].

3. RESULTS

3.1 Results for Bacterial Identification

Cellulase producing microorganisms was identified from the soil sample of sugarcane waste both microbiological and biochemical protocols. The microorganism was identified as *Pseudomonas flourescens* as seen in Table 1 and Fig. 1. Microbiological analysis reveals the isolates has a rod shape morphological appearance and gram negative when gram stained.

Table 1. Results for Biochemical characteristics of the bacterial isolates from the sugar cane waste soil

S/N	Biochemical test	Result
1	Starch hydrolysis	Negative
2	Gelatin liquefaction	Positive
3	Fluorescent pigment	Positive
4	Catalase test	Positive
5	Oxidase test	Positive

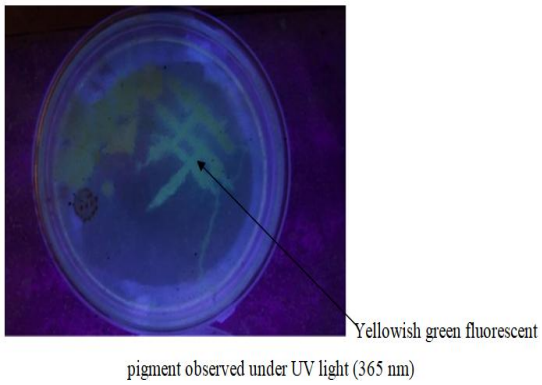

Fig. 1. Fluorescent test under UV ray

3.2 Result for Total Protein Determination using Bradford Method

The total protein of the sample fractions after serial doubling dilution was determined using Bradford standard method, which shows fraction 1 has the highest protein content of 0.68mg/ml, while fraction 1 has the lowest (Fig.. 2).

3.3 SDS PAGE Electrophoresis of Cellulose Enzymes Isolated from Bacterial Soil Isolate

Fig. 3 shows the SDS-PAGE Electrophoresis of the Cellulose Enzymes isolated from bacterial culture. The estimated molecular weight of this enzyme is shown in lane 1, with an extrapolated weight of 13.5KDa. Lane 2 is for the ladder.

3.4 Effect of Change in Temperature on Cellulase Activity

Enzyme activity recorded at different temperatures revealed that the bacteria yielded maximum cellulase production at 40˚C, with an activity of 3.12mg/ml as seen in Fig. 4. The temperature was found to influence extracellular enzyme secretion, possibly changing the physical properties of the cell membrane.

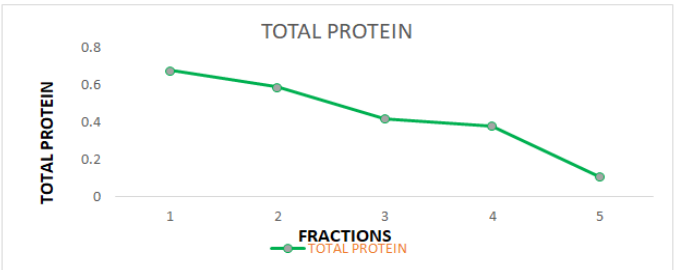

Fig. 2. Results for total protein content of the individual fractions

3.5 Effect of Change in pH on Cellulase Activity

The isolate was allowed to grow at different pH and the maximum enzyme activity was observed in the medium of pH 7.0 – 9.0. The analysis reveals that the enzymes was most active at a neutral pH of 7.0.

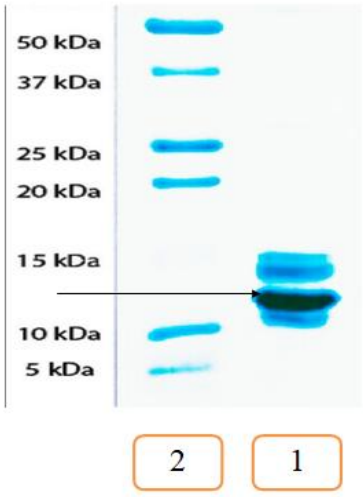

Fig. 3. SDS-PAGE of Cellulase Enzyme Isolated from Bacterial Soil Isolate
Key: Lane 2 = Molecular weight marker, Lane 1 = Isolated Cellulase Enzyme

3.6 Effect of Change in Enzyme Concentration on Cellulase Activity

An increase in enzyme concentration leads to increase in cellulase activity, because by increasing enzyme concentration the maximum reaction rate increases greatly. When the enzyme concentration was increased, the activity of the enzymes also increased as seen in Table 2.

3.7 Effect of Change in Substrate Concentration on Cellulase Activity

Different concentration of substrate was used on the isolated enzyme and it was found out that an increase in substrate concentration increases the rate of the reaction and enzyme activity this is because more substrate molecules will be colliding with more enzyme molecules and more products will be formed as seen in Table 3.

4. DISCUSSION

The vast use of Cellulase enzyme in both domestic and industrial sector is one of the main reasons why the enzyme demand is always high. This enzyme aids in catalyzing cellulolysis, the decomposition of cellulose and of some related polysaccharides. In which it break down the cellulose molecule into monosaccharides such as beta-glucose or shorter polysaccharides and oligosaccharides [21]. Many researchers have documented that, this enzyme can be found in sugarcane waste soil due to the presence of the bacteria like *P. fulorescens*. The presence of that bacteria leads to the production of cellulase. In this research cellulose producing bacteria was isolated from soil around sugarcane waste dumping area, which was identified to be *P. fulorescens* after biochemical and microbiological analysis. Microbiological analysis reveals the isolates has a rod shape morphological appearance and gram negative when gram stained. Biochemical analysis of the bacteria gave positive result for Catalase test, Gelatin liquefaction, Fluorescent pigment and Oxidase test but negative result for starch hydrolysis test.

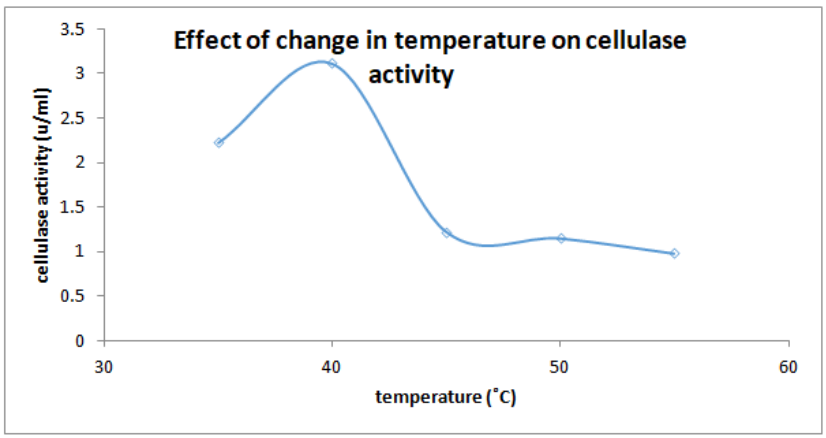

Fig. 4. Effect of change in temperature on Cellulase activity

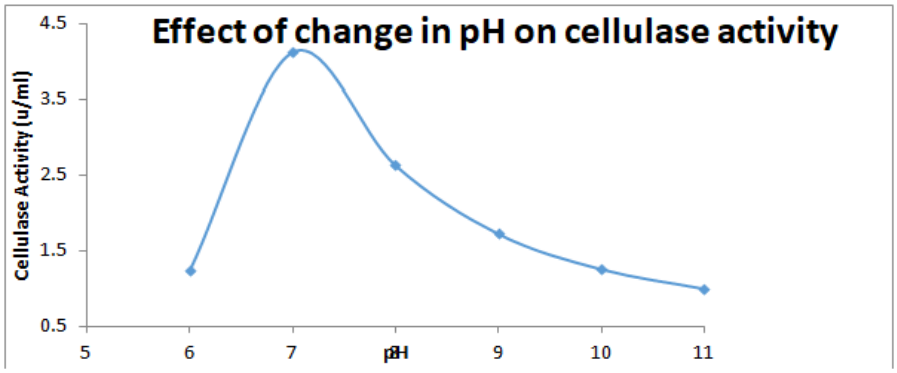

Fig. 5. Effect of Change in pH on Cellulase activity

Table 2. Effect of change in enzyme concentration on cellulase activity

Substrate concentration (mg/ml)	Enzyme concentration (mg/ml)	Enzyme activity (mg/ml)
0.50	0.20	0.95
0.50	0.40	1.48
0.50	0.60	2.67
0.50	0.80	3.83
0.50	1.00	6.13

Table 3. Effect of change in substrate concentration on cellulase activity

Enzyme concentration (mg/ml)	Substrate concentration (ml)	Cellulase activity (mg/ml)
0.2	0.50	1.10
0.2	1.00	1.58
0.2	1.50	2.58
0.2	2.00	3.64
0.2	3.00	5.89

These bacteria were grown and use to ferment certain biomass, with the aim of using the organisms to produce the cellulase enzyme. The total protein/cellulase enzyme activity of the medium was determined using Bradford method/other standard protocols [22]. Optimization for maximum cellulase activity was done by varying the temperature, pH, enzyme concentration and substrate concentration. SDS-PAGE electrophoresis was carried out to determine and reconfirm the presence and molecular weight of the isolated enzyme. The estimated molecular weight of this enzyme is shown in lane 1, with an extrapolated weight of 13.5KDa as seen in Fig 3. This correspond to a research done by [23]. The total protein determination done on the five fractions after serial doubling dilution shows that the first stock fraction has the highest total protein while the last dilution power fraction has the least protein content.

Effect of change in temperature on the isolated enzyme was ascertain by varying the temperature of the production medium. The enzyme activity was recorded at different temperatures, which shows that the bacteria yielded maximum cellulase production at 40˚C, with an activity of 3.12mg/ml as seen in Fig 4. This optimum temperature of 40˚C was found to influence the most production of the extracellular cellulase enzyme, possibly changing the physical properties of the bacterial cell membrane. This result is close to that of [4] on 'Purification and characterization of cellulase from wild type and two improved mutants of *Pseudomonas flourescens'*, who found that cellulase enzymes produced by *P. flourescens* was activated at 30 to 35˚C showing an optimum production at 35˚C. Another parameter that was analyzed in this research is the pH, in which the isolates was allowed to grow at different pH and the maximum enzyme activity was observed in the medium of

pH 7.0 – 9.0. The analysis reveals that the enzyme were most active at a neutral pH of 7.0. This shows that the enzyme will not be viable at an acidic pH, rather than a neutral pH will be most suitable for maximum production and potent activity. [7] Also reported similar finding in their research on isolation and characterization of Cellulase-producing Bacteria from Sugar Industry Waste.

An increase in the enzyme and substrate concentration leads to the increase in the activity of the enzyme, probably by increasing enzyme concentration which in turns give a maximum reaction rate. When the enzyme concentration was increased, the activity of the enzymes also increased. Different concentration of substrate was used and it was found out that an increase in substrate concentration increases the rate of the reaction and enzyme activity this is because more substrate molecules will be colliding with more enzyme molecules and more products will be formed. This result often in this research is somehow in conformity with the research done by [24] on Isolation, Screening and Identification of Cellulolytic Bacteria from Soil.

5. CONCLUSION AND RECOMMENDA-TION

P. flourescens isolated from the sugarcane waste soil do have the ability to produce cellulase enzyme and it's yield, appears to depend on a complex relationship involving a variety of factors like inoculum size, pH, temperature, presence of inducers, aeration, growth time, enzyme and substrate concentrations. However 16S ribosomal sequencing should be done on these bacteria to ascertain the kind/type of strain/serotype the organism is and to fine out the genes responsible for this enzyme production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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