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Effects of Some Variable Factors on Cellulase Production by *Aspergillus Niger* using Solid State Fermentation

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Abstract

Several agro-wastes end up in landfills in Nigeria. there has been a renewed interest in the recycling of such agricultural waste for value-addition through innovative methods. This process will reduce environmental pollution and generates additional useful products. Corn cob is one of the largest farm-originating underutilized waste nuisances in Nigeria. Despite their high metabolizable sugars, not all microbial strains utilize them in the same way and rates. Cellulase is an industrially important products, essential for human activities. This study aimed to use corn cobs as substrates of cellulase enzyme production, determine the effects of the *Aspergillus niger* strains, (cellulosic or non-cellulosic) as well as the time of fermentation on the enzyme yields. Cellulosic and non-cellulosic niches were screened for Aspergillus strains. Aliquot 0.1ml of the serially diluted samples were pour-plated on Streptomycin-containing potato dextrose Agar (PDA). The *Aspergillus niger* obtained were identified by microscopic examination using lactophenol cotton blue staining and then screened in a cellulose-containing medium for their cellulase activity. Strains with the highest zones of clearance were selected for further studies. The enzyme activity of *Aspergillus niger* strains was from day 2 to the 10th day of fermentation. The highest performance across the strains was on the 6th day by *Aspergillus niger* RA23, 1.032 U/ml) and the lowest was 0.372 U/ml by *Aspergillus niger* RA05. Enzyme production was influenced by fermentation time and microbial strain agreeing with previous literatures that fermentation process is strain-dependent. The microbial strain used for bioprocessing has important function in the metabolism and transport of nutrients. Therefore, autochthonous factor needs to be considered in strain selections.

Keywords: Cellulase, Enzymes, Corn Cobs, Bio-Processing, Wastes and Fermentation.

Introduction

Agriculture is the main sustaining practice in Nigeria and many parts of the world. Several Agricultural wastes are therefore generated from the different forms of agriculture. These wastes constitute environmental nuisance even though they are disposed of in one form or the other. Several of the solid wastes that are disposed by burning produces greenhouse gases (such as methane, carbon dioxide, nitrogen) known to deplete the ozone layer (Jha and Mishra, 2021). Aside this, emissions during the burning of such wastes also including stench, soot, noxious gases, and the release of dioxins and furan compounds which have earlier been reported to be implicated in some types of cancer, reproductive damage as well as birth defect (Ghasemi *et al*., 2022). As long as these agricultural practices continues, so will pollution of air in this manner will continue with the consequential cause of respiratory symptoms such as chronic bronchitis, allergic rhinitis, acute exacerbation of asthma, scarring of the lungs, and lung cancer in long term (Fapetu, 2000). Land fill refer to as dump sites in the local setting are randomly situated around residential places and with some located close to the water bodies without government approvals or regulations. This practice is also laced with potential health risks one is that when dumpsites get overloaded with different forms of wastes materials that breed both pests and harmful microorganisms (Aliyah *et al*., 2017). Harnessing these wastes for industrial and agricultural fertilizers is one of the few methods of waste management with positive environmental consequences (Akkarachaneeyakorn *et al*., 2018). Several enzymes are required in the industries for food and pharmaceutical applications. Cellulase is one of these enzymes which are produced by microorganisms. The enzyme has an important bio-conversion roles in organic waste into glucose processes as well as other important applications like the production of single cell protein, animal feed, ethanol, etc. (Anto *et al*., 2006). The enzyme has the ability to hydrolyzes β (1-4) in the cellulose. Although cellulase enzymes can be obtained from various sources such as plants and insects, microorganisms such as fungi is the main microorganisms that produce cellulase enzymes of commercial interest (Almanaa *et al*., 2020). For example, the filamentous fungi Trichoderma and Aspergillus are often reported to be highly relevant in the production of cellulase.

Waste disposal as seen in the disposal of agricultural wastes ends up in direct and indirect environmental impacts, like land occupation, resource depletion, greenhouse emissions, water intoxication and cytotoxic effects on the environment especially as combustion takes place (Fadel *et al*., 2020). Direct impacts of this waste represent a share of global climatic change and resource depletion has indirect environmental effects. Waste management including those of agricultural origin is outlined because the totally different approaches and procedures designed and enforced to spot and handle the various sorts of waste generation till disposal can be complex (Díaz *et al*., 2020).

It is interesting to note that interest in the implementation of sustainable as well as efficient processes for enzyme production through waste management processes has grown significantly in recent times. In this regards, chemical catalysts are being replaced by biochemical catalysts and several enzymes have been identified and applied in

industrial and biotechnological processes. Consequently, both the efforts in research and the production of enzymes have increased in recent years (Hegde *et al*., 2018). Higher demands for enzymes are as a result of the need for clean and renewable energy, such as the production of biofuels. Also, cellulase is the most commonly applied enzyme in the treatment of lignocellulosic biomass. It is often applied in industries that produce food, detergents, beverages such as juices, wines and beers, paper, jeans, pharmaceuticals, fine chemicals, biofuels such as ethanol and biogas, in wastewater treatment and for bioremediation purposes (Levin *et al*., 2012).

It is an enzyme complex composed of endoglucanases, exoglucanases or cellobiohydrolases and β-glycosidases, in which each enzyme plays an important role in the hydrolysis of cellulose. Enzymatic hydrolysis is preferred over other chemical treatments because it both offers an efficient bioconversion process under simple and moderate operating conditions, as well as avoids the generation of harmful by-products to fermentative microorganisms (Movahedpour *et al*., 2022). *Aspergillus niger* species is a fungal that has been used by industries for years due to its efficiency in the secretion of cellulolytic enzymes and their being able to adapt to several environmental conditions. For instance, the type of strain, the culture conditions, the nature of the substrate and the availability of nutrients in the medium are factors that can be modified to improve cellulase production and activities in that the charges and the permeability of the cell membrane are either positively or negatively affected (Msarah *et al*., 2020). The current high cost of enzyme production and sustainability due to cost of raw material and low stability demand that alternative methods and sources be looked into in order to overcome the challenges bedeviling enzyme application in our local industries.

In Nigeria, many agro-wastes end up in landfills. In recent years, however, there has been a renewed interest in the recycling of such agricultural by-products as cheaper source of substrate for the production of many valuable products of economic importance. However, this waste can be transformed into valuable resources through innovative methods. This process not only reduces environmental pollution but also generates additional useful products (Rajesh and Gummadi, 2020). Corn cob is one of the largest farm-originating underutilized waste materials that have become nuisance in Nigeria. Despite their contents being high in metabolizable sugars in the production of cellulase to increase economic value, not all Aspergillus strains utilize them in the same way and rates. With certain modern pretreatment techniques, agricultural nuisance-causing wastes could become major raw materials for enzyme production Rizk *et al*., 2019. Therefore, the aim of this study was to use corn cob as substrates of cellulase enzyme production, determine the effects of the *Aspergillus niger* strain, (cellulosic or non-cellulosic) as well as the time of fermentation on the enzyme yields.

Materials and Methods

Selection of Materials

The corn cobs used in this experiment was obtained from National Biotechnology research and development Agency farm sites. All the standard products were purchased from commercial suppliers in the right packages and conditions.

Pre-treatment of substrates

The procured corn cobs cellulosic substrate was dried to constant weight, pulverized to fine powder and passed through a 60-mesh sieve. Thereafter, substrate was treated with 1% (w/v) NaOH solution in the ratio of 1:10 (substrate: solution) for 1h and was brought to neutral pH by washing thoroughly with distilled water and dried at room temperature. The treated substrates were autoclaved at 121°C for 1 h (Sahnoun *et al*., 2015).

Microorganism

The fungus, *A. niger* strains were isolated from buried roasted corn, decayed liters, soil sample laboratory air and rotten tomatoes in Lugbe, federal capital territory, Abuja, Nigeria. The isolate was grown on CMC agar medium and screened according to standard method (Sahnoun *et al*., 2015). The culture was thereafter identified based on colony morphology and basic microscopic examination. Strains with the highest zones of clearance were selected for further studies. The isolated fungal colony was individually subsequently sub-cultured and maintained on potato dextrose agar (PDA) slants and maintained at 4 °C in a refrigerator, until further needed.

Preparation of fungal inoculum

The inoculum prior to the fermentation was prepared by growing the strains in 250-ml Erlenmeyer flask with 100ml of Czapek-Dox broth containing g/l of sucrose, 30; sodium nitrate, 3; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, trace; agar, 15. The medium was inoculated from the Czapek-Dox agar slants and incubated at 30 °C for 3 days in a shaker (200 rpm) before it
was used for the fermentation process (Sheela *et al*., 2021).

Fermentative production of cellulase by Aspergillus strains using corn cobs

Twelve (12) Aspergillus strains were used in solid state fermentation of corn cobs after prior culturing on PDA medium at 28 ℃ for 3 days in slant bottles. The fungal spores were collected using inoculating loop to make spore suspension in sterile normal saline with tween80. A 10 mL spore suspension was inoculated into 100 mL PDY medium and incubated in the incubator at 28℃ for 5 days. The fermentation medium consisted of corn cob powder 40.0g, beef peptone 10.0g, yeast extract 6.0g, KH_2PO_4 2.0g, K_2HPO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, natural pH, moistened with distilled water, sterilized at 121℃ for 20 min. Afterwards, 10 mL (10%) of the seed solution was inoculated into 250mL of moisten fermentation medium (Singh *et al*., 2012). Mixed well and incubated at 30℃ in a humidified incubator for 96 h with periodic gentle shaking and mixing.

Enzyme extraction

After the fermentation time, the crude enzyme was extracted from the corn cob waste by first homogenizing the mixture with distilled water in ratio 1:10 w/v with agitation on a rotary shaker (120 rpm) at 30°C with a contact time of 1h. Afterwards, dampened cheese cloth was used to filter the extract which was pooled by centrifuging at 6000 rpm for 15min and the clear

supernatant and were used as a source of the extracellular enzyme for subsequent test (Singh *et al*., 2016).

Enzyme assay and analysis

Carboxymethyl cellulase (CMCase) activities were assayed according to standard method (). One unit of enzyme activity (CMCase) is defined as the amount of enzyme which releases 1 µmole of reducing sugars per min with glucose as standard under the assay conditions.
The values of enzymatic activity were expressed as U/ml for SmF and U/g of dry mycelial bran (DMB) for SSF (Naik *et al*., 2022).

Optimization of process parameters in SSF

Evaluation of enzyme yield by optimizing the culture conditions viz; the *Aspergillus niger* strains and the length of fermentation. The fermentation time was varied from day 1 to 10 where as 3 *Aspergillus niger* strains viz; FT23, SL13 and TT06 obtained from Department of Microbiology, Ahmadu Bello University, Zaria were used along with the most cellulase-producing strain (RA23) in this work to optimise for cellulase production based on differences in strains.

Results and Discussion

Isolation of cellulase-producing *Aspergillus niger* for SSF cellulase production

Fifty-three fungal isolates were isolated from some samples; buried roasted corn, decayed liters, soil sample, laboratory air and rotten tomatoes in around Lugbe, FCT, Abuja, Nigeria. Samples were serially diluted in sterile normal saline respectively and was spread on to the media as stated earlier. The results of the *Aspergillus niger* isolated and purified in relation to the source and performance on CMC agar are shown on Table I.

Table 1: Cellulosic activity of Isolated Fungal strains

S/N	Isolate	Source	Zone of clearance (mm)
1	RA12	DL	13.2
2	RA23	BC	15.4
3	RA02	RT	11.7
4	RA17	SS	9.6
5	RA05	BC	17.6
6	RA27	LA	10.5
7	RA14	BC	16.4
8	RA28	BC	18.1
9	RA39	SS	7.4
10	RA63	RT	8.9
11	RA09	BC	19.5

Key: Source of Aspergillus; Buried roasted corn (BC), Decayed liters (DL), Soil sample (SS), Laboratory air (LA) and Rotten tomatoes (RT).

The fungal Isolates were identified based on their morphological characteristics. The morphological features of the isolates were similar to those of *Aspergillus* sp. reference strain. After 7 days of incubation on potato dextrose agar (PDA) supplemented with chloramphenicol (to suppress the growth of bacteria growth). The isolates produced creamy white substrate mycelia with black conidial areas (Fig. I).



Fig. 1. Morphological features of fungal isolate on a PDA plate

Microscopic examination of the isolates

Under the microscope, revealed by staining with lactophenol cotton blue, the spores had a width of approximately 3µm. Therefore, the isolates were identified as member of the genus *Aspergillus*. Based on morphological and microscopic analysis, the strains were identified and used for the experiment.

Of the fifty-three isolates, twelve isolates exhibited cellulolytic activity (zone of clearance) on CMC agar plates based on their high ratios of clear-zone diameter to colony diameter which ranged from 7.4mm to 19.5mm (Table 1). However, strains with clear zone greater than 15mm (RA23, RA05, RA14, RA28 and RA09) were further selected for cellulase production test (Table 1 and Figure I).

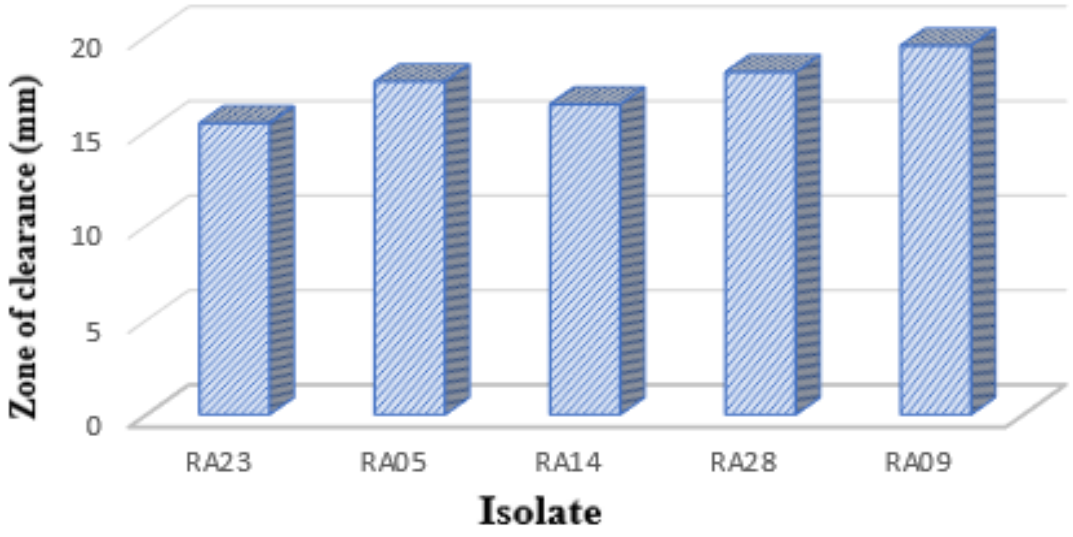


Figure I: Zone of clearance diameter on CMC agar for the five selected strains

Enzymatic activity of strains

Fungal cellulase production through SSF has been reported previously by several researchers (Shamala and Sreekantiah, 1986).

The five isolates, capable of producing cellulase, were cultured in supplemented corn cob substrate and fermented using SSF method. After 7 days of fermentation, the cellulase produced from the five fungal isolates was extracted and the total cellulase activity was analyzed. Fungal isolate RA09 exhibited the highest CMCase activity of 1.032 U/ml substrate (Table 2). The fungal isolate that exhibited the highest activity was selected for optimization of the SSF parameters for cellulase production.

Table 2: Cellulosic activity of Isolated Fungal strains

S/N	Isolate	Source	Enzyme activity (U/ml)
1	**RA23**	**BC**	**1.032**
2	**RA05**	**BC**	**0.372**
3	**RA14**	**BC**	**1.021**
4	**RA28**	**BC**	**1.015.**
5	**RA09**	**BC**	**0.925**

Optimisation of fermentation parameters

In this work, the protocol adopted for the standardization of fermentation parameters was to evaluate the effect of an individual parameter; effect of length of fermentation (1 to 10 days) and the strain of the fungus used RA23.

Effect of extending fermentation time on enzyme activity

The effect of the optimization showed that the enzyme activity of the the most cellulase-producing (RA23) strain was on the 6th day (Fig. II).

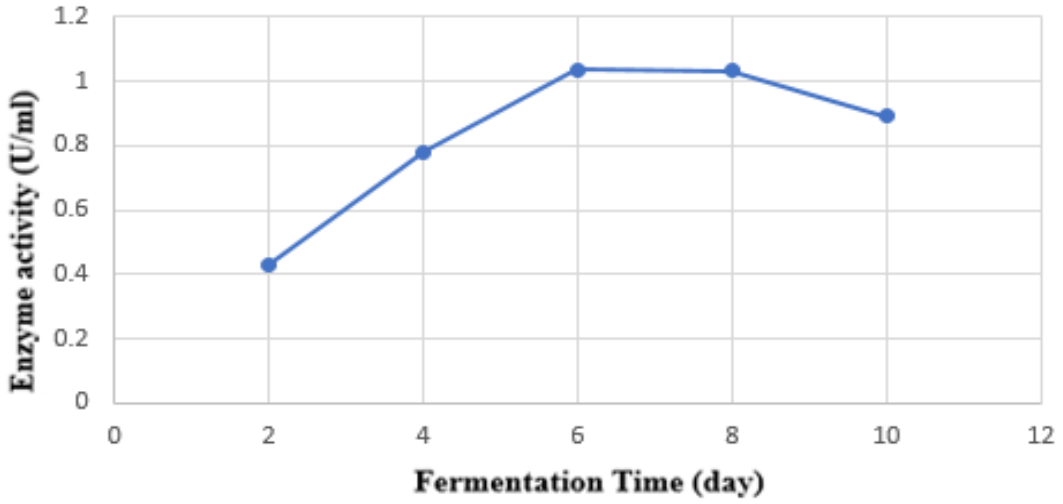


Fig. II: The effects of fermentation time on enzyme activity by Aspergillus niger RA23 strain

The cellulase production increased till the 6th day of incubation after which it significantly reduced. This could be due to nutrient deficiency or the accumulation of other byproducts in

the fermentation medium. However, we did not observe a significant change in CMCase activity afterwards. It was therefore suggested that 6day fermentation will make the process more economical and efficient as additional days would not add any value. An incubation period of 6 days was selected as the optimal time. Bueno *et al.,* 2014 reported the optimal incubation time for maximum cellulase production by *A. niger* and *A. flavus* to be 6days. The cellulase activity of both strains decreased after 7 days of incubation. These results do not agree with the findings of Coradi *et al*., (2012) who reported the highest cellulase production after 4 days of incubation. Malilas *et al*., (2013) reported maximum cellulase production after 3 days of incubation. In this study, the highest level of cellulase production was observed after 6 days of incubation, which may be due to the cessation of growth and release of cellulase into the medium during the later growth phase of *Aspergillus* sp. RA23. The decreased enzyme activity observed after prolonged incubation may have resulted from irreversible absorption onto the substrate, feedback inhibition, or degradation of the enzyme. This was attributed to changes in environmental factors possibly brought by the changes in media composition over time (Prasad, 2014). It was observed that before the 5th day of fermentation, optimal conditions were recorded and maximum activity was achieved probably because of nutrient consumption and the production of other metabolites in the fermentation medium (Romero *et al*., 2012). This agree with several previous researches that enzyme production depends on biomass during the exponential phase of growth which are produced during the exponential growth phase, being primary metabolic product. Thereafter, production decreases till the growth enters the death phase where activities completely ceased. This agrees with **Huyen et al., (2019)** in their reseach on 'Fermenting rice straw with the fungus *Pleurotus eryngii* increased the content of crude protein and the digestibility of the straw '.

The effects of Aspergillus strains on cellulase activity

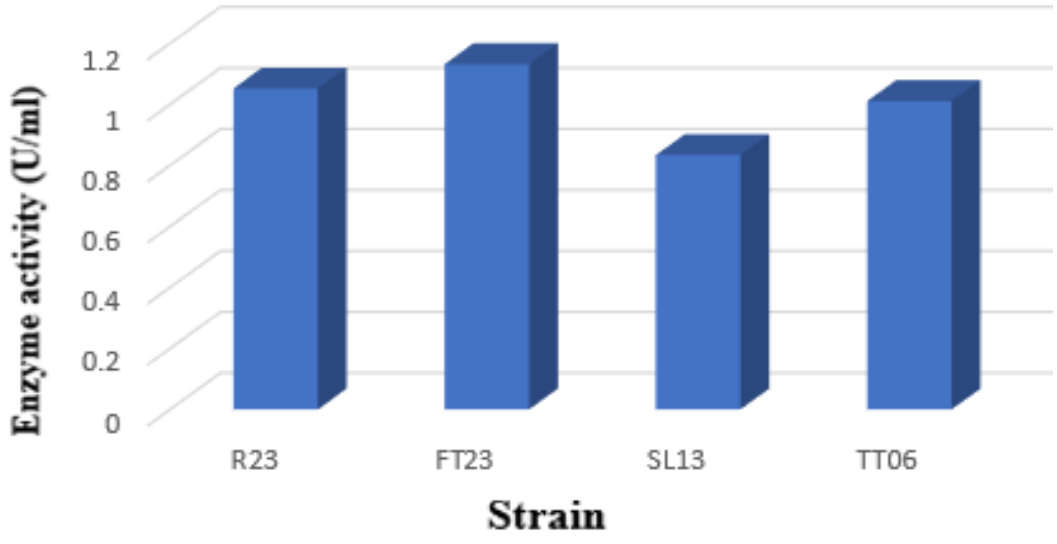


Fig. III: The effects of varying *Aspergillus niger* strains on enzyme activity

The effects of varying fungal strain on cellulase production was studied using Aspergillus strains RA23, FT23, SL13 and TT06 respectively (Fig. III. The maximum amylase activity found among the fungi strains was reported to be 1.132 U/mL by strain *Aspergillus niger* FT23 using corn cobs as the substrate. This was followed by 1.032 U/ml by RA23 and the lowest recorded was by *Aspergillus niger* SL13 where 0.834 U/ml was recorded. Varying the strains of Aspergillus niger fungus during cellulase production affected the yield of the enzymes. This analysis revealed the role of strains in cellulase enzyme production using corn cobs as substrate.

Generally, one of the most important variables to consider in the selection of an appropriate agricultural residue for bio-conversion for value addition is substrate and the strain of microorganisms that are capable of utilizing it. In recent years, different substrates have been screened for high yields of enzyme production using SSF method (Chimata *et al*., 2010). This overall result agreed with several suggestions, based on research findings, that availability and cost of the raw material are important factors that need to be considered for simultaneous saccharification and fermentation (SSF). In this research, the corn cob might have facilitated the growth and development of the fungal strains used that led to the synthesis of the metabolites (cellulase). This is in accord with the work of Elegbede, *et al*., (2021) in their research on Microbial valorization of corncob: Novel route for biotechnological products for sustainable bioeconomy

Inoculum size also affects the maximum cellulase enzyme production. To check the effect of inoculum size, as shown in Figure 4, we used 5, 10, 15 and 20 discs of 8 mm size and cellulase activities were 0.06414, 0.09867, 0.03577 and 0.05427 IU/mL, respectively. Thus, by using 10 discs of 8 mm size of A. niger maximum cellulase activity of 0.09867 IU/mL was found. When high fungal mass was inoculated, initially cellulase activity and reducing sugar production decreased. But when low load of fungal mass was inoculated, increase in sugar production and cellulase activity was continuously observed up to 96 h

Conclusion and Recommendations

In this experiment, we employed a novel fungal isolate, *Aspergillus* sp. RA23, which was found to be a highly efficient cellulase producer. We optimized some parameters, including extension of fermentation time and the use of difference strains, to maximize cellulase production. After optimization, the best settings were determined to be 6-day fermentation using FT23 strain. With these, higher cellulase enzyme of 1.032 IU/ml was produced. Fungal cellulases derived from *Aspergillus* sp. RA23 have practical applications in the enzymatic hydrolysis of pretreated corn cobs and can serve as an alternative means of hydrolyzing the waste into important economic product as well as a form of environmental clean-up in several agricultural waste disposal system. This ca greatly contributes to the degradation of lignocellulosic materials and presenting promising implications for sustainable agricultural feedstock management in both rural and urban settings.

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