International Journal of Microbiology and Biotechnology
2023; 8(3): 54-61
http://www.sciencepublishinggroup.com/j/ijmb
doi: 10.11648/j.ijmb.20230803.12
ISSN: 2578-9678 (Print); ISSN: 2578-9686 (Online)

Isolation and Characterization of Cellulose Producing Fungi from Soil in Abraka, Delta State, Nigeria

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To cite this article:

Adomi Patience Ogheneochuko, Anozie Anthony Jude. Isolation and Characterization of Cellulose Producing Fungi from Soil in Abraka, Delta State, Nigeria. *International Journal of Microbiology and Biotechnology*. Vol. 8, No. 3, 2023, pp. 54-61.
doi: 10.11648/j.ijmb.20230803.12

Received: March 1, 2023; **Accepted**: March 24, 2023; **Published**: August 31, 2023

Abstract: Cellulase is an important enzyme which is essential for industrial production of paper, animal feeds and other products useful for humans. Previous study showed that bacteria and fungi from different sources produce cellulase. Few reports exist on the production of cellulase from soil fungi. The purpose of the study was to isolate fungi from soil and screen fungi for cellulase production. Soil samples collected from different sites were serially diluted and plated out into standard mycologic media. Pure isolates were screened for cellulase production using carboxylmethyl cellulase agar. Effects of temperature, incubation time, carbon source, nitrogen source, agricultural wastes and other factors were determined for cellulase production. Eighteen fungal isolates were isolated. Fourteen hydrolysed the medium for cellulase production however isolates 1FG and 8FG that produced higher zones of inhibition were selected for further study. Morphology, lactophenol staining and molecular characterization indicated these isolates as *Aspergillus* sp and *Fusarium* sp. Arabinose and yam peels showed better results among the other carbon sources. Peptone and temperatures at 45°C and 55°C respectively were best nitrogen source and conditions for enzyme production. This investigation showed that fungi isolated from soil have potential for cellulase production, which could be used for large scale production of the enzyme.

Keywords: Cellulase Producing Fungi, Arabinose, Peptone

1. Introduction

Enzymes are proteinous catalysts that speed up chemical reactions. Cellulose is one of the most abundant polymer available on earth, usually present in plant materials and their wastes. Cellulose can be detected in dry plant materials and approximately 50% from agricultural wastes [19]. Cellulases depolymerize cellulose into fermentable sugar. Cellulose is a polymer of glucose linked by ß-1,4 glucosidase bond having a crystalline structure, stabilized by intermolecular and intramolecular hydrogen bonds. Cellulase as a multicellular enzyme system comprises of complexes: endo-ß-1,4-glucanase (CMCase; E. G, EC3.2.1.4), exo-ß-1,4-glucanase (celliobiohydrolase: CBH: EC3.2.1.91) and ß-1,4 glucosidase (GH, EC3.2.1.21) [20].

Cellulolytic microrganisms in nature are able to breakdown cellulose with the aid of the multienzyme system. The complete breakdown of cellulose to glucose is achieved by the synergistic activity of the three complexes that make up the multicellular enzyme system [21]. ß-1,4endoglucanase randomly attacks the internal ß-1,4 glycosidic bonds along a cellulose chain, yielding glucan chains of different sizes and lengths, then ß-1,4-exoglucanase reacts with ends of the cellulose chains and releases ß-cellobiose as the end product. The ß-1,4-glucosidase hydrolysis ß-cellobiose to two glucose molecules [17].

Microorganisms play tremendous role in degradation and production of cellulose in nature. Fungi are important in degradation of cellulose and are capable of producing various cellulytic enzymes such as endonuclease, exonuclease and ß-glucosidase [13]. Cellulase produced from fungi is advantageous due to the high quantity compared with other microorganisms [18]. Cellulolytic organisms have been isolated and characterized in different studies. *Bacillus*

cereus, B. subtilis and *B. thuringensis* were isolated from soil [23], *B. subtilis* from cellulosic waste [9] and *B. subtilis* from rice bran [20].

Application of cellulase in different industries have been reported. Cellulases have found application in biotechnology in terms of feeds and food production and account for more or less 20% of the worlds enzyme market [7, 8]. It's application in animal feeds include improvement of easy digestible animal feeds [12, 15]. Also, in food sciences and industry, the enzyme is used for drying beans, cleansing juices, clarification and liquefaction of fruits and vegetables. In paper and pulp industries, the enzyme is used for improvement and fibre modification, it has application in the textile industry as well, where it is used for cotton softening, in laundry detergents, for cleaning, colour care and cotton handling. The enzyme is important for waste management; paper reusing, bioremediation and wastewater treatments [5]. For research purposes it is used for isolation of plant protoplast, plant viruses investigations, and genetic and metabolic modification studies [14, 25]. In agriculture, cellulase is important in degrading plant pathogen cell walls and to improve quality of the soil [10, 27, 28], thereby producing health plants and enhancing food production. In view of the usefulness of cellulase enzyme in area of food, animal feeds, paper and pulp, textile and other industries; gives the driving force to search for more organisms which can produce cellulase. Therefore, this study was undertaken to isolate and characterize cellulase producing fungi from the soil and determine the physiologic parameters necessary for production of cellulase.

2. Materials and Methods

Soil samples (100g) were collected from four different locations in Abraka town into sterile containers and transported to Microbiology laboratory, Delta State University, Abraka. Each soil sample was serially diluted. Then 0.1ml of each dilution (10^2, 10^3, 10^5) was spread out on Potato dextrose agar and incubated for 3-7days at 28°C. Discrete colonies were subcultured and allowed to grow and identified by morphologic and molecular features [11].

2.1. Cellulase Screening

Cellulose production was determined by method described by Arotupin, [6] hoswever modified. The basal medium; carboxymethycellulose containing NaNO3, 2.5g, MgSO4.7H2O, 0.75g, KH_2PO4, 1.75g, $CaCl_2$. H_2O, 2g, CMC 10g as carbon source, 1 litre distilled water, and agar-gar was prepared. The mixture was dissolved, sterilized and poured out into sterile Petri dishes. The fungal isolates were plated out using methods of Aurotupin and Akinyosoye (2001) and incubated at 30^{o}C for 3-5days. Halo formation around the colony after flooding with 0.1 percent congo red solution and washed with 0.1 M NaCl showed cellulose production. Two fungi that produced larger clear zones were selected for molecular identification.

2.2. Molecular Identification

Pure isolates of fungi were identified. DNA was extracted according to the protocol prescribed by Zymo kit Primers. For the PCR reaction, ITS1F: 5'-TCCGTAGGTGAACCTGCGG-3' ITS2R: 5' GCTGCGTTCTTCATCGATGC3' Primers were used to amplify the internal transcribed spacer region with thermocycler. PCR cocktail mix (10µl) included: 10× PCR buffer (1.0µL), 25mM, $MgCl_2$ (1.0µL), 5p Mol each of forward and reverse primer (0.5µL), DMSO (1.0µL), 2.5Mm DNTPs (0.8µL), taq 5u/ul (0.5µl) 10ng/µL), DNA (2.0µl) and wáter (3.1µL). The PCR program was as follow initial denaturation 94°C for 4 minutes, Denaturation 94°C for 30 seconds, anneal temperature 58°C for 40 seconds. Extension 72°C for a minute. Number of cycles in 45 final extension of 72°C for 10 minutes. Hold temperature 4°C. The PCR products were sequenced and edited using the BLAST tool of NCBI database.

2.3. Cellulase Assay

Cellulase activity was determined as described by Mandel [22]. The assay mixture of a total volume of 2 mL, contained 1 mL of 1 mM of carboxylmethyl cellulose (CMC) in 0.1M sodium acetate buffer (pH 5.0) and 1 mL crude enzyme. The mixture was incubated at 50°C for 30 min. The reaction was stopped by the addition of DNS reagent. The treated samples were boiled for 10 min, cooled and the optical density was measured at 550 nm. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

Culture conditions for cellulase production

Method of Azzaz [8] was adopted for studying fungal cellulase production. Variable conditions including substrate source, incubation period, initial pH and nitrogen source and temperature were investigated. The general procedure included use of triplicate of 1000 mL conical flasks each containing 100 mL of CMC Agar. Effect of substrate source was investigated through replacing of cellulose powder in CMC by 20% (w/v) of different waste materials including yam peel, plantain peel, rubber (*Hevea brasiliensis*) seed and egg shell. The fermented substrate for each flask was mixed with 25 mL of 0.02 M acetate buffer (pH 5.0) by shaking in a rotary shaker (120 rpm) for one hour at room temperature to extract the enzyme and the extracted mixture was filtered and collected for cellulase activity assay. The effect of incubation period was studied through determination of cellulase activities after 24, 48, 72 and 96 h. Effect of the initial pH of growth medium was studied through adjusting the initial pH values in a range between 3 and 9 using either NaOH or HCl 0.1 N. Effect of nitrogen source included the use of inorganic salts (potassium nitrate, urea, peptone and casein). Effect of different temperature was also studied. Flasks were incubated in a rotary shaker at 150rpm at different temperatures (15°C, 25°C, 35°C, 45°C and 55°C).

Table 1. *Results showing fungi with halo region round the colonies and colony diameter.*

Site	Fungal Isolates	Fungal Colony diameter (cm)	Fungal colony +Clear zone (cm)	Clear zone diameter (cm)
1	1FG	1.0	4.3	3.3
	2 FG	0.5	2.0	1.5
	3 FG	1.0	3.0	2.0
	4 FG	0.5	2.0	1.5
2	5 FG	-	-	-
	6 FG	0.8	2.8	1.2
	7 FG	1.2	3.0	1.8
	8 FG	0.5	3.5	3.0
	9 FG	0.2	3.0	2.8
3	10 FG	1.1	2.0	0.9
	11 FG	-	-	-
	12 FG	0.4	2.0	1.6
	13FG	0.6	2.2	1.4
	14 FG	0.2	2.0	1.8
4	15 FG	0.6	2.0	1.8
	16 FG	-	-	-
	17 FG	1.2	3.0	1.8
	18 FG	-	-	-

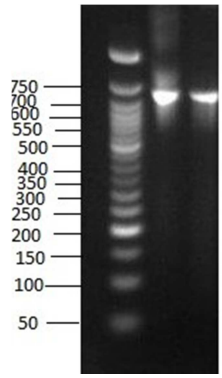

Figure 1. *PCR Gel image 750bp (fungi).*

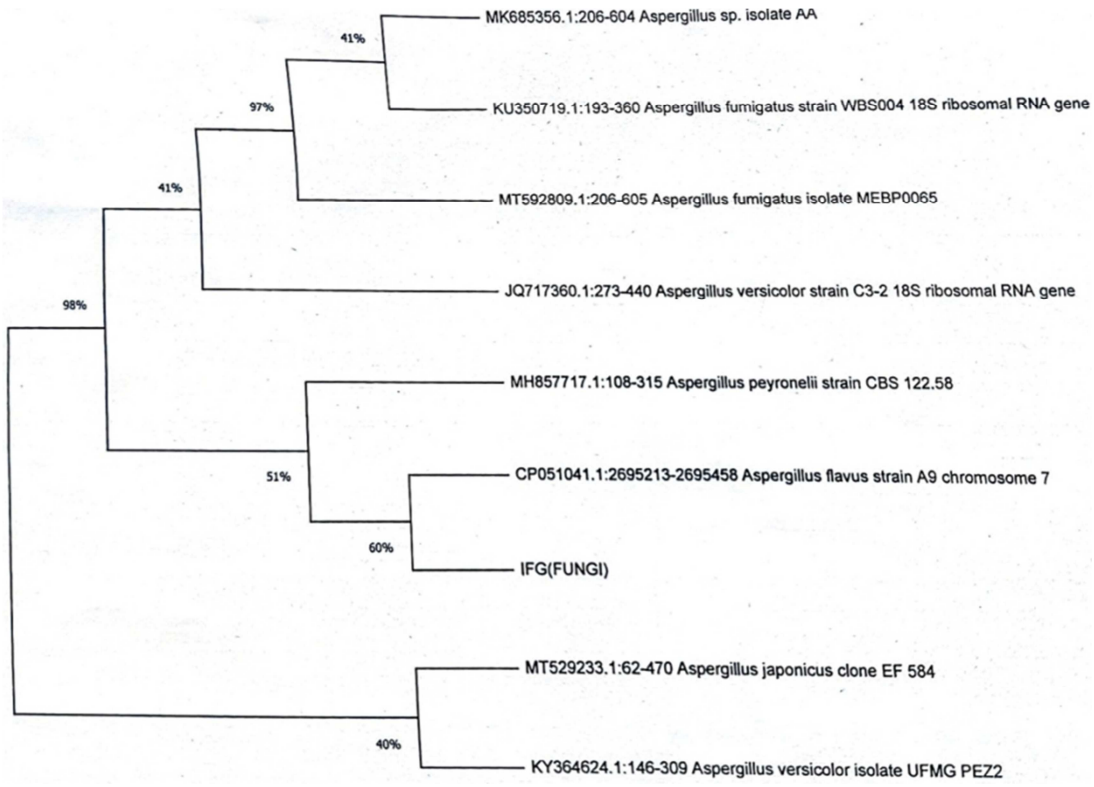

Figure 2. *Phyylogenetic tree for Aspergillus sp.*

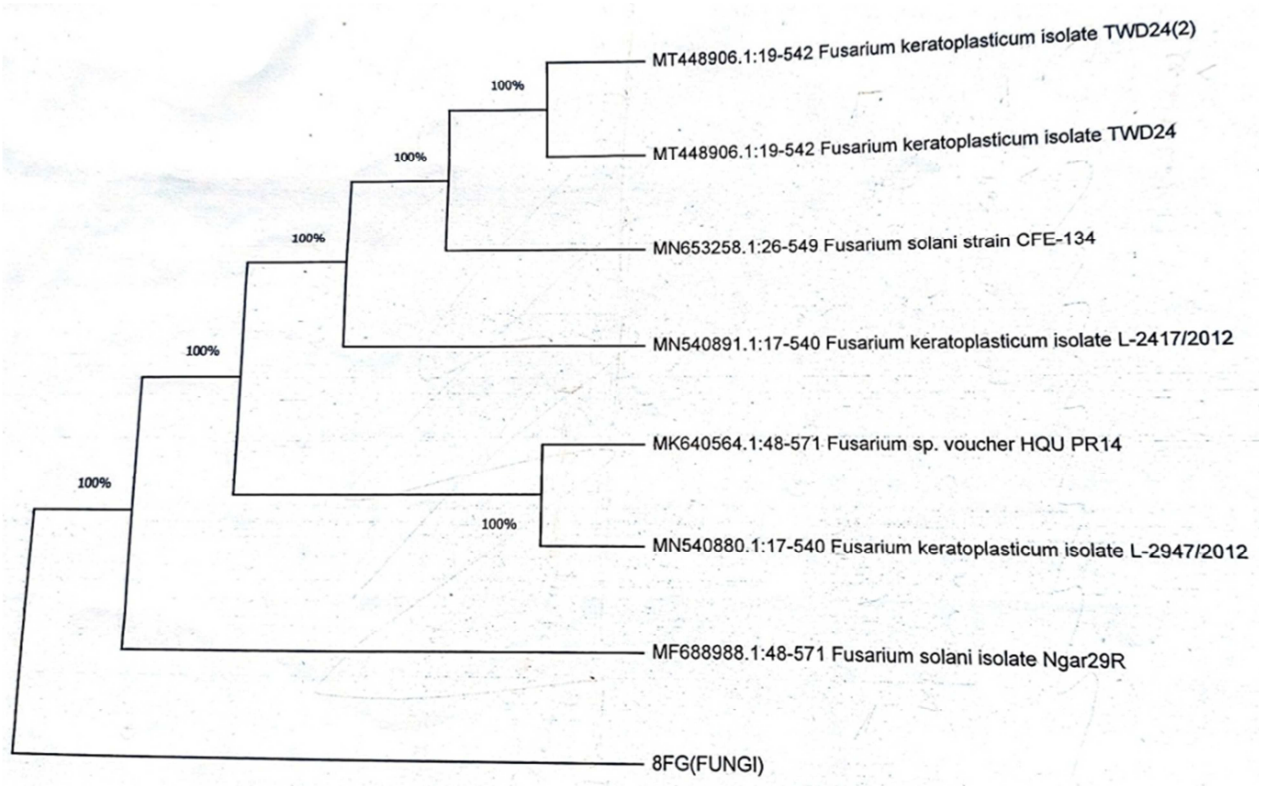

Figure 3. *Phylogenetic tree of Fusarium sp.*

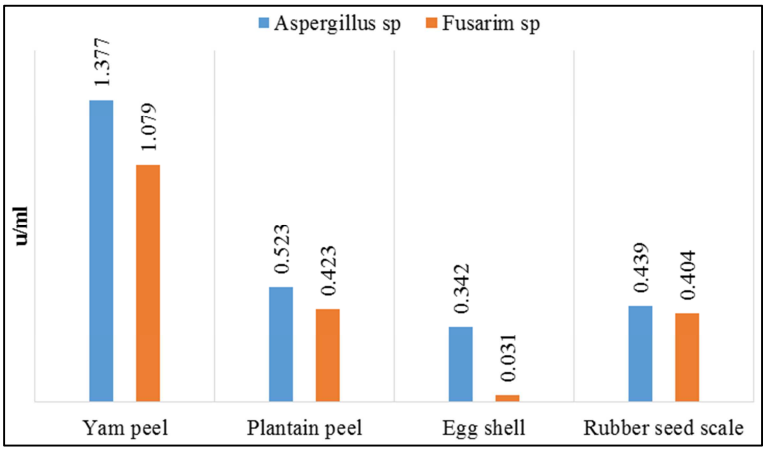

Figure 4. *Cellulase production based on agricultural wastes.*

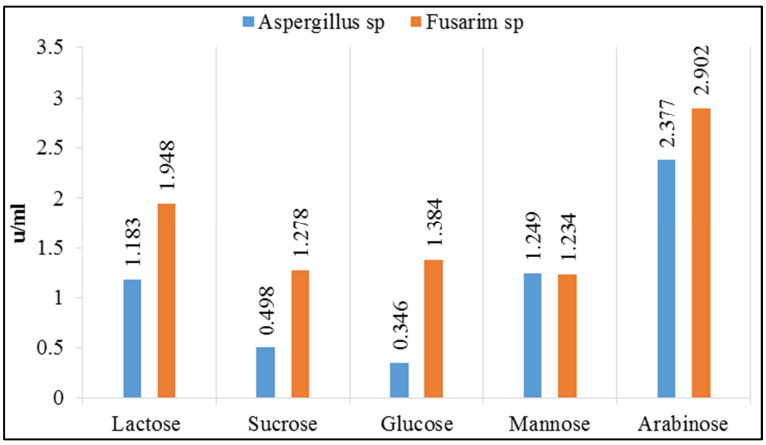

Figure 5. *Cellulase production based on carbon source.*

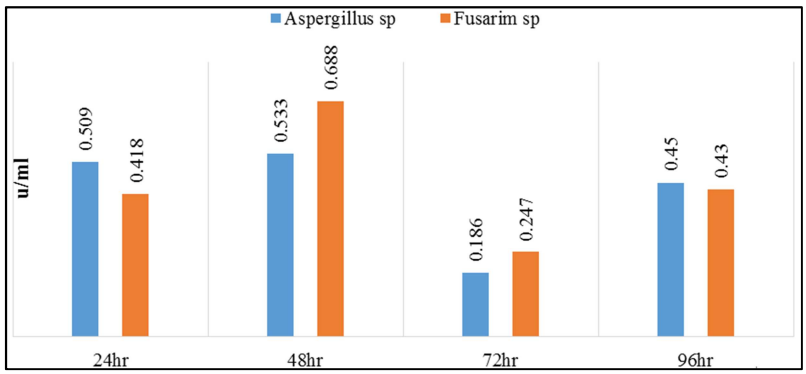

Figure 6. *Cellulase production based on incubation time.*

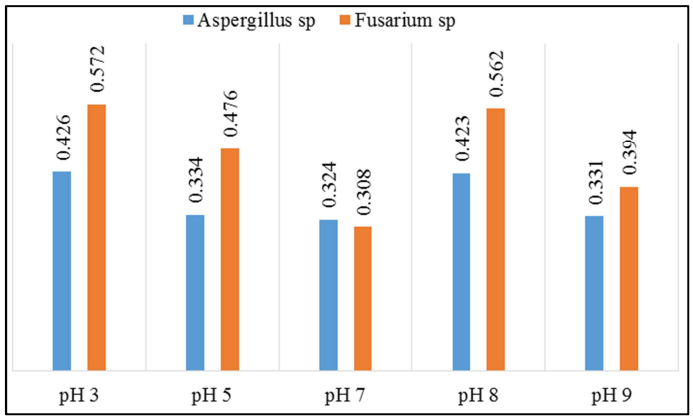

Figure 7. *Cellulase Production Based on PH.*

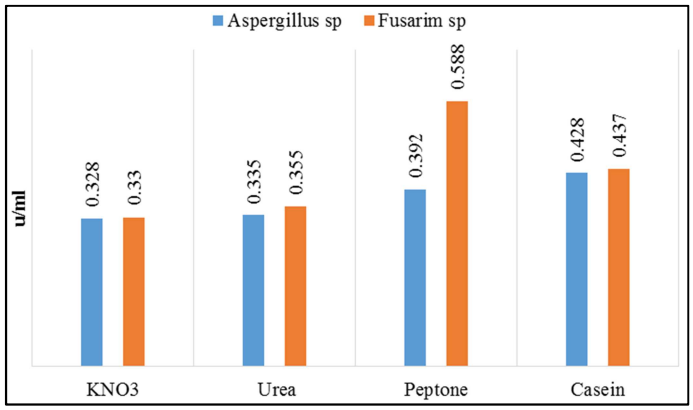

Figure 8. *Cellulase production based on nitrogen source.*

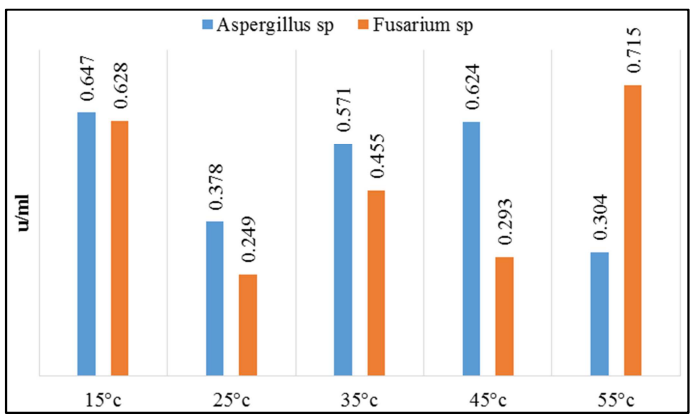

Figure 9. *Cellulase production based on temperature.*

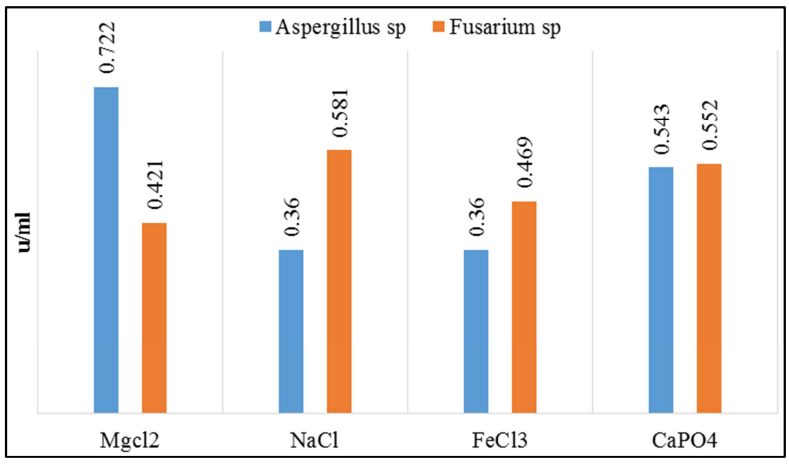

Figure 10. *Cellulase production based on ion.*

3. Results and Discussion

A total of 18 fungal isolates were isolated from the soil samples collected from the four sites in Abraka town. Screening experiment for cellulose production showed that 14 isolates produced cellulase, of which two isolates 1FG and 8FG produced appreciable halo zones around the fungus colony. These isolates were identified using cultural and microscopic features and confirmed molecularly as *Aspergillus* sp and *Fusarium* sp respectively (figures 1-3).

Optimization experiments using different environmental conditions were carried out and varied results were obtained. Figure 4 indicated cellulase production using agricultural wastes. Yam peels produced more cellulase by the two isolates than other agricultural wastes. Arabinose showed a more promising source of carbon for both fungi for cellulase production (figure 5). Figure 6 showed that cellulase production peaked at 48hours for the two organism, however, *Fusarium* sp produced more cellulase than *Aspergillus* sp at that same incubation period. Cellulase production was higher for p H3.0 than pH7.0 and pH 9.0 (figure 7). For nitrogen sources, peptone was the best nitrogen source as more enzyme was produced by *Fusarium* sp while casein yielded more cellulase by *Aspergillus* sp (figure 8). Figure 9 showed cellulase production based on different temperatures. Results showed varying quantity of cellulase production. *Fusarium* sp. produced more cellulase at 55°C however, appreciable quantity was produced at 15°C by same organism compared with 25°C while *Aspergillus* sp produced more cellulase at 15°C through apprecable quantitity was produced at 45°C (figure 8). While $MgCl_2$ was best for *Aspergillus* sp and NaCl was best for *Fusarium* sp respectively (figures 9, 10).

Fungi isolates from soil samples screened for cellulytic activity showed varied results, however IFG and 8FG identified and characterized as *Aspergillus* sp and *Fusarium* species showed higher zone of hydrolysis during screening. Molecular analysis showed that these fungi were similar to *Aspergillus versiculor* and *Fusarium solani* previously isolated by other researchers. A wide range of fungi have been shown to have cellulytic property. *Aspergillus fumigatus, A terrous* and *A. flavus* isolated from soil showed higher cellulytic activity [26]. Initial studies have established the production of cellulase from agricultural wastes and soil by *Fusarium graminearum*; *Aspergillus niger*, *Trichoderma viride* and *Fusarium solani* [8, 24]. Arabinose as a carbon source was the best for cellulose production compared to others. Other reports indicated sucrose produced more cellulase by *Fusarium solani* and *Trichoderma* sp [24] which contrasted this study probabily because the fungal isolates for this study is different from their own.

The optimal time for cellulase productin was 48 hours contrasting the 72 hours for enzyme production from previous report [8]. This variation in time may be due to the different species of fungi. A close observation of results indicated that higher cellulase were produced at temperature of 15°C though the two fungi also showed ability to produce enzyme at temperatures 45°C and 55°C for *Aspergillus* sp and *Fusarium* sp respectively. This may indicate the thermophilic nature of these fungi which make them able to utilize agricultural wastes for cellulase production. Previous researcher have reported cellulase production from agricultural wastes like rice straw, palm fronds, peapods, corn strover [1, 8, 16, 27] which concurred with this study. Though, different agricultural wastes were used in this research than the previous studies. Yam peel produced more cellulase in this study while in a previous study, plantain peel produced more enzyme than other agricultural wastes [2]. Apart from enzyme production, agricultural wastes from peels are capable of supporting microbial growth [4].

Aspergillus sp and *Fusarium* sp produced higher cellulase at pH3.0 which contrasted other study with temperature 32°C and incubation period of 72hours [18]. Also, maximum cellulase production by *Fusarium graminearum,* and *F. solani* respectively at pH5.0 were reported by other researchers [8, 24] while pH 3.5 for *Aspergillus niger* strains [1, 15, 6]. However, results from this study showed maximum cellulase production at pH 3.0. Fungi thrive more in acidic environment and besides the source of these organisms were from soil which previous study showed was acidic [3].

The best nitrogenous source for maximum cellulase

production as noted in this study was peptone for *Fusarium* sp) (figure 5) while casein was the next and the least was potassium nitrate. The results obtained from this study corresponds with previous study where peptone produced more cellulose [8]. However contrasted other reports where sodium nitrate was the best nitrogen source for *A. niger* and *F. solani* and meat extract for *A. niger* for more cellulase production [8, 24].

4. Conclusion

The result of study showed that *Aspergillus* sp and *Fusarium* sp isolated from soil produced more cellulase enzyme. The best temperatures for enzyme production were 55°C and 45°C for *Fusarium* sp and *Aspergillus* species respectively. Though the isolates also produced appreciable quantity of cellulase at 15°C at the second day of fermentation, arabinose and yam peel were the best carbon sources. These organisms are promising for large scale production of cellulases from cheap source of substrates like yam peel.

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