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Full Length Research Article

OPTIMIZATION OF CELLULASE PRODUCTION BY *ASPERGILLUS NIGER* ISOLATED FROM SOIL SAMPLES IN IBADAN, NIGERIA

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ABSTRACT
Cellulolytic microorganisms have found relevance in various industrial applications. The genus *Aspergillus* is among the cellulase-producing organisms that have been studied over time. Hence, this study aimed to establish the optimal conditions for cellulase production by *Aspergillus niger* isolated from soil. Soil samples were aseptically collected in Ziploc bags from four locations in Ibadan: Bashorun, Akobo, University of Ibadan, and Bodija. Isolation, cellulose degradation, and screening for cellulase production by the isolated *A. niger* were done on Potato Dextrose Agar (PDA), Carboxyl Methylcellulose (CMC) agar, and Congo red agar, respectively. Furthermore, cellulase production by A. niger was optimized using standard procedures. The results showed that ten of the fifteen isolated A. niger were cellulase producers, and two isolates (A. niger ISOd and A. niger ISOg) exhibited the largest zones of clearance (24 mm and 21 mm, respectively). Optimizing temperature, pH, carbon and nitrogen sources, % CMC concentration, and incubation time of *A. niger* ISOd demonstrated the highest cellulase yield of 0.32 U/mL at 37 ^{0}C, 1.28 U/mL at 4.5, 0.93 U/mL using glucose, 0.26 U/mL using peptone, 0.39 U/mL using 5% CMC, and 1.20 U/mL at 72 h, respectively, compared to other isolates. In conclusion, *A. niger* ISOd is a good microbial cellulase producer that may be harnessed for useful food and industrial applications. In addition, this study supports Sustainable Development Goal 3, which targets good health and well-being.

Keywords: Sustainable development goal, spectrophotometer, conidiophore, potato dextrose agar, 3,5- dinitrosalicylic acid.

INTRODUCTION

Cellulose is one of nature's most abundant biopolymers, comprising a significant portion of plant cell walls. It is characterized as a homopolymer of anhydroglucose, with glucose residues bonded in a ß-1,4 configuration. Cellulose structural characteristics allow it to maintain a semi-crystalline state, even in aqueous environments (a trait uncommon to polysaccharides) (Prasanna *et al*., 2016; Dina *et al*., 2021). As a natural long-chain polymer, cellulase boasts diverse applications across numerous industries (Kumar & Shrama, 2017). Products derived from cellulose are among the essential commodities we use every day, including foods and feeds, drugs, paper, paperboard, cellophane, and crayons (Li *et al*., 2018; Oliveira *et al*., 2019). Organisms proficient in cellulose degradation produce a diverse array of enzymes, such as cellulase, lipase, and amylase, with specificities that complement one another and act synergistically (Naher *et al*., 2021). Studies have shown that the cost of making cellulase depends on how much the microbes can produce, how strong the enzyme is in the mix, and what kind of substrate they use to grow the microbes (Dina *et al*., 2021; Sukumaran *et al*., 2021). According to Vieira et al. (2021), microbial species reported as cellulase producers include Aspergillus, Trichoderma, Penicillium, Fusarium, Cladosporium, Pseudomonas, Cellulomonas, Bacillus, Firobacter, Clostridium, and *Ruminicoccus*. The study by Sukumaran et al. (2021) reported that at 1,4-D-glucan linkages, cellulases hydrolyze cellulose to produce cellobiose and other cello-oligosaccharides, including cellotetrose, glucose, and cellohexaose. Some of the industrial functions of cellulose are: (i) They enhance compressibility in tablets, (ii) act as thickening agents and stabilizers in liquid dosage forms, (iii) serve as binders in granules and tablets, (iv) serve as gelling agents in food (Naher *et al*., 2021). For example, in contemporary food biotechnology, cellulases are recognized as valuable assets due to their wide-ranging applicability across various processes, as they play vital roles in fruit and vegetable juice clarification, viscosity reduction in nectars, puree concentration, alteration of fruit sensory attributes, carotenoid extraction, olive oil extraction, and enhancement of bakery products fluffiness quality on a global market scale (Saheed *et al*., 2016; Dina *et al*., 2023). Cellulolytic microbes, which include fungi, play crucial roles in human life across various domains. While commonly associated with causing disease and food spoilage, they significantly influence human well-being by contributing to ecosystem nutrient cycles, such as facilitating the recycling of cellulose (the primary carbohydrate produced by plants) (Han et al., 2018).

According to Coherent Market Insights, the textile industry emerged as the primary market for cellulases in 2017. Additionally, numerous enzyme market research reports from 2018 highlight food and beverages, the textile industry, animal feed, and biofuels as major areas of application (Naher *et al*., 2021). In a separate Global Cellulase Market Research Report published in 2018, it was noted that Asia-Pacific was the largest consumer of cellulase, accounting for approximately 32.84% of the market share by revenue by 2016. The report also indicates significant demand for cellulase in animal feed (29.71%), food and beverages (26.37%), and the textile industry (13.77%) in 2016 (Singh *et al*., 2019; Vieira *et al,* 2021). Projections from the same report suggest that cellulase applications will reach a value of 2300 million USD by the end of 2025, with a "Compound Annual Growth Rate" (CAGR) of 5.5% during the 2018–2025 period. This data underscores the escalating annual adoption of cellulases across diverse industries (Alexandria *et al*., 2019). The key producers of cellulase, including Novozymes and DuPont from Denmark, supply these enzymes to the global market for industrial applications (Kumar & Christopher, 2017). Therefore, this study aimed to establish the optimal cultural and physicochemical conditions that maximize cellulase yield from

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A. niger isolated from soil, enabling its utilization for various industrial applications.

MATERIALS AND METHODS

Sample collections

Soil samples were collected from four locations in Ibadan: Bashorun, Akobo, University of Ibadan, and Bodija. The soil samples were collected using a sterilized spatula inside Ziploc bags and immediately taken to the Industrial Microbiology Laboratory of the Microbiology Department, University of Ibadan, for analysis.

Isolation of *A. niger*

One (1) g of each soil sample was diluted by transferring it into 9 ml of sterile distilled water inside test tubes. The mixtures were vigorously shaken using an orbital shaker for 10 min at 300 rpm. Afterward, the mixtures were serially diluted by transferring 1 mL of each into 9 mL of sterile distilled water. Dilution factors 10^{-5} and 10^{-6} were used for culturing. A 0.1 ml of the 10^{-5} and 10^{-6} diluted samples was spread plated on sterile potato dextrose agar (PDA) plates and incubated at 28±2.0 ^{0}C for 5-7 days (Bakare *et al*., 2022). The plates were observed for fungal growth, and colonies were subsequently sub-cultured to obtain pure cultures. The obtained pure cultures were maintained on PDA slants and stored at 4 °C in the refrigerator for future use.

Characterization and identification of *A. niger*

The fungal isolates were macroscopically characterized by observing the color, shape, and mycelial arrangements using the fungal compendium (Alexopoulos 4th Edition, 2007 as a guide. Furthermore, the isolates were microscopically identified by staining pure isolate mycelium with lactophenol blue on a sterile, grease-free microscope slide and viewing under the x40 objective lens.

Screening for cellulase production by the identified *A. niger*

The plate assay method described by Dina *et al*. (2023) was used to screen for cellulase production by the identified *A. niger* species. One (1) ml of 7-day-old suspension of *A. niger* was inoculated into 1% carboxymethyl cellulose and incubated at 25 ^{0}C for 5-7 days. After the incubation period, 0.1% Congo red was used as an indicator stain, then the slide was counterstained with 1 M NaCl and left on the laboratory bench for 15 min. The presence of clear zones around the streak lines indicates cellulose hydrolysis. Thereafter, the clear zones were measured using a calibrated ruler and reported in mm, as the A. niger isolate with the highest zone of clearance (isolates 4 and 7) was used for further studies.

Production of cellulase by the *A. niger* isolates with the highest zone of clearance.

One (1) ml of *A. niger* suspension with the highest zone of clearance was inoculated into Mandel's Weber medium (MWM) and incubated at 28 ^{0}C using an orbital shaker incubator at 110-120 rpm for 24 h as described by Jasani *et al*. (2016).

Harvesting of cellulase

After incubation, the *A. niger* suspension was filtered to separate the culture broth from the mycelia. The culture broth was then homogenized at 8000 rpm for 10 min at 4 ^{0}C to remove any remaining mycelia. The supernatant was aseptically harvested into a sterile test tube, corked, and stored in the refrigerator at 4 °C to prevent contamination. This crude cellulase was thereafter used for further studies (Dina *et al*., 2023).

Cellulase optimization processes

Effect of different temperatures on cellulase production

The effect of different temperatures on cellulase production was assessed using MWM. One (1 M) citrate buffer was added to the MWM, and 0.5 ml of the harvested crude cellulase was inoculated into sterile tubes. The inoculated tubes were thereafter incubated at different temperatures (^{0} °C, 25, 30, 37, 50, and 60 °C) for 1 h. Five (5) ml of DNS solution (3,5-dinitrosalicylic acid) was added to the inoculated test tubes and allowed to heat inside a water bath at 80 ^{0}C for 10 min. Color change was observed in all the inoculated test tubes, and cellulase activity was measured at 540 nm using a UV spectrophotometer (Naher *et al*., 2021).

Effect of different pH on cellulase production

The effect of initial pH on cellulase production was assessed using the method of Dina et al. (2023). The crude cellulase was aseptically dispensed into the inoculated culture broths, and the pH was adjusted to 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 using HCl and NaOH. The mixtures were incubated for 1 h and read for cellulase activity at 540 nm using a UV spectrophotometer.

Effect of different carbon and nitrogen sources on cellulase production

One (1) % of different carbon sources (dextrose, sucrose, maltose, glucose, and lactose) and nitrogen sources (peptone, yeast extract, urea, and ammonium sulfite) were supplemented separately into the medium containing A. niger, which produced crude cellulase, and incubated at 30 ^{0}C for 1 h. Cellulase activity was thereafter measured at 540 nm using a UV spectrophotometer.

Effect of different substrate concentrations on cellulase production

The crude cellulase was introduced into a medium containing carboxymethyl cellulose (CMC) at different concentrations (1%, 2%, 3%, 4%, and 5%) and incubated at 30 °C for 1 h. Again, cellulase activity was measured at 540 nm using a UV spectrophotometer.

Effect of different incubation times on cellulase production

One (1) ml of the crude cellulase was inoculated into MWM and incubated at different times (h), which are: 24, 48, 72, 96, and 120. Cellulase activity was measured at 540 nm using a UV spectrophotometer (Thermo Scientific Genesys 30).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS version 17) was used to analyze the data, with descriptive statistics used to determine means and standard errors.

RESULTS AND DISCUSSION

Characterization and identification of *A. niger*

The results of the colony morphology and micrograph of isolated *A. niger* are documented in Plates 1 and 2 below. The growth of *A. niger* on PDA was characterized by a filamentous colony with brownish chocolate color, in addition to the growth of black patches

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on the entire agar plate. Moving forward, the A. niger micrograph showed clearly the conidiophore characterized by a smooth-walled, long, unbranched conidiophore that holds the conidia at the head. The conidia were observed under the microscope to be round, numerous, and black.

Plate 1: *A. niger* growth on PDA

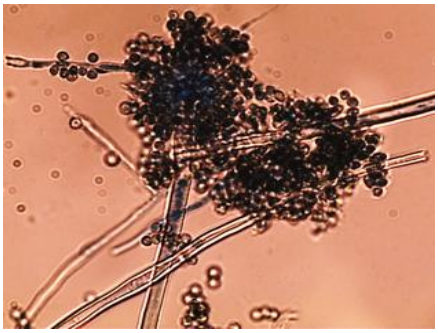

Plate 2: micrograph of *A. niger* (Mag x40).

Screening for cellulase production by *A. niger* isolates on Congo red agar.

Cellulase activity of the isolated *A. niger* was assayed using Congo red agar (Plate 3). The zone of clearance produced by the *A. niger* isolates differs from one isolate to another. However, isolates with the highest zones of clearance (ISOd and ISOg) were selected for further analyses.

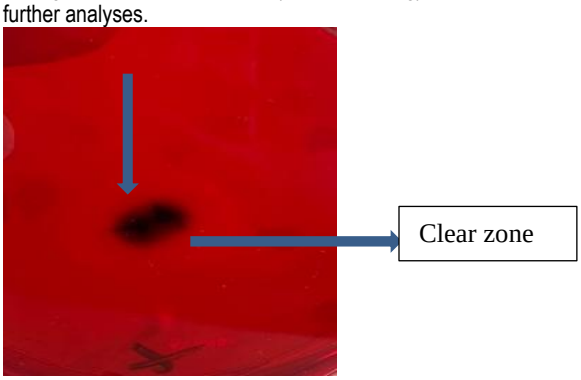

Plate 3: Zone of clearance produced by *A. niger* on Congo red agar

Zones of clearance produced by *A. niger* isolates for cellulase production

s/no	Isolate code	+/- for cellulase production	Colony size (mm)	Zone of clearance (mm)
1	ISOa	-	-	-
2	ISOb	+	10	19
3	ISOc	+	8	10
4	ISOd	+	12	24
5	ISOe	+	8	12
6	ISOf	-	-	-
7	ISOg	+	10	21
8	ISOh	+	6	11
9	ISOi	-	-	-
10	ISOj	+	10	18
11	ISOk	+	11	20
12	ISOl	+	10	16
13	ISOm	-	-	-
14	ISOn	-	-	-
15	ISOp	+	5	9

Key: += positive for cellulase production; -=negative for cellulase production

The highest and lowest zones of clearance indicating cellulase production were recorded in isolates ISOd and ISOp, with 24 mm and 9 mm zones of clearance, respectively. Furthermore, some *A. niger* isolates were observed not to produce any zone of clearance, and these isolates are ISOa, ISOf, ISOj, ISOm, and ISOn, as documented in Table 1.

Cellulase production optimization processes by *A. niger* ISOd and ISOg

The results of the effect of temperature on cellulase production by *A. niger* ISOd and ISOg are presented in Figure 1. It was observed that *A. niger* ISOd demonstrated the highest (0.32 U/mL) and lowest (0.24 U/mL) cellulase production at 37 °C and 60 °C, respectively, while *A. niger* ISOg demonstrated highest (0.32 U/mL) and lowest (0.22 U/mL) cellulase production at 50 ^{0}C and 60 ^{0}C respectively.

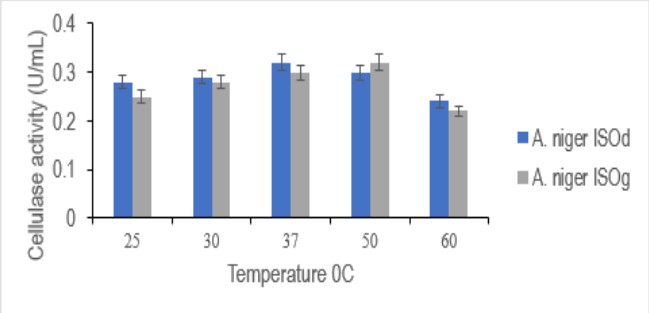

Figure 1: Effect of temperature (^{0}C) on cellulase production by *A. niger* ISOd and ISOg

Figure 2 documents the effect of pH on cellulase production by *A.*

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niger ISOd and ISOg. At pH 4.5, *A. niger* ISOd produced the highest cellulase activity (1.28 U/mL), while at pH 3.5, the lowest cellulase activity was recorded. Furthermore, *A. niger* ISOg produced the highest cellulase activity (0.28 U/mL) at pH 4.5, while the lowest (0.23 U/mL) was recorded at pH 3.5.

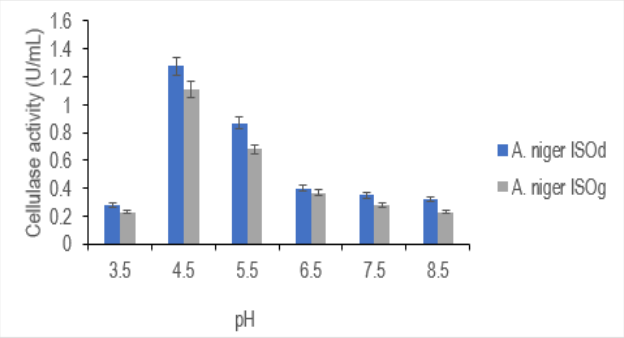

Figure 2: Effect of pH on cellulase production by *A. niger* ISOd and ISOg

Figure 3 presents the effect of different carbon sources on cellulase production by *A. niger* ISOd and ISOg. It was recorded that *A. niger* ISOd produced the highest (0.93 U/mL) and lowest (0.16 U/mL) cellulase using glucose and maltose, respectively, as the carbon source, while *A. niger* ISOg also demonstrated the highest (0.82 U/mL) and lowest (0.82 U/mL) cellulase production using glucose and maltose, respectively.

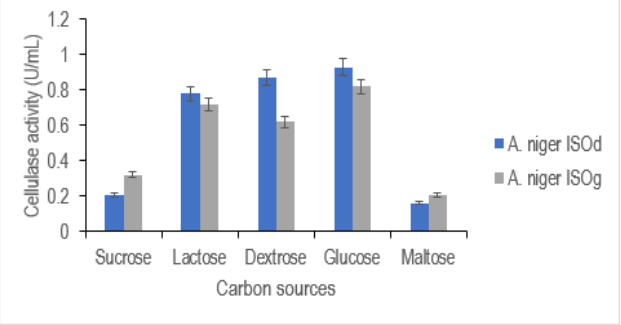

Figure 3: Effect of carbon sources on cellulase production by *A. niger* ISOd and ISOg

The results for the effect of nitrogen sources on cellulase production by *A. niger* ISOd and ISOg are documented in Figure 4. It was observed that using peptone as the nitrogen source produced the highest cellulase activity (0.26 U/mL) by A. niger ISOd, while yeast extract produced the lowest (0.21 U/mL) by the same isolate. In addition, *A. niger* ISOg produced the highest (0.26 U/mL) cellulase using ammonium sulfate $(NH_4)_2SO_4$, while yeast extract also produced the least (0.21 U/mL).

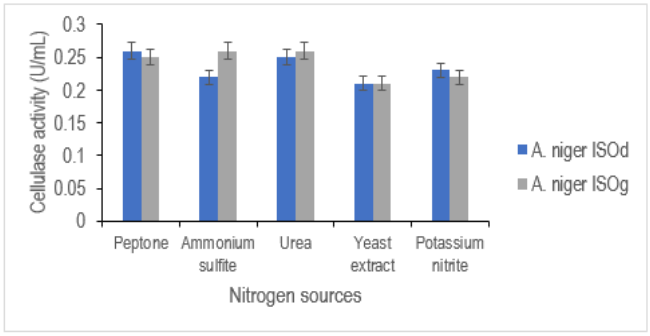

Figure 4: Effect of nitrogen sources on cellulase production by *A. niger* ISOd and ISOg

Figure 5 documents the effect of different CMC concentrations on cellulase production by *A. niger* ISOd and *A. niger* ISOg. It was recorded that at 5% and 1% concentrations, *A. niger* ISOd produced the highest (0.39 U/mL) and lowest (0.18 U/mL) cellulase, respectively, while at 2% and 1% concentrations, A. niger ISOg produced the highest (0.38 U/mL) and lowest (0.17 U/mL) cellulase, respectively.

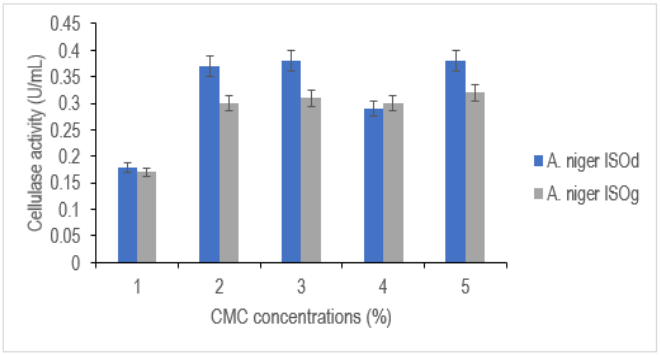

Figure 5: Effect of different concentrations of CMC on cellulase production by *A. niger* ISOd and ISOg

The effect of incubation time on cellulase production by *A. niger* ISOd and ISOg is presented in Figure 6. At 72 h incubation time, *A. niger* ISOd produced the highest cellulase (1.20 U/mL), while the lowest (0.25 U/mL) was recorded at 24 h. In addition, *A. niger* ISOg demonstrated the highest (1.00 U/mL) and lowest (0.22 U/mL) cellulase production at 72 h and 24 h, respectively.

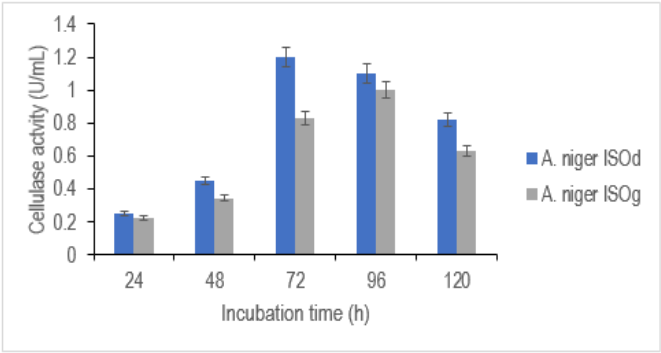

Figure 6: Effect of incubation time on cellulase production by *A. niger* ISOd and ISOg

This research provides information on optimizing cellulase production by A. niger isolated from soil samples in Ibadan, Nigeria. Cellulases are enzymes of industrial importance with a wide array of applications, including food and feed processing, biofuel production, biopolishing, etc. (Dina *et al*., 2023). The ability of *Aspergillus* species to produce cellulase has been reported in previous studies. However, other species of microorganisms capable of producing cellulase include bacteria (Bacillus, Cellulomonas, Pseudomonas, Clostridium, Ruminococcus, and Fibrobacter) and fungi (Fusarium, Penicillium, and *Cladiosporum*) (Behera & Ray, 2016; Bajaj & Mahajan, 2019). The production of cellulase from *A. niger* offers a promising path for sustainable bioprocessing and industrial utilization. Ten of the fifteen isolates in this study produced cellulase on Congo red agar, indicating that not all A. niger isolates produce cellulase. This observation is contrary to the earlier submission by Bakare et al. (2022), who reported that all A. niger isolates in their study produced cellulase. In view of this, the discrepancy in the *A. niger* isolates' ability in this study to produce cellulase or not may be attributed to factors such as the media/substrate used for growth, genetics, growth conditions, and catabolite repression (Chysirichote, 2018). According to the reports of Yoon *et al.* (2007), Remazol Brilliant Blue xylan (RBBX) agar, which is a selective medium for degrading xylan, was used for cellulase screening, unlike the Congo red agar, which is also a selective medium for cellulase detection that binds to its indicator dye (Congo red), may be a pre-determining factor for cellulase production (Yoon *et al*., 2007; Bakare *et al*., 2022). Earlier studies by Septiani et al. (2029), Siva et al. (2022), and Dina et al. (2023) also reported that organisms with high zones of clearance during the cellulase screening assay indicate potential for cellulase production.

Moving forward, this study investigated the effect of physicochemical factors (temperature and pH) on cellulase production by *A. niger*. Optimum cellulase production was observed at 37 ^{0}C and pH 4.5 in this study. This occurrence closely aligns with the previous report by Akinyele et al. (2014), who documented optimal cellulase production by A. niger at 37 °C and pH 5.5. In addition, Abou-Taleb et al. (2009) documented that optimum cellulase production was achieved at a temperature range of 30-45 °C and a pH range of 7.0-7.5, while Guowei et al. (2011) reported optimum cellulase at 30 °C and a pH range of 6.0-8.5. This study also monitored the effect of carbon and nitrogen sources on cellulase production. It was observed that optimum cellulase production was achieved using glucose as the carbon source. This finding is similar to the previous results of Abou-Taleb *et al*. (2009), Dashtban et al. (2011), Naher et al. (2021), and Dina et al. (2023). Thus, establishing the fact that glucose, among other carbon sources, is easily assimilated by *A. niger* for cellulase production. Furthermore, peptone and ammonium sulfite were recorded as the best nitrogen sources for cellulase production by *A. niger*. This occurrence is in tandem with the earlier reports of Sasi et al. (2012), who stated that optimal cellulase production by *A. niger* was achieved using ammonium sulfite as the nitrogen source. Moving forward, Mehboob *et al*. (2014) reported optimal cellulase production using peptone rather than urea and ammonium sulfite. This observation, however, differs somewhat from what Shahriarinour et al. (2011) reported, which found that peptone and yeast extract enhanced cellulase production compared to other nitrogen sources. It can, therefore, be suggested that the peptone concentration should be considered when using it as a nitrogen source for cellulase production. The effect of substrate (CMC) concentration on cellulase production by the isolated *A. niger* was also monitored. It was observed that a CMC concentration of 5% produced the highest cellulase activity, although 2-3% CMC also generated substantial amounts of cellulase. Ja'afaru and Fagade stated this. (2010) documented a contrary report in their study in which they submitted that optimum cellulase production was recorded using 1% CMC, and a decrease in cellulase production was observed as CMC concentrations increased. However, Septiani *et al*. (2019) also support the claim in this work that an increase in cellulase production is directly proportional to an increase in CMC concentration.

Finally, the effect of incubation time on cellulase production was also monitored in this study, as optimum cellulase production was obtained at 72 h and 96 h. This corresponds to the findings of Guowei *et al.* (2011), who stated that cellulase optimum production by A. niger was reached within the incubation time range of 72 h - 120 h. Therefore, it can be suggested that an incubation time range of 72 h-120 h is suitable for cellulase optimum production.

Conclusion

This study concluded that *A. niger* ISOd and *A. niger* ISOg are good cellulase producers readily isolated from the environment. In addition, future studies may be tailored towards the bioengineering of optimization processes to produce more cellulase for various industrial applications. Furthermore, this study also suggests that microbial cellulase may be used as an alternative to chemically synthesized (synthetic) cellulase, which is less toxic, more eco-friendly, and more biodegradable.

Authors Contribution

IFF, FTA, and JOB conceptualized the study. IFF, FTA, and JOB designed the study. JOB, PTO, and FTA participated in fieldwork and data collection. IFF, JOB, and PTO performed the data analysis; IFF, JOB, and PTO interpreted the data. JOB and PTO prepared the first draft of the manuscript, which IFF and FTA reviewed. All authors contributed to the development of the final manuscript and approved its submission.

Conflict of interest

The authors declare no conflicts of interest.

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