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Original Research

Optimization of Crude Cellulase Production by *Fusarium oxysporum* Isolates from Native Environments

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ABSTRACT

Cellulase, a key enzyme in breaking down cellulose, has significant applications in biomass, biofuel production, and environmental pollution control. This research investigated optimization of crude cellulase production from *Fusarium oxysporum* isolates using solid-state fermentation (SSF). For cellulase production optimization, the fungal isolates were cultured to obtain pure cultures and identified based on genus characteristics. Inoculum was prepared by harvesting spores from Sabouraud dextrose agar (SDA). Solid-state fermentation was conducted with agro-based waste materials, including crushed agro-residues: date cores, wild reed, peanut shell, sunflower scales, corn cobs, banana peel, and sawdust, as substrates. Various parameters, including solid substrates, carbon and nitrogen sources, moisture content, incubation temperature and periods, and inoculum size, were optimized for cellulase production. Enzyme activity was measured by carboxymethyl cellulase (CMCase) and filter paperase (FPase) assays. The results showed that ten isolates of *Fusarium* spp. were identified, with isolate F4 demonstrating superior cellulase production compared to the others. This isolate was identified as *Fusarium oxysporum*. F4 isolate exhibited the highest cellulase index (CI) and specific activities for CMCase (17.33 U/mg) and FPase (8.62 U/mg). The optimal SSF conditions included corn cobs as the substrate, 60% moisture, and ammonium sulfate as the nitrogen source, yielding specific activities of 22.93 U/mg (CMCase) and 10.61 U/mg (FPase). The optimal temperature for cellulase production was 25°C, with peak enzyme activity observed after 120 h of incubation. The study's findings demonstrated the

potential of *F. oxysporum* for efficient cellulase production, particularly from inexpensive agro-residues, highlighting its industrial and environmental significance.

INTRODUCTION

Cellulose $(C_6H_{10}O_5)_n$ makes up the main part of plant cell walls. It is also one of the most vital polymers found on the surface of the earth. The cellulose chain consists of hundreds or thousands of monosaccharide glucose molecules linked together to form this chain (Khan *et al*., 2021). Cellulose comprises β-D-glucopyranoside units that are joined side by side by β-D-glucosyl bonds (Butnariu and Flavius, 2022). Cellulose is formed by linking glucose subunits via β(1→4)-glycosidic linkages between D-glucose units (Gomes *et al*., 2021). Cellulases are enzymes that are normally secreted outside living cells and break down natural cellulosic materials. There are three types of cellulases that differ in the type of bonds they attack: β-glucosidase, endo-1,4-β-D-glucanase (endoglucanase), and exo-1,4-β-D-glucanase (exoglucanase). They break down cellulosic materials into fermentable sugars such as glucose or larger units. The resulting sugars are used for the production of biomass and the production of alcohols and fuels. Cellulases are produced on a large scale through various fermentation processes, depending on the type of organism involved. These enzymes are induced by the presence of materials containing the β-1,4 bond. It can also be induced by cellobiose and similar substances such as lactose (Gomes *et al*., 2021). For commercial production, various microorganisms are utilized through continuous submersible cultivation methods. The production process is influenced by several factors, including carbon and nitrogen sources, pH, ventilation, and incubation period (Suwannarach *et al*.,2022).

Fungi are the main producers of the cellulase enzymes, as an estimated 80% of the breakdown of cellulose in nature is done by species of fungi of the division Ascomycota (Corbu *et al*., 2023). *Fusarium* is a large genus of filamentous fungi, widely distributed in soil and associated with plants, especially in agricultural crops. *Fusarium oxysporum* strains are soil inhabitants that have the ability to exist as saprophytes and degrade lignin and complex carbohydrates associated with soil debris (Ekwomadu and Mwanza, 2023). Many researchers have found that cellulase can be produced from many aerobic fungi (Singh *et al*., 2021). Fungi are the preferred source of cellulase for industrial applications since they secrete large quantities of cellulase to culture medium (Borthakur *et al*., 2024). Despite the vast number of fungi capable of producing cellulase enzymes, only a few have been extensively studied due to their ability to secrete these enzymes in large quantities extracellularly (Nehad *et al*., 2020). Therefore, most of the studies focused on cellulase production from fungi. One of the most important methods of enzyme production used in recent decades is solid-state fermentation (SSF). This method relies on natural, water-insoluble materials, the absence of free water, and maintaining humidity above 12%. The key natural materials used in this method include grains, wheat bran, corn stalks, and agricultural waste such as straw. These materials effectively absorb water, providing microorganisms with the necessary moisture for growth and metabolic activities (Mattedi *et al*., 2023). Environmental

pollution is steadily increasing, significantly impacting living organisms, including humans. However, it can be mitigated by microorganisms with biosynthetic pathways that enable the degradation or accumulation of pollutants in soil and water (Iqbal *et al*., 2023).

This study aimed to optimize the production conditions for crude cellulase enzyme employing the solid-state fermentation technique with *Fusarium oxysporum* isolated from native environments. Also, it highlights the most important applications of cellulases and provides a potential approach for addressing environmental pollution.

MATERIALS AND METHODS

Isolation of Cellulase-Producing Fungi

Ten samples for fungal isolation were collected from various sources, including wilted vegetables, infected wheat ears, decaying plant roots, rotting agricultural waste, diseased melon seeds, and field soil down to a depth of 5 cm. Each sample was placed in sterile plastic bags, separately, and transported to the laboratory. Then, the samples were cultured on Sabouraud dextrose agar (SDA) until a pure culture of each isolate was obtained. According to Summerell *et al*. (2003), all the isolates were identified based on genus characteristics.

Maintenance of Fungal Cultures

All isolated fungal strains were preserved on slants of potato dextrose agar (PDA). The pH of the culture medium was adjusted to 6.0, and the culture was incubated at 30°C for 7 days and kept in the refrigerator at 4°C. Subculturing was done once every 2 months.

Inoculum Preparation

The isolates of *Fusarium* spp. were subcultured on SDA Petri plates and incubated at 30°C for 7 days until good sporulation occurred. The spores were harvested by suspending them in sterile saline 0.085% of NaCl containing 0.1% of Tween 80. After 30 minutes, the mixture was filtered through sterile gauze to collect spores, which were then counted using a hemocytometer (Kumar et al., 2014) using the following equation:

$$\text{Spores/mL} = (n) \times 10^4$$

where n = the average cell counts per square of the four corner squares

Qualitative Assessment Of Cellulase Activity in *Fusarium* spp.

A qualitative examination was carried out by culturing the isolated *Fusarium* spp. on Czapeck-Dox agar medium supplemented with 1% of carboxymethyl cellulose (CMC). The dishes were incubated at 30°C for 5 days. Then 10 mL of Congo red reagent with a concentration of 2.5 g/l was added to each dish. Congo red staining was used for the identification of amyloids. After 15 minutes, the reagent was discarded, and all fungal cultures were washed with 10 mL of NaCl (1 mol/L). The clear

zone region around each fungal colony was considered positive for cellulase activity. The experiment was conducted with three replications. To calculate the cellulase index (CI), the following mathematical relationship was used (Avin 2020):

$$Cl = \frac{Diameter\ of\ Hydrolysis\ Zone(clear\ zone)}{Diameter\ of\ Colony}$$

Estimation of Dry Biomass

Ten isolated fungi were cultured separately in a 250 mL conical flask containing 100 mL of Nakamura broth medium. For each liter of distilled water (D.W.), the medium was prepared by adding 10 g of carboxymethyl-cellulase (CMC), 20 mL of corn steep liquor, 12 g of $NH_4H_2PO_4$, 0.7 g of KCl, 0.5 g of MgSO4.7H2O, and 0.01 g of FeSO4.7H2O, with an initial pH of 4.8. One milliliter of $1x10^5$ spores per milliliter was added to the broth medium, and it was then incubated in a rotating shaker incubator with shaking speed pf 140 rpm at 30ºC for 5 days. The flasks were withdrawn from the incubator, and the biomass was separated from the culture medium containing the enzyme solution by using a Buechner funnel containing a Whatman No. 1 filter paper previously weighed with a sensitive balance. After the filtration process was completed, filter paper containing the biomass was dried in an electric oven at 50°C for 24 h and then weighed to estimate the weight of the biomass. The experiment was conducted with three replications.

Measurement of Soluble Protein And Enzyme Activity

The above filtrate was centrifuged at 15000 rpm for 15 min at 4°C to collect solid materials. The supernatant was filtered through Whatman No. 1 filter paper, and the clarified supernatant was assayed for soluble protein and enzyme activity by measuring the reducing sugar released. The specific activity was calculated as follows:

$$Specific\ Activity\ \left(\frac{U}{mg}\right) = \frac{Enzyme\ Activity\ (\frac{U}{mL})}{Protein\ Concentration\ (\frac{mg}{mL})}$$

Identification of a Fungal Isolate With the Largest Clear Zone and Highest Specific Activity

The fungal isolate exhibiting the largest clear zone and highest specific activity of carboxymethyl cellulase (CMCase) and filter paperase (FPase) was selected and identified based on colony characteristics, including colony color, reverse color, margin, and texture, on PDA plates. Microscopic characterization was accomplished using the slide culture technique and staining by lactophenol cotton blue (Summerell *et al.*, 2003).

Preparation of Substrate Materials

Seven types of agro-based waste materials were used for cellulase production by *F. oxysporum*, namely, date cores (Dc), wild reed (Wr), peanut shell (Ps), sunflower scales (Ss), corn cobs (Cc), banana peel (Bp), sawdust (Sd). These waste materials were collected locally. Substrates were washed with water to free them from dust. The samples were dried under sunlight for two weeks depending on the moisture content and crushed and sieved through 1 mm mesh and used as substrates for further studies.

Pre-treatment of Substrates

According to the procedure of Haddadin *et al.* (2009), all substrates were oven-dried at 50°C for 48 h. Then, dried substrates were milled into fine particles using a mechanical grinder and then separated through a sieve with a porosity of 1.25 mm. According to Bansal *et al.* (2012), all substrates were treated by soaking in 1% NaOH solution in the ratio of 1:10 (substrate: NaOH solution) overnight at 25°C. Then, the samples were filtered and washed with D.W. until the pH became neutral. The washed samples were oven-dried at 65°C for 48 h. Finally, treated samples were then kept in clean plastic containers separately until use.

Extraction of Crude Enzyme

Fifty mL of acetate buffer solution (0.1M, pH 5.8) was added to a conical flask that contained 10 g of fermented solid substrate. The mixture was placed in a shaking incubator with a shaking speed of 120 rpm at 30°C for 1 h. Then the mixture was filtered with a clean piece of gauze. The filtrate was divided into 10 mL tubes and centrifuged at 6000 rpm under refrigerated conditions for 20 min. The supernatant was filtered using Whatman No. 1 filter paper. The filtrate was used to measure the enzyme activity and estimate the amount of protein.

Measurement of Carboxymethyl Cellulase and Filter Paperase Activities

Carboxymethyl cellulase activity was determined following the procedures described by Dutta *et al*. (2008). The reactants consisted of 0.5 mL of crude enzyme solution with 0.5 mL of 1% CMC in 50 mM citrate buffer (pH 5). The mixture was placed in a water bath at 40°C for 20 min. The reaction was stopped with the addition of DNS reagent. The sample was boiled for 10 min, then 1 mL of 40% (w/v) sodium potassium tartrate solution was added to maintain the colour reaction. A spectrophotometer was used to measure the absorbance at 540 nm. Carboxymethyl-cellulase activity was determined using a calibration curve for glucose. Filter paperase activity was measured according to Silveira *et al*. (2014), by mixing 0.5 mL of citrate-phosphate buffer (0.05 M, pH 4.8) with 0.5 mL of the crude enzyme. The mixture was incubated at 50°C for 10 min. The Whatman No. 1 filter paper strips, each weighing about 50 mg (1×6 cm), were added to the test tubes. The mixture was incubated at 50°C for 60 min. The reducing sugars released after the enzymatic reaction were terminated by adding 1.5 mL of DNS reagent. The tubes were then placed in boiling water for 5 minutes, followed by the addition of 10 mL of distilled water to each tube. The absorbance was measured at 540 nm.

Protein Estimation

The protein samples were estimated using the method described by Bradford (1976).

Optimization of Solid-State Fermentation Parameters

Optimal conditions for cellulase production were determined using different influencing factors, such as solid substrate materials, carbon sources, moisture content, nitrogen sources, incubation temperatures, incubation periods, and inoculum size. Each experiment was performed with three replicates. To determine the effect of different solid substrate materials, 10 g of each substrate was separately placed in a 250 mL conical flask. The moisture content was adjusted to 20% using distilled water, and the mixture was autoclaved at 121°C for 20 minutes at 15 psi pressure. After the flasks cooled, they were inoculated with 1 mL of $1x10^5$ spore/mL. The contents of the flasks were thoroughly mixed and incubated at 30°C for 6 days. The effect of different carbon sources, including fructose, glucose, lactose, maltose, xylose, CMC, cellobiose, and Avicel, each at a concentration of 1%, was tested, while all other previous conditions were kept constant. The effect of the water content on the cellulase production was determined at seven different levels of distilled water (20% - 80%), while other previous conditions were fixed.

The effect of different nitrogen sources was examined by supplementing solid substrates with various organic nitrogen sources, such as tryptone (T), casein (C), peptone (P), urea (U), and yeast extract (YE), as well as inorganic nitrogen sources like ammonium sulfate (AS), ammonium chloride (AC), and sodium nitrate (SN), all at a concentration of 1%. Other conditions from previous experiments were kept constant to investigate their impact on cellulase production. The effect of optimum temperature was determined by testing a temperature range of 20–45°C, while other conditions from previous experiments were kept constant. To determine the optimal incubation period for cellulase production, the incubation period range of 24-168 h was considered, while other previous conditions remained constant. The effect of inoculum size on cellulase production was tested with concentrations ranging from $1x10^2$ to $1x10^8$ spores/mL, while other conditions from previous experiments were kept constant.

Statistical Analysis

Each experiment was performed in three replicates. The Excel program was used to calculate the mean and standard deviation of each experimental value.

RESULTS AND DISCUSSION

Cellulase Index of *Fusarium* spp. Isolates

The results (Table 1) revealed that all isolated fungi belonging to the genus *Fusarium* degraded CMC at varying rates. This variation was due to the secretion of cellulase in the culture medium, where the Congo red reagent remained attached only to the regions with β-1,4-D-glucanohydrolase bonds (Lamb and Loy, 2005). It was observed that isolate F4 produced the largest diameter of the clear zone, which was determined by calculating the CI ratio. The diameter of the clear zone is a vital indicator for fungal isolates that effectively degrade cellulose, which helps in choosing the isolates that have a great ability to produce cellulase.

Table 1: Isolation sources and cellulase index (CI) of *Fusarium* spp. isolates.

Reference ID	Isolation source	CI (cm)
F1	Field soil	1.14±0.02
F2	Wilted cucumber plant	1.29±0.09
F3	Field soil	1.20±0.06
F4	Infected wheat ears	1.57±0.11
F5	Rotting agricultural waste	1.31±0.03
F6	Rotting plant roots	1.22±0.01
F7	Wilted tomato plants	1.39±0.04
F8	Field soil	1.08±0.03
F9	Infected melon seeds	1.19±0.16
F10	Field soil	1.12±0.05

Values are expressed as mean + standard deviation (SD) of three replicates.

Cellulase-Specific Activity from *Fusarium* spp. Isolates

The results in Table 2 indicated that all fungal isolates are cellulase producers. Among them, the F4 isolate was the most efficient one. The results also showed that the specific activity of CMCase and FPase was 17.33 and 8.62, respectively, while the specific activity for the other isolates ranged between 11.32 and 14.65 U/mg.

Table 2: Dry biomass weight and specific activity of *Fusarium* spp. isolates.

Isolate symbol	Dry biomass weight (gm/l)	Specific activity (U/mg)	
		CMCase	FPase
F1	8.77±0.04	11.67±0.07	2.55±0.08
F2	9.69±0.13	13.79±0.01	3.47±0.12
F3	9.22±0.05	13.08±0.04	2.89±0.05
F4	12.48±0.09	17.33±0.03	8.62±0.06
F5	10.78±0.11	14.26±0.15	3.64±0.09
F6	9.32±0.06	13.15± 0.01	2.14± 0.06
F7	10.91±0.08	14.65± 0.14	2.94± 0.05
F8	8.54±0.02	11.32± 0.08	2.47± 0.08
F9	8.95±0.04	12.27± 0.12	3.15± 0.04
F10	8.29±0.07	11.54± 0.09	2.71± 0.07

Values are expressed as mean + standard deviation (SD) of three replicates.

Based on the results presented (Tables 1 and 2), isolate F4 showed the highest ratio of cellulase index (CI) and the highest specific activity, respectively. It was selected and identified as *Fusarium oxysporum* and used in subsequent experiments to determine the optimum conditions for crude cellulase production.

Optimal Conditions for Solid-State Fermentation

Effect of different substrates

The results (Fig. 1) revealed that the seven substrate materials screened varied in their ability to induce CMCase and FPase production. Corn cobs (Cc) resulted in the maximum CMCase and FPase production with specific activities of 19.46 U/mg and 9.52 U/mg, respectively. Meanwhile, a low level of CMCase and FPase production was recorded for sawdust (Sd) with specific activities of 4.82 U/mg and 2.26 U/mg, respectively, when fermented with *F. oxysporum* under SSF.

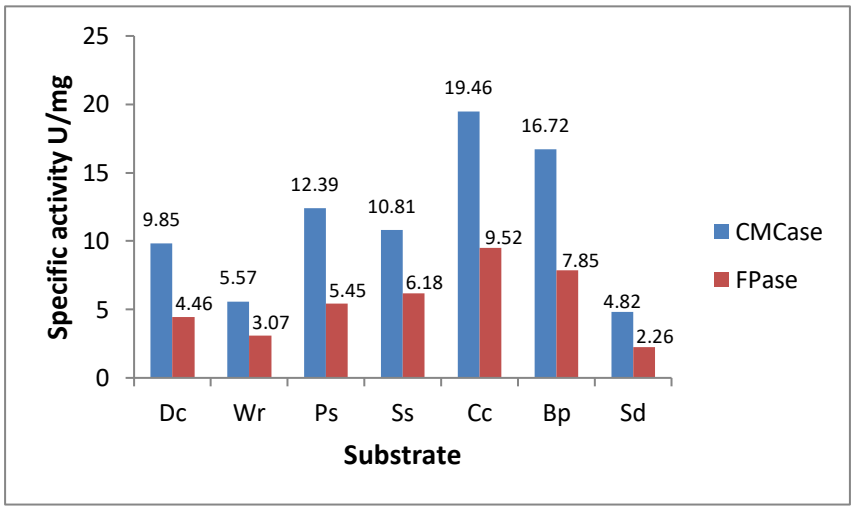

Fig. 1: Effect of different solid substrate materials on cellulase production by *Fusarium oxysporum* using 40% moisture content, inoculum size of $1x10^5$ spores/mL, and incubation at 30°C for 6 days**.** The bars represent mean values (n = 3).

One of the important determining factors for the production of enzymes by microorganisms is the cheap source of lignocellulosic biomass (Bajaj *et al*., 2013). With the advancement of scientific research methods, the abundant plant residues left after harvesting have been increasingly exploited as cost-effective substrates for the production of microbial enzymes (Ravindran *et al*., 2018). Numerous studies have shown through chemical analysis of corn cobs that it contains a higher percentage of cellulose than other plants (Harini *et al*., 2014). The results of this study agree with what was reported by Ire *et al*. (2018), who reached the conclusion that optimal cellulase productivity from *Penicillium* sp. of 15.787 IU/mL was obtained with the utilization of corn cobs as the substrate. The results of this study

also agree with what was discovered by Harini *et al*. (2014), who explained that corn cobs gave a greater yield of cellulase enzyme than wheat bran when using *Aspergillus niger* because corn cobs contain a higher percentage of cellulose, which was estimated to be about 66%.

Environmental pollution is an international threat to public health. This has led to the human invention of new means to preserve the environment. In spite of the huge utilization of lignocellulose materials, there are still huge amounts of cellulose accumulating in the environment that contain raw materials that are not used in an efficient manner. The problem is to create sustainable and cost-effective processes. For economic and environmental reasons, biodegradation has become a successful alternative for treating agricultural, industrial, organic, and toxic wastes. These wastes, if not disposed of properly, will lead to environmental pollution (Chandra *et al*., 2015). Cellulases are inducible enzymes produced by a large number of different microorganisms, which may be aerobic, anaerobic, mesophilic, or thermophilic (Lee and Koo, 2001). Production of cellulase by various microorganisms in the fermentation process has gained more consideration, and the cost has proven to be high due to the high cost of process engineering. Therefore, producing cellulase using locally available sources will reduce import costs. Municipal solid waste includes large amounts of cellulose, which is a perfect organic waste for the microorganism's growth in addition to composting by possible microorganisms. Municipal solid waste contains 40–50% cellulose, 9–12% hemicelluloses, and 10–15% lignin, based on dry weight. A great number of microorganisms have been found in municipal solid waste. Municipal solid waste is appropriate for composting due to the presence of a very large percentage of organic matter in it (Gautam *et al*., 2010). Agro-residues and industrial waste resulting from agricultural processes are one of the most important energy resources, as it is possible to convert this waste into products of great value through enzymes produced by microorganisms (Bala *et al*., 2023).

Agro-residue lignocellulosic biomass is a sustainable resource that stores sunlight energy in its chemical bonds (McKendry, 2002). It is composed of a high amount of cellulosic matter, which is simply decayed by a confluence of physical, chemical, and biological processes (Iyiola*et al*., 2024). Humans have the potential to produce pure ethanol fuel through the production of decomposing enzymes and microbial fermentation of agro-residues, which is more cost-effective than using starch-based materials. Saccharification of lignocellulosic biomass results in non-polluting bioethanol, biofuel, and other chemical molecules derived from cellulose materials (Bajaj *et al*., 2014). Recycling plant waste has been developed as a method for preventing environmental degradation due to pollution and rising food supply. The possible advantages from a successful reuse of lignocellulosic wastes are huge. Cellulose and hemicellulose are compounds found in all plants and are the parts rich in sugars that are of interest for use in fermentation processes, as microorganisms can use the sugars for growth and production of new chemical compounds of great importance. The disposal of plant residues represents a major environmental problem. However, with the participation of various microorganisms specializing in cellulosic ecosystems and the development of scientific and engineering methods, humans have

been able to overcome the resistance of plant materials to decomposition. This process allows the conversion of plant residues into useful chemical compounds, such as biofuels, at a low cost (Bayer *et al.*, 2007).

Effect of Different Carbon Sources

As presented in Fig. 2, the carbon sources examined vary in their ability to induce cellulase production. CMC gave the highest yield of CMCase and FPase production, with specific activities of 21.92 U/mg and 10.06 U/mg, respectively. In contrast, a low level of CMCase and FPase production was recovered when using lactose, with specific activities of 13.51 U/mg and 3.16 U/mg, respectively, when fermented with *F. oxysporum* under SSF.

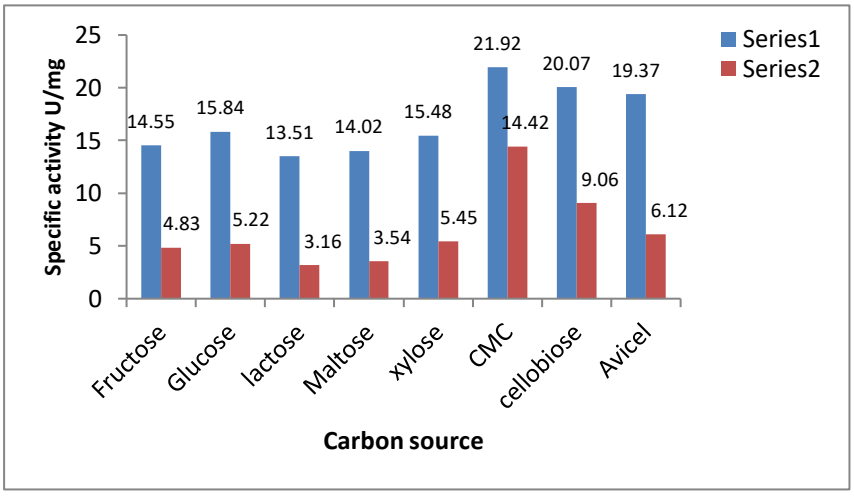

Fig. 2: Effect of different carbon sources on cellulase production by *Fusarium oxysporum* using corn cobs, 40% moisture content, inoculum size of $1x10^5$ spores/mL, and incubation at 30^oC for 6 days. The bars represent mean values (n = 3).

Carbon sources play an essential function in the cellulase synthesis by cells. The biosynthesis of cellulase is of great importance for breaking down complex substrates. It is inhibited in the presence of easily metabolizable carbon sources, such as glucose, and is induced by low molecular weight compounds like cellobiose (Blanchette 1998). The results of this study are consistent with the findings of Gautam *et al*. (2011), who reported that CMC was observed to be the best carbon source for enzyme production by *A. niger*. Also, the results of this study are in agreement with those of Olajuyigbe *et al*. (2016), who found that CMC supported the highest yield of β-glucosidase produced from *F. oxysporum* with specific activity up to 177.5 U/mg at pH 6.0, with the liberation of 2.121 µmol/mL glucose. In the present study, low specific activity was observed in the presence of glucose and xylose, while substrates like CMC and cellobiose were confirmed as the primary inducers of cellulase production. The cellulase

activity in Avicel-containing medium was slightly less compared with the activity in the presence of CMC and cellobiose (Lynd *et al.*, 2002).

A number of studies have reported that the production of glucose from cellulose results in a decrease in cellulase activity (Sherif *et al.*, 2005). They found that both CMC and Avicel showed a decrease in cellulase activity towards day 10, while the cellulase activity for the cellobiose-containing medium started decreasing after 8 days. This may occur as a result of the accumulation of glucose, an end product of cellobiose phosphorylase, which inhibits further production of the cellulase. They also reported a decrease in cellulase activity earlier with the cellobiose substrate, as it is an intermediate in the conversion of cellulose to glucose. The accumulation of glucose occurred sooner in that medium, leading to an earlier decline in cellulase activity (Niranjane *et al.*, 2007).

Effect of Moisture Content

It was found that the best moisture content was 60% for the production of CMCase and FPase with specific activities of 22.93 U/mg and 10.61 U/mg, respectively. Any decrease or increase in the percentage of moisture content resulted in a reduction in the specific activity of the enzyme (Fig. 3).

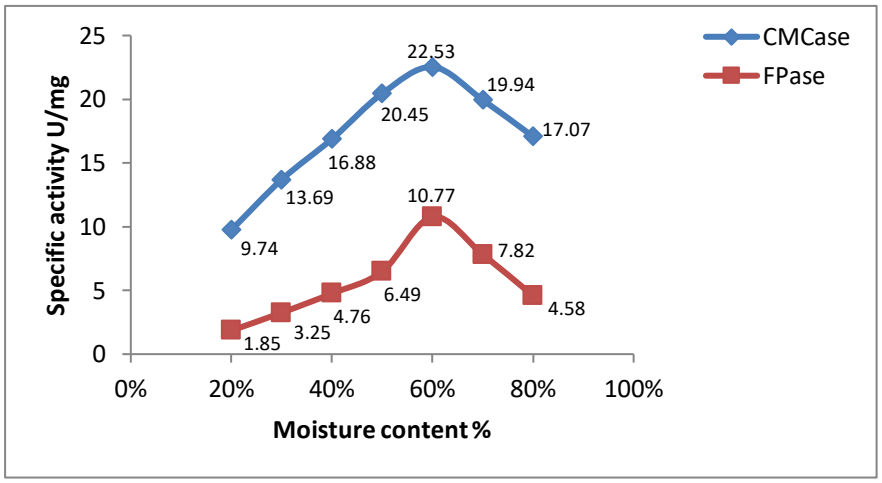

Fig. 3: Effect of different moisture contents on cellulase production by *Fusarium oxysporum* using corn cobs, inoculum size of $1x10^5$spore/mL, and incubation at 30°C for 6 days. The bars represent mean values (n = 3).

One of the major factors affecting SSF technology is the moisture content of the substrate. This factor affects the growth of the microorganisms through its effect on the biosynthesis of compounds inside the cell and secretion of enzymes (Wonoputri *et al.*, 2018). One of the significant effects of decreased substrate moisture content is the reduced solubility of compounds, making them less accessible. This leads to increased water tension and a decrease in substrate swelling. A high percentage of moisture content in the substrate decreases enzyme yield. This occurs because excessive moisture

reduces the size of interstitial voids in the substrate, leading to poor growth by hindering oxygen transport (Lopez-Gomez and Venus, 2021). The results of this study are consistent with those of Liu and Yang (2007), who reached the conclusion that the optimum moisture content when using SSF technology to produce cellulases is 40%-60%. They also reported that an increase in moisture ratio beyond a certain level decreases cellulase enzyme production. This occurs because higher moisture reduces the substrate's surface area, limiting air access and affecting microbial growth by disrupting metabolic processes.

Effect of Different Nitrogen Sources

The effect of supplementation of nitrogen sources on the cellulase production is shown in Fig. 4. All the nitrogen sources enhanced cellulase production. Among them, ammonium sulphate supported maximum CMCase and FPase production with specific activities of 24.88 U/mg and 11.96 U/mg, respectively. Nitrogen is an important element for microbial protein and enzyme synthesis. It is one of the major cellular proteins, and the stimulation of cellulase activity by ammonium sulfate may be attributed to its direct involvement in protein synthesis (Mandels and Reese, 1999). The results of this study are in agreement with those of Sadida and Manchur (2021), who recorded that maximum extracellular cellulase production by actinomycete occurred when yeast extract followed by peptone was used as nitrogen sources. Also, the results of this study align with the findings of Li *et al.* (2021), who reported that ammonium sulfate as a nitrogen source supported maximum cellulase production by *Penicillium oxalicum* R4. Additionally, the study agrees with Kumar *et al.* (2012), who found that ammonium sulfite at a concentration of 1.5% was the optimal nitrogen source for CMCase production.

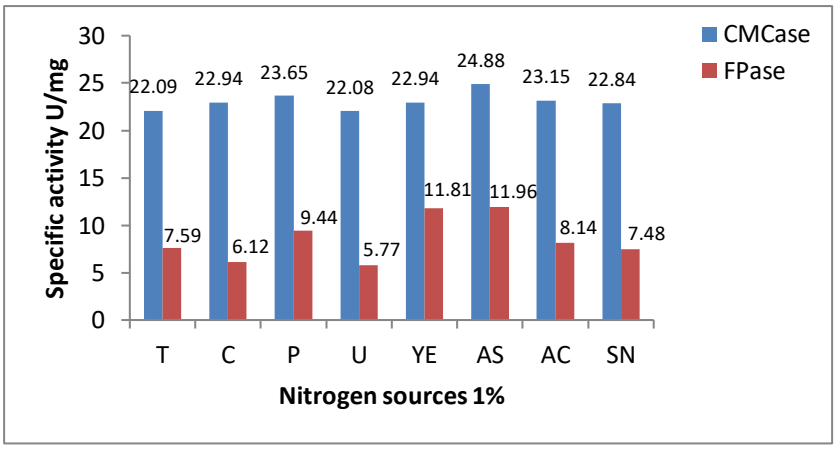

Fig. 4: Effect of different nitrogen sources on cellulase production by *Fusarium oxysporum* using corn cobs, 60% moisture content, inoculum size of $1x10^5$ spores/mL, and incubation at 30°C for 6 days**.** The bars represent mean values (n = 3).

Effect of Incubation Temperature

The results (Fig. 5) revealed that the optimal temperature for the production of crude cellulase enzyme by *F. oxysporum* was 25°C with specific activities of 26.78 U/mg and 12.13, respectively, for CMCase and FPase. It has been shown that any increase or decrease in incubation temperature causes a decrease in enzyme production.

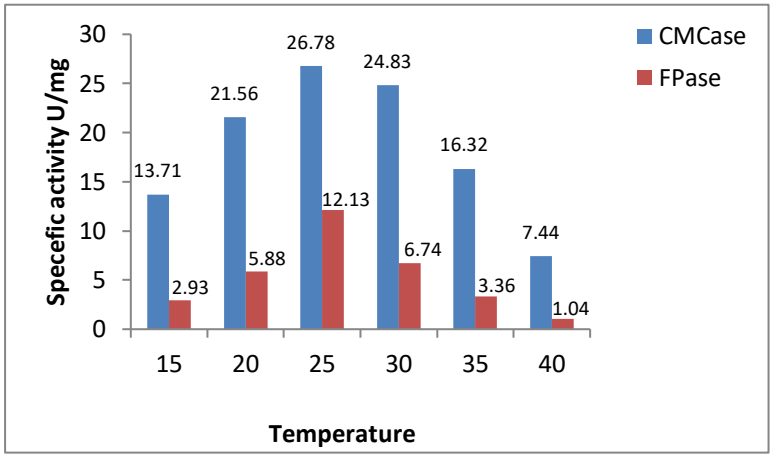

Fig. 5: Effect of different incubation temperatures on cellulase production by *Fusarium oxysporum* using corn cobs, ammonium sulphate, 60% moisture content, inoculum size of $1x10^5$ spores/mL, and incubation for 6 days. The bars represent mean values (n = 3).

The findings revealed that *F. oxysporum* can degrade cellulose across a broad temperature range of 15 to 40°C. The metabolic activities of all microorganisms are affected by temperature changes. Some studies indicated that the optimal temperature for the production of cellulase enzyme varies according to the studied fungal species, such as *Penicillium*, *Trichoderma*, *Aspergillus*, and white mold fungus (Navaneethapandian *et al*. 2020). Other studies found that the optimum temperature for the production of this enzyme differs according to the strains of the microorganisms (Harini and Kumaresan, 2014). The findings of this study agree with those of Evelyn *et al*. (2020), who found that the maximum production of cellulase enzyme by the fungus *Eupenicillium javanicum* by the SSF technique using pineapple crown leaves as a substrate was at 25°C. Also, the findings of this study are consistent with those of Abd Elrsoul and Bakhiet (2018), who found that 28°C was the optimal temperature for maximum enzyme yield from *F. solani* using submerged fermentation. However, the results differ from those of Ramanathan *et al*. (2010), who reported that the optimum temperature for producing maximum cellulase from *F. oxysporum* by submerged fermentation was 50°C.

Effect of Incubation Periods

Cellulase production was low during the first 24 h but increased significantly between 72 and 120 h. The highest CMCase and FPase production occurred at 120 h, with specific activities of 30.57 U/mg and 12.68 U/mg, respectively, before beginning to decline (Fig. 6).

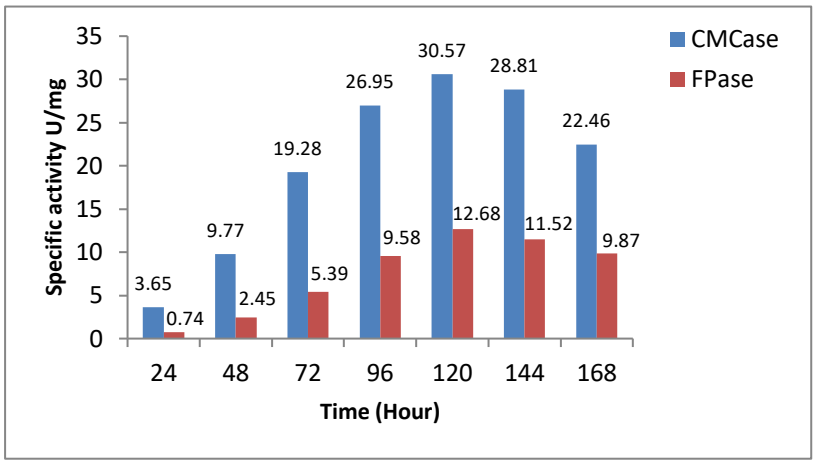

Fig. 6: Effect of incubation periods on cellulase production by *Fusarium oxysporum* using corn cobs, ammonium sulphate, 60% moisture content, inoculum size of $1x10^5$ spores/mL, and incubation at 30°C. The bars represent mean values of ± standard deviation of duplicates of three independent experiments.

Generally, one of the important factors when producing enzymes using SSF is the incubation period. Cellulases are primary metabolites produced by microorganisms during the exponential growth phase, with their production declining during the death phase (Ray, 2016). The findings of this study agree with those of Kumar (2016). Kumar discovered that a 5-day incubation period was ideal for cellulase production by *A. niger* and *A. flavus* strains. Additionally, the study found that enzyme activity begins to decrease after more than 5 days of incubation. However, during this period, the biomass of the fungi studied continues to increase. The findings of the present study differ from those of Darabzadeh et al. (2019), which indicated that the best incubation period was 4 days using a mutant strain of *T. reesei*. The findings of this study contrast with those of Ramanathan et al. (2010), who reported that the optimal incubation period for cellulase production by *F. oxysporum* in submerged fermentation was 12 days. Similarly, they differ from the results of Yakubu et al. (2021), who observed maximum CMCase (3.52 U/mL) and FPase (4.07 U/mL) production after 8 days of incubation at pH 8, a temperature of 30°C, and 1.0 g/L ammonium sulfate.

Effect of Inoculum Size

As shown in Fig. 7, maximum specific activities of cellulase enzyme for both CMCase and FPase were 33.86 U/mg and 12.67 U/mg, respectively, when the inoculum size was $1x10^6$ spores/mL. Any increase or decrease in the inoculum size led to a reduction in enzyme activity.

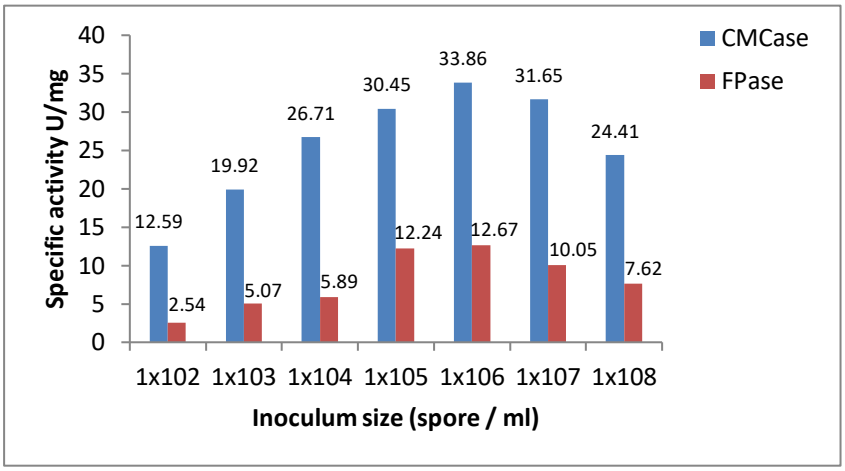

Fig. 7: Effect of inoculum size on cellulase production by *Fusarium oxysporum* using corn cobs, ammonium sulphate, 60% moisture content, and incubation at 30°C for 120 h**.** The bars represent mean values (n = 3).

The inoculum size is a critical factor in enzyme production. A low inoculum concentration may be insufficient to initiate growth and enzyme synthesis. Conversely, increasing the inoculum size accelerates biomass proliferation and enhances enzyme production (Sahrawat and Garg, 2022). However, beyond a certain threshold, enzyme production may decline due to nutrient depletion caused by increased biomass, leading to a reduction in metabolic activity. Stability between the proliferating biomass and available substrate material would give in maximum production of enzyme (Usman *et al.*, 2021). The results of this study are consistent with those of Muhammad *et al*. (2019), who reported that an inoculum size of $1x10^6$ spore/mL of *A. niger* was the best for cellulase production using sugar cane bagasse as a substrate, while Sulymana *et al*. (2020) discovered that the best concentration of spores for the cellulase production by *A. niger* and *Arachis hypogaea* was 4.5×10^6 spore/mL and 3×10^5 CFU/mL, respectively.

Applications of Cellulases

Cellulase is an enzyme of significant industrial importance, accounting for approximately 20% of the global enzyme market. Asia-Pacific is the biggest user of cellulase, with an income market share of about 32.84% in 2016. In 2016, the cellulase market demand was distributed as follows: 29.71% in animal feed, 26.37% in the food and beverage industry, and 13.77% in the textile industry. The same report projects

that cellulase applications will reach 2,300 million USD by the end of 2025, with an annual growth rate of 5.5% from 2018 to 2025. These findings suggest a significant yearly increase in the industrial use of cellulases. The Danish company Novozyme is one of the most important cellulase enzyme producers, providing these enzymes to the world market for industrial applications (Jayasekara and Ratnayake, 2019).

Biofuel Production

The enzymatic conversion of lignocellulosic materials into biofuels offers an eco-friendly and sustainable alternative to fossil-derived fuels. Moreover, the demand for cellulase is expected to rise significantly, driven by the expansion of commercial biofuel production industries in the near future. The use of biomass in the production of biofuel from lignocellulosic waste falls under the second generation of biofuels. Lignocellulosic biomass can be considered one of the most abundant polymers on the surface of the earth, as the annual production rate of this compound reaches approximately 200 billion tons. Lignocellulosic biomass is of great importance as a renewable carbon source that can be converted into many important products with great industrial and consumer value, such as chemical reagents and biofuel. These products play an important role in preventing environmental pollution and cleaning it from plant residues. The complex structure of lignocellulosic biomass consists of three biopolymers: cellulose (35-50%), hemicellulose (25-30%), and lignin (15-20%). The efficiency of converting lignocellulosic biomass to biofuel depends on many factors such as the amount of lignin in the biomass and the efficient depolymerization of cellulose and hemicellulose into sugars. Therefore, cellulose and hemicellulose, which constitute about two-thirds of cellulosic biomass, are preferred as potential feedstocks for second-generation biofuel production (Rodionova *et al*., 2022).

Bioactive Compounds

Many biologically active compounds can be produced by SSF using various cellulose wastes. One example is the production of tetracycline by bacteria growing on cellulose substrates. Another is the production of gibberellic acid by the fungus *Giberella fujikuroi* and *Fusarium moniliforme*, which grow on corn cobs. Oxytetracycline can also be produced by the bacterium *Streptomyces rimosus* growing on corn cobs. Additionally, the fungus *Metarhizium anisopliae* produces destruxins A and B while growing on rice husks. Finally, the fungus *A. niger* produces ellagic acid when growing on pomegranate peels (González-Aguilar *et al*., 2008).

Organic Acids

Many organic acids can be produced by transforming cellulose. These include citric acid, succinic acid, acetic acid, and lactic acid. Lactic acid, for example, is produced from lignocellulose substances through several steps. The first step is chemotherapy, which facilitates the enzymes' access to cellulose. This is followed by the enzymatic glycolysis step, which generates solutions containing glucose. The final

step is water fermentation, where microorganisms, such as bacteria from the genus *Lactobacillus*, are involved in the process (Mussatto *et al*., 2008). The method of converting cellulosic biomass to acetic acid involves an initial stage of enzymatic degradation of the substrate or acidolysis, after which yeast fermentation and oxidation to acetic acid by *Acetobactor* sp. (Ravinder *et al*., 2001). The fungus *Aspergillus niger* has been extensively utilized for acid production, using crop residues as a substrate (Vandenberghe *et al*., 2000).

Utilizing Cellulase for the Bioremediation of Lignocellulosic Wastes

There are three main components that make up lignocellulose biomass: cellulose, hemicellulose, and lignin. These contents vary depending on the plant genus, species, soil fertilization, soil fertility, and climate (Martinez *et al*., 2009). Agro-residues are currently used in modern and innovative scientific ways to produce materials with great market value. The concept of cellulose biorefining has received significant attention in recent years. This is because cellulose has unique chemical properties that make it a compound of enormous biotechnological value. Additionally, cellulose can be recycled into various products with great added value (Demirbas, 2008). Agricultural fields and natural forests contain huge amounts of cellulose that is not fully utilized or not sufficiently utilized. Failure to properly utilize and dispose of plant waste continuously will result in its accumulation in the environment, leading to pollution and potentially causing significant damage in the future.

Enzymatic Textile Waste Degradation

Cotton sludge, denim scraps, and various cotton residues from different stages of the cotton processing are examples of textile waste. The extensive waste streams produced by the cotton textile industry can be broken down by cellulase, but its application is limited by temperature and pH. It provides an alternative to landfilling or long-term storage of contaminated cellulosic waste. Under controlled conditions, cellulase is not significantly impacted by the presence of hydrocarbons, low amounts of heavy metals, lanthanides, and actinides (Chandrabose and Kumar, 2007).

Saccharification of Pretreated Hemp Biomass by Enzyme

Fiber companies generate a significant amount of cellulosic waste. The value of this material can be enhanced by utilizing the waste to produce bioenergy. Because hemp (*Cannabis sativa*) is widely used in the fiber business, it is readily available. According to Sipos *et al.* (2010), hemp is an annual herbaceous crop that has both bast fiber and a woody core. The former finds numerous uses in industry. According to Rehman *et al.* (2013), the residual woody core is usually regarded as a waste product, which makes it a perfect candidate supply of easily available, inexpensive cellulose for the synthesis of fermentable sugars that yield ethanol. *Trichoderma reesei* cellulase was employed for hydrolysis after being immobilized through covalent binding on an active magnetic support (Abraham *et al*., 2014).

Enzymatic Biodegradation of Radioactively Contaminated Cellulose

Cellulases may break down cellulose-containing sorbents and other cellulose-based wastes that are tainted with uranium or crude oil. They are used in denim manufacturing and detergent formulations. The volume of cellulose substrates contaminated with PuO_2 and lanthanide surrogates was reduced by a commercially available cellulase. After digestion, the residual radioactivity predominantly remained with the solid residue. This suggests that enzyme digestion could be a promising technique for reducing the volume of low-level and transuranic wastes (Heintz *et al*., 1999).

Treatment of Environmental Pollution

Environmental pollution is constantly increasing due to the unregulated or regulated production of harmful and hazardous substances. These substances often accumulate in the environment, making it difficult to remove them. This accumulation increases their negative impact on other living organisms, including humans. Researchers have devoted their efforts in this field to finding radical solutions that provide modern treatments, provided that they are low-cost, easy to apply, environmentally friendly, and capable of reducing or even eliminating sources of pollution in various environmental aspects, including soil, water, and air. Enzymes are among the biological agents with a great ability to decompose and remove harmful substances that pollute the environment. They do this effectively without causing harm to other forms of life and can help restore polluted environments. Scientific research has shown that enzymes have a high ability to convert some types of pollutants into harmless products, which are often beneficial. There are many types of pollutants in the environment that affect various types of life, such as humans, animals, plants, and beneficial microorganisms. Pollution sources vary from one another. They may include agricultural activities, such as plant residues, fertilizers, or pesticides. Pollution can also result from industrial activities, such as paper industries, dye industries, mining, metal processing, petrochemical products, industrial waste, or chemical weapons production. Also, the source of pollution may be due to various human activities. All of the above-mentioned pollutants may affect human and animal health and the environment in different ways. As a result, there has been a growing need and increasing interest in exploring new, effective technologies to restore contaminated sites, either partially or fully. Therefore, many modern strategies have been used and applied in this field, including engineering and biological strategies. Biological remediation strategies seem to be more acceptable than other techniques that rely on the production of compounds from living organisms, such as enzymes, the use of which represents a good and safe alternative to overcome most environmental pollutants. The most representative enzyme classes in the remediation of contaminated environments are oxidation-reduction enzymes and hydrolase enzymes such as the cellulase enzyme under study.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

In conclusion, this study successfully optimized crude cellulase production by *Fusarium oxysporum* isolates obtained from native environments. The findings highlighted the significant impact of substrate type, carbon and nitrogen sources, moisture content, temperature, incubation period, and inoculum size on cellulase yield. Among the various factors, corn cobs proved to be the most effective substrate, and CMC was identified as the best carbon source for enzyme induction. Furthermore, the study demonstrated that ammonium sulfate was the optimal nitrogen source, with an incubation temperature of 25°C and moisture content of 60% supporting maximal enzyme production. These results underscore the potential of F. oxysporum in producing cellulase for industrial applications, offering a sustainable approach to lignocellulosic biomass conversion.

Recommendations

Based on the findings of this study, it is recommended that *Fusarium oxysporum* isolates, particularly F4, be explored for large-scale cellulase production due to its superior performance in cellulase index and specific activity. The optimization of SSF conditions, such as the use of corn cobs as the substrate, moisture content around 60%, and ammonium sulfate as the nitrogen source, should be considered for maximum cellulase yield. Additionally, it is crucial to maintain an inoculum size of 1×106 spores/mL and an incubation temperature of 25°C for efficient enzyme production. Further studies could explore the scalability of these conditions in industrial applications, especially in the biodegradation of lignocellulosic waste and biofuel production.

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Conflict of Interest

The author declares no conflict of interest.

Author Declaration

The author hereby declares that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by him.

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