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Optimization of cellulase production by *Nigrospora oryzae* (berk and br.) petch and its application in biomass saccharification and ethanol production

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ABSTRACT

The increasing demand for sustainable biofuel alternatives has intensified the search for new microbial sources of cellulolytic enzymes. This study aim to evaluate the cellulolytic potential of *Nigrospora oryzae* and to optimize its cellulase enzyme production using low-cost lignocellulosic substrates, specifically maize cobs and sugarcane bagasse, under solid-state fermentation. Additionally, the study assess the efficiency of crude cellulase enzymes in biomass saccharification and bioethanol production. Molecular identification confirmed the isolate as *N. oryzae* through ITS sequencing and phylogenetic analysis. *N. oryzae* exhibited significant cellulolytic activity on carboxymethylcellulose-Congo red agar. Maize cobs and sugarcane bagasse were used as primary substrates for enzyme production. The cultural parameters were optimized using a one-variable-at-a-time (OVAT) approach. The peak filter paperase (FPase) activity reached 11.3 ± 0.94 IU/ml for maize cobs and 8.9 ± 0.47 IU/ml for bagasse on day 9. Additionally, maximum endoglucanase activity was recorded at 19.7 ± 1.74 IU/ml and 15.5 ± 0.76 IU/ml on day 12, respectively. Exoglucanase activity peaked at 3.46 ± 0.25 IU/ml for maize cobs and 2.06 ± 0.11 IU/ml for bagasse. The optimal pH for enzyme secretion ranged from 5 to 6. Nitrogen supplementation with ammonium nitrate, urea, and peptone significantly enhanced enzyme yields. Among the carbon sources tested, fructose, mannitol, and sucrose markedly improved enzyme production compared to glucose, suggesting a partial relief from carbon catabolite repression. An enzyme loading of 5% optimized saccharification efficiency. Simultaneous saccharification and fermentation (SSF) using *Saccharomyces cerevisiae* achieved maximum ethanol concentrations at substrate levels between 5% and 15%, demonstrating the bio-conversion potential of this system. These findings position *Nigrospora oryzae* as a promising non-conventional cellulase producer for lignocellulosic bioconversion, with significant implications for sustainable ethanol production.

Introduction

The increasing demand for energy, combined with urgent need to address climate change and environmental degradation, underscores the imperative shift towards sustainable and low-carbon energy alternatives. Continued reliance on fossil fuels has led to

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unprecedented levels of greenhouse gas emission, with global energy-related CO_2 emissions reaching an alarming 37.4 gigatons (Gt) in 2023, reinforcing the urgency to transition to renewable energy systems [1]. Biomass, as a renewable and carbon-neutral resource, holds vast potential for mitigating environmental impacts while contributing to energy security. Among renewable options, lignocellulosic biomass emerges as a particularly sustainable and non-competitive feedstock for liquid biofuels and value-added bioproducts, given its extensive availability and lack of overlap with food resources [2,3]

Lignocellulosic biomass such as maize cobs and sugarcane bagasse, represents industrially significant agricultural residues due to their high abundance and favorable biochemical composition. These materials are primarily composed of cellulose, hemicellulose, and lignin, with cellulose typically accounting for 35–50 % of dry biomass [4]. While their abundance makes them attractive feedstocks, the complex structure of lignocellulose limits enzymatic accessibility, creating a major bottleneck in efficient bioconversion. Overcoming this recalcitrance requires effective pretreatment strategies and robust enzyme systems capable of hydrolyzing cellulose into fermentable sugars [5].

In natural ecosystems, microorganisms degrade lignocellulose through the secretion of hydrolytic and oxidative enzymes. Microbial cellulase systems consist of three main enzyme groups that act synergistically: endoglucanases, which cleave internal bonds in cellulose chains; exoglucanases (cellobiohydrolases), which release cellobiose from chain ends; and β-glucosidases, which convert cellobiose into glucose [6]. Efficient saccharification therefore depends on the coordinated activity and balance of these enzymes. Recent studies also highlight the importance of accessory enzymes such as lytic polysaccharide monooxygenases (LPMOs), which further augment this process via oxidative cleavage, circumventing some of the limitations posed by conventional hydrolytic mechanisms [7]

Although fungal genera such as *Trichoderma* and *Aspergillus* dominate industrial cellulose enzyme production, increasing interest is being given to lesser-explore fungal species with unique enzyme potentials. *Nigrospora oryzae*, traditionally known as a plant endophyte and pathogen, has recently been recognized as a promising cellulolytic agent based on genomic evidence showing abundant carbohydrate-active enzyme (CAZyme) genes [8]. Nevertheless, the empirical verification of its cellulolytic efficacy under optimized production conditions has been limited [9].

Among fermentation methods, solid-state fermentation (SSF) has attracted significant attention for large-scale cellulase production owing to its lower water demands, efficient utilization of lignocellulosic substrates, and relatively higher enzyme productivity compared to submerged fermentation (SmF) [10–12]. For example, agricultural residues such as sugarcane bagasse and palm waste have demonstrated remarkable results in SSF setups, with enzyme yields up to 196.8 U/g for xylanase alone under certain conditions [11]. However, achieving economically viable cellulase production necessitates further optimization of process parameters, including substrate selection, moisture content, pH, temperature, incubation time, and nutrient supplementation [13].

The use of computational tools such as response surface methodology (RSM) or artificial intelligence-guided modeling to streamline this optimization process is also becoming more prominent [14]. In addition,the drive for cost-efficient enzymatic systems also incorporates approaches such as feedstock diversification, enzyme recycling, and consolidated bioprocessing (CBP) [15]. CBP integrates cellulase production, substrate hydrolysis, and fermentation processes into a single step, effectively reducing operational costs and increasing overall process efficiency [16,17].

This study aims to investigates the potential of *Nigrospora oryzae*, an endophytic fungi isolated from decaying wood, as a novel source of cellulases. Similarly, the intergration of maize cobs and sugarcane bagasse in SSF was utilized to enhance enzyme production economically and sustainability. Following enzymatic hydrolysis, the sachharified biomass hydrolysates were further evaluated for their bioethanol yield using simultaneous saccharification and fermentation (SSF) with *Saccharomyces cerevisiae*.

Materials and methods

Sample collections

Decaying wood samples were collected from Kenyatta University Main Campus, Nairobi County, Kenya (1.1811°S, 36.9272°E). Samples were placed in a sterile, well-labeled sterile polythene zip bag and transported to the Laboratory of Microbial Biotechnology, Kenyatta University within the same day of collection for processing.

Surface sterilization of sample material

Surface sterilization was performed according to the method described by Cheruiyot et al. [18]. The sample material was rinsed using sterile distilled water to eliminate any surface debris and contaminants. The sterilization procedure was carried out within a biosafety cabinet (level 2) through a sequential immersion of the samples in 70 % ethanol for three minutes, succeeded by immersion in 3.5 % sodium hypochlorite (NaOCl) for five minutes. Subsequently, the samples were rigorously rinsed five times with sterile distilled water to remove any residual sterilizing agents. To assess the efficacy of the surface sterilization, the final rinse solution was inoculated onto a potato dextrose agar (PDA) medium plate, which was then incubated for a period of 6 to 12 days at room temperature under a 12-hour light/dark cycle. The absence of fungal growth on the PDA medium plate serves as an indicator of the successful application of the sterilization protocol.

Isolation of celullase-producing fungi

Isolation was carried out following the method described by Cheruiyot et al. [18]. The sterilized samples were dried in a sterilized

biosafety cabinet, and small tissue segments, each approximately 5 mm^2, were cut using a sterilized scalpel. Five sterilized fragments (one segment per Petri plate) were evenly spaced on gentamicin-supplemented potato dextrose agar (PDA) media. The prepared plates were wrapped with Parafilm and then incubated at 28 °C for five to seven days under a regimen of 12 h of light and 12 h of darkness. Distinct fungal colonies that emerged were sub-cultured repeatedly on fresh PDA plates until pure isolates were obtained. These isolates were preserved on PDA slants at 4 °C for subsequent experiments.

Screening of cellulolytic-producing fungi

The cellulolytic acitivity of the isolated fungal colony (4 mm agar disc) was screened on carboxymethylcellulose (CMC) following a protocol described by Demissie et al. [19]. The medium contained (g/L): CMC 10, $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, yeast extract 0.2, agar 20, adjusted to pH 6.0, and autoclaved at 121 °C for 15 min. Mycelial discs (4 mm diameter) were inoculated at the center of plates and incubated at 28 °C for 5 days. After incubation, plates were flooded with 0.1 % (w/v) Congo Red for 15 min, followed by destaining with 1 M NaCl for 15 min. The formation of a clear halo around the fungal colony indicated cellulase production. The fungal isolate was then subsequently sub cultured in PDA media for morpho-molecular characterization.

Molecular identification of cellulolytic fungi

DNA extraction

Genomic DNA extraction was performed using pure culture of cellulase-producing fungi. A 200 mg sample of fungal mycelium was aseptically excised from the PDA medium using a sterile scalpel, followed by washing with sterile distilled water to remove residual PDA agar. Subsequently, the sample was freeze-dried and pulverized with a pestle and mortar in liquid nitrogen. The resultant fine powder of mycelium was employed for DNA extraction using the protocol established by Cheruiyot et al. [18].

PCR amplification and sequencing

Polymerase chain reaction (PCR) amplification of the internally transcribed spacer (ITS) regions of fungal DNA was conducted utilizing the primers ITS 4 (R) (5′TCCTCCGCTTATTGATATGC-3′) and ITS 86 (F) (5′GTGAATCATCGAATCTTTGAA-3′), as described by Kamande et al. [20], within a PCR thermocycler. A master mix of 20 µl was prepared, consisting of 12.2 µl of DNase-free H2O, 0.4 µl of the forward primer, 0.4 µl of the reverse primer, 4 µl of Taq polymerase, and 3 µl of DNA template. The PCR amplification protocol consisted of the following thermal cycling conditions: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, concluding with a final extension at 72 °C for 7 min. A holding temperature of 4 °C was maintained throughout the process.

Upon completion of the amplification, the PCR products were analyzed via gel electrophoresis utilizing a 1 % agarose gel stained with GelRed, and visualized using a UV transilluminator. Following the detection of positive bands on the gel, the amplified DNA was purified and subsequently sent to Microgen in the Netherlands for sequencing in both directions using the Sanger Dideoxy method.

Sequence alignment and phylogenetic analysis of cellulolytic fungi

Obtained ITS sequences were edited using BioEdit v7.2 and compared with related sequences using the NCBI BLASTn tool. Multiple sequence alignment was carried out with ClustalW. A phylogenetic analysis of fungal isolates' internal transcribed spacer (ITS) region was performed utsing MEGA11 software ([21]. The phylogenetic tree was generated based on a matrix of pairwise distances computed using the Neighbor-Joining method. The stability and reliability of the tree topology were rigorously assessed through bootstrap analysis, incorporating 1000 resamplings of the sequence alignment to ensure robust statistical support for the observed phylogenetic relationships [22].

Solid-State fermentation (SSF)

Substrate preparation

The production of cellulases was carried out using the locally available substrates: maize cobs and sugarcane bagasse, which served as carbon sources in the solid-state fermentation process. These substrates were washed, sun-dried and ground into a fine powder using an electric mill, then sieved with a 2 mm sieve. Five (5) grams of each substrate powder were weighed into 350 mL Erlenmeyer flasks and moistened with 10 mL of a Bushnell-Haas medium solution, which contained the following components (g/L): 0.02 $CaCl_2$, 0.2 $MgSO_4{\cdot}7H_2O$, 1 KH_2PO_4, 0.1 $FeCl_3$, 1.0 NH_4NO_3, and 1.0 peptone, with a pH of 5.3. The flasks were then sealed with cotton wool and autoclaved at 121 °C and 15 psi for 15 min.

Inoculum preparation

A four-day-old pure culture of *N. oryzae*, grown on PDA medium at room temperature, was scraped from the plate and homogenized in 10 mL sterile 0.85 % NaCl. The spore suspension was then blended using a sterile blender for 30 s and adjusted to 1×10^7 spores/mL using a Neubauer hemocytometer. This suspension was utilized as the inoculum for solid-state fermentation, based on previous findings that indicate optimal sporulation and biomass accumulation.

Fermentation of untreated maize cobs and sugarcane bagasse

The sterile substrates prepared as shown in section 2.7, were inoculated with 2 ml of the spore suspension and incubated at 28 °C for 6 days. After incubation, 50 mL of 50 mM citrate buffer (pH 4.8) was added, the flasks were shaken at 150 rpm for 2 h, and the mixture was filtered through muslin cloth. The filtrate was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant (crude enzyme extract) was collected and analyzed for cellulose activity.

Optimization of cultural conditions

Cellulase production was optimized using the one-variable-at-a-time (OVAT) approach. Various parameters evaluated to enhance cellulase production, includes: incubation times (3, 6, 9, 12, 15, and 18 h), pH levels (2, 3, 4, 5, 6, 7, and 8), nitrogen sources (such as peptone, urea, ammonium nitrate, asparagine, yeast extract, and sodium nitrate), and carbon sources (including glucose, fructose, galactose, mannitol, sucrose, and maltose. Enzyme and substrate loading were also assessed. Co-substrates (carbon sources) were supplemented at concentrations of 20 mM and 80 mM. All fermentations were performed in triplicate at 28 °C, using 5 g substrates moistened with 10 mL of a Bushnell-Haas medium solution.

Enzyme extraction and activity assay

The extraction of crude cellulase was performed as described by Kamande et al. [20], by adding 50 mL of a 50 mM citrate buffer at pH 4.8 to each fermeted samples. The flasks were vigorously agitated to facilitate enzyme release and then allowed to equilibrate at room temperature for 2 h. The mixture was filtered through muslin cloth to obtain the filtrate, which was subsequently centrifuged at 10,000 rpm for 10 min. The resulting crude enzyme extract, containing endoglucanase, FPase, and exoglucanase, was analyzed using the dinitrosalicylic acid (DNS) assay. The reducing sugars was measured at 540 nm((UV–Vis spectrophotometer, Shimadzu UV-1800). All assays were conducted in triplicate. Enzyme activity was quantified, with one unit defined as the amount of enzyme that liberates 1 μmol of reducing sugar per minute under standard assay conditions.

Concentration of crude enzymes for saccharification

The concentration process was carried out by sieving the mixture using a muslin cloth to remove larger particulate matter. The enzymes was the centrifiged at 10,000 rpm for 10 min. The resultant crude extracts were concentrated five-fold via freeze-drying, using a BIOBASE™ tabletop freeze dryer, following a methodology outlined by Kamande et al. [20]. Samples were frozen at −80 °C for 2 h before lyophilization.

Yeast inoculum preparation for ethanol fermentation

The yeast inoculum preparation for ethanol fermentation process was carried out as described by Kamande et al. [20]. Yeast activation commenced with inoculating 1 g of dried yeast (*Saccharomyces cerevisiae)* into 20 mL of a sterile 5 % (w/v) glucose solution in a 50 mL Erlenmeyer flask. The culture was incubated at 25 ± 0.5 °C with orbital shaking at 150 rpm for 24 h to facilitate optimal cellular revival. To evaluate yeast viability, a loopful of the activated yeast culture was streaked onto YPD agar plates, which were subsequently incubated at 25 °C for 24 h. The absence of contaminating microorganisms was confirmed through microscopic examination and colony morphology analysis. Cell concentration was quantified using an improved Neubauer hemocytometer with 0.4 % (w/v) trypan blue exclusion staining, effectively distinguishing between viable and non-viable cells. Before fermentation, the yeast cell concentration in the maize cob hydrolysate (20 mL working volume) was systematically standardized to 1.0 × 10^6 cells/mL through appropriate dilutions with sterile 0.9 % (w/v) saline solution.

Saccharification of maize cobs and sugar bagasse utilizing isolated fungal cellulases

The enzymatic saccharification of maize cobs and sugar baggase were conducted according to Kamande et al. [20], with modifications. Untreated maize cob/sugar bagasse particles were prepared at various concentrations (1 %, 5 %, 10 %, and 15 % w/v) and suspended in 18 mL of 50 mM citrate buffer at pH 4.8. The suspension was sterilized at 121 °C for 15 min. After cooling to ambient temperature (25 ± 1 °C), each reaction mixture received 1 mL of crude fungal cellulase, 0.5 mL of gentamicin solution (200 mg/L), and 0.5 mL of griseofulvin solution (200 mg/L) to mitigate microbial contamination, achieving a total reaction volume of 20 mL in a sterile 50 mL Falcon tubes. The experimental design encompassed positive controls comprising commercial cellulase and β-glucosidase (100 IU/mL), sourced from Sigma-Aldrich, and negative controls containing only citrate buffer. The saccharification process was executed at 25 °C under continuous agitation at 150 rpm for 72 h. Throughout the incubation interval, 1 mL aliquots were aseptically collected at 12-hour intervals (0 to 72 h), immediately centrifuged at 10,000 rpm for 5 min, and the resultant supernatants were analyzed for reducing sugar content using 3, 5-dinitrosalicylic acid (DNS) method.

Simultaneous saccharification and fermentation (SSF)

Ethanol was produced from saccharified maize cobs and sugarcane bagasse through sequential saccharification and fermentation in 50 ml Falcon tubes, as described by Kamande et al. [20]. A total volume of 25 ml was used, along with an inoculum concentration of 1 × 10^6 CFU of *Saccharomyces cerevisiae*. The substrate concentrations included 1 %, 5 %, 10 %, and 15 % (w/v), suspended in 18 ml of 50 mM citrate buffer at pH 4.8. The tubes were incubated at 25 °C with shaking (150 rpm) for 72 h, and samples collected at 0, 12, 24, 36, 48, 60, and 72 h. The ethanol concentration was measured spectrophotometrically at 600 using acidified potassium dichromate

and quantified from a standard ethanol calibration curve.

Statistical analysis

Absorbance data were converted to IU/mL using glucose standard curves. Ethanol concentrations were calculated in mg/mL. All analyses were performed in triplicate using R software (version 3.5.1). A one-way ANOVA ($p \leq 0.05$) and Tukey's HSD post hoc test were applied. The assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene's tests, respectively. Molecular sequences were analyzed via BLAST and aligned using CLUSTAL. Phylogenetic trees were constructed in MEGA X. The results were presented in tables and figures.

Results and discussion

Screening of nigrospora oryzae

In this study, the initial screening of *Nigrospora oryzae* on 1 % carboxymethyl cellulose (CMC) agar revealed significant cellulolytic potential, as shown by the distinct hydrolysis zones observed (Fig. 1). The isolate produced clear hydrolysis zones, which were confirmed through Congo red staining. These visible zones indicate the effective secretion of extracellular endoglucanase, which is capable of hydrolyzing β−1,4-glycosidic bonds in CMC. This finding is consistent with established indicators of cellulase production as reported by Roy et al. [23]. Furthermore, the Congo red assay was effective in detecting low concentrations of endoglucanase activity, resulting in the formation of visible halos around the colonies.

Molecular characterization and phylogenetic analysis of nigrospora oryzae

The fungal isolate was identified as *Nigrospora oryzae* based on molecular characterization of the internal transcribed spacer (ITS) region. Sequencing of the 574 bp ITS fragment, which includes partial regions of the 18S rRNA, complete ITS1, the 5.8S rRNA, ITS2, and partial 28S rRNA genes, revealed a significant identity of 97.5 % with reference strains of *N. oryzae* available in the NCBI GenBank (Accession: PQ896652). This high degree of sequence similarity supports its taxonomic classification within the *Nigrospora* genus [24]. It also affirms the ITS region's utility as a reliable molecular barcode for fungal identification, given its variability across species while maintaining sufficient conservation within taxa.

Phylogenetic reconstruction using the Neighbor-Joining method, supported by 1000 bootstrap replicates, illustrated strong support for the clustering of this isolate with known *N. oryzae* strains (Fig. 2). The evolutionary distances calculated using the Kimura 2-parameter model indicated 0.02–0.05 substitutions per site between this isolate and other *N. oryzae* sequences. This level of genetic divergence reflects the typical intraspecific variation observed within this genus [24]. The bootstrap values observed at critical branch nodes support the phylogenetic placement, aligning with established thresholds for confidence in fungal phylogenetic studies. In this study, both BLAST-based comparisons and tree-based phylogenetic methods were employed, confirming the species-level identity of this isolate [24].

Effect of incubation time on the production of cellulose enzymes by nigrospora oryzae

The enzymatic hydrolysis profiles of maize cobs and sugarcane bagasse demonstrated distinct patterns in cellulase production during an 18-day incubation period (Table 1). For maize cobs, the production of filter paper activity (FPase) exhibited a characteristic growth-associated pattern, peaking on day 9 at 11.3 ± 0.94 IU/ml, followed by a sharp decline to 2.8 ± 0.36 IU/ml by day 12 ($p < 0.05$). This trend aligns with established fungal cellulase production kinetics, whereby initial rapid growth is succeeded by enzyme inhibition, likely attributable to catabolite repression from the accumulation of cellobiose as supported by studies on *Trichoderma reesei*

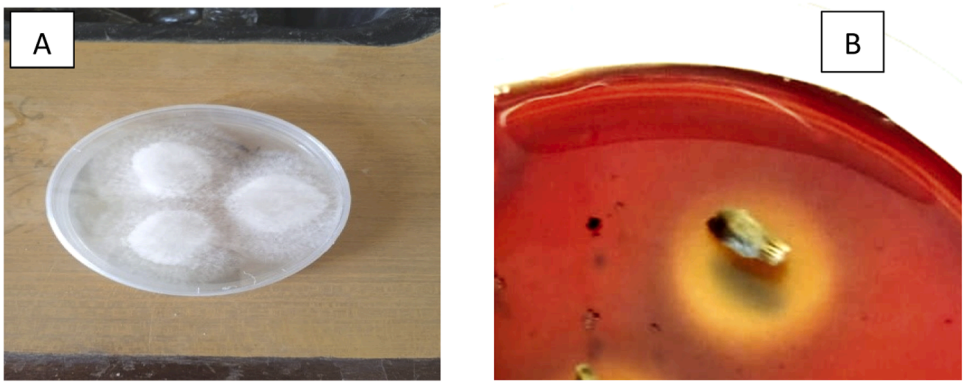

Fig. 1. Growth *of Nigrospora Oryzae* on Carboxymethyl cellulose (B) and Potato dextrose agar (A).

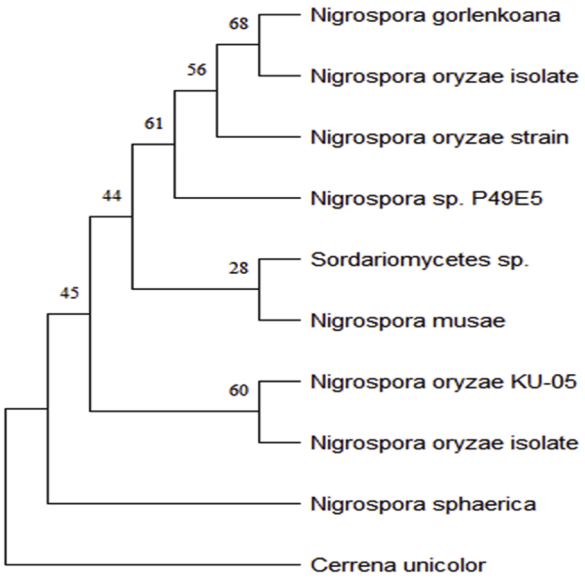

Fig. 2. Phylogenetic analysis of ITS sequence of *Nigrospora oryzae* KU-05.

Table 1
Time of incubation on FPase, endoglucanase, and exoglucanase activities.

Substrate	Day	Fpase	Endoglucanase	Exoglucanase
	3	8.3 ± 0.64^{b}	13.7 ± 1.79^{b}	3.31 ± 0.25^{a}
	6	10.3 ± 0.87^{a}	12.3 ± 1.01^{b}	3.11 ± 0.16^{a}
	9	11.3 ± 0.94^{a}	12.5 ± 1.73^{b}	3.46 ± 0.25^{a}
Maize cobs	12	2.8 ± 0.36^{c}	19.7 ± 1.74^{a}	2.19 ± 0.15^{b}
	15	4.0 ± 0.11^{c}	18.9 ± 1.44^{a}	2.53 ± 0.08^{b}
	18	4.4 ± 0.18^{c}	7.0 ± 0.16^{c}	2.42 ± 0.15^{b}
	3	8.7 ± 0.26^{a}	14.6 ± 0.97^{a}	1.77 ± 0.11^{a}
	6	8.8 ± 0.36^{a}	10.4 ± 0.72^{b}	1.80 ± 0.10^{a}
	9	8.9 ± 0.47^{a}	15.2 ± 0.74^{a}	1.72 ± 0.10^{a}
Sugarcane	12	2.8 ± 0.11^{c}	15.5 ± 0.76^{a}	1.96 ± 0.16^{a}
Bagasse	15	2.5 ± 0.05^{c}	14.1 ± 1.90^{a}	2.06 ± 0.11^{a}
	18	4.6 ± 0.04^{b}	11.1 ± 0.37^{b}	1.40 ± 0.06^{b}

Note: Treatments with different letters in the same column for each substrate are significantly different at $p \leq 0.05$ (Tukey's HSD).

[25] or the depletion of readily hydrolyzable cellulose fractions ([26]; Da Silva [27]). The 75 % reduction in Fpase activity after a peak suggests the activation of protease or the influence of end-product inhibition, phenomena that have been well-documented in *Trichoderma* species [28]. Endoglucanase activity in maize cobs exhibited a delayed peak production on day 12, recording 19.7 ± 1.74 IU/ml. This finding is consistent with the sequential expression patterns of cellulase components, where endoglucanases function synergistically with other enzymatic activities to degrade amorphous cellulose regions [29]. Literature on *Aspergillus niger* ITV02 corroborates such sequential kinetics, with cellulase components achieving optimal synergies post initial cellulose solubilization [30]. In addition, the 60 % decline in activity by day 18 may reflect either shifts in fungal metabolism or physical barriers posed by residual lignin [29].

In contrast, exoglucanase activity remained relatively stable throughout the incubation period, ranging from 2.19 to 3.46 IU/ml, with a modest peak at day 9 (3.46 ± 0.25 IU/ml), indicating coordinated yet limited production in conjunction with FPase. This trend is common in fungi like *Rhizomucor miehei*, where exoglucanase remains steady over time due to its specific targeting of crystalline cellulose domains [31]. Conversely, sugarcane bagasse manifested fundamentally different degradation kinetics. FPase maintained stable activity, ranging from 8.7 to 8.9 IU/ml, through day 9 before experiencing a gradual decline. This stability may be attributed to the uniform lignocellulose structure of bagasse, which likely provides sustained substrate availability.

Furthermore, the endoglucanase profile exhibited similar temporal dynamics as observed in maize; however, it yielded a peak activity that was 21 % lower (15.5 ± 0.76 IU/ml on day 12) and demonstrated superior preservation of residual activity (67 % remaining at day 18 versus 36 % in maize). These differences may reflect the higher silica content within bagasse, which could provide physical protection for enzymes against denaturation [32].

Effect of pH

Table 2 presents data on the activities of FPase, endoglucanase, and exoglucanase during the solid-state fermentation of maize cobs and sugarcane bagasse mediated by *Nigrospora oryzae* at various pH levels (ranging from 2 to 8), measured on days 3 and 6 of fermentation. Concerning maize cobs, the FPase activity on day 3 remained relatively stable throughout the pH range (15.9–19.0 IU/ml), with a notable peak at pH 7 (19.0 ± 1.33 IU/ml). By day 6, FPase activity exhibited a significant increase at pH levels 5 (18.6 ± 1.62IU/ml), 6 (18.8 ± 0.68 IU/ml), and 7 (18.4 ± 0.45 IU/ml), all of which were statistically higher than the values recorded at more extreme pH levels.

Endoglucanase activity was significantly enhanced at pH 6 and 7 on day 3 (39.4 ± 3.09 and 38.8 ± 0.49 IU/ml, respectively), while on day 6, the highest activity was again observed at pH 6 (36.2 ± 0.17 IU/ml). Exoglucanase activity peaked at pH 6 on both measurement days, with a substantial increase observed on day 6 (7.85 ± 0.21 IU/ml), which was significantly higher than in most other treatments. FPase activity displayed consistency across all pH levels on both days for sugarcane bagasse, revealing no statistically significant differences ($p > 0.05$). Endoglucanase activity increased with pH, attaining maximum values at pH 5 and 6 on day 6 (37.9 ± 1.00 and 38.1 ± 0.96 IU/ml, respectively). A similar trend was observed for exoglucanase activity, which reached its highest levels at pH 6 on both measurement days (8.57 ± 0.40 and 8.23 ± 0.54 IU/ml), suggesting that slightly acidic conditions are conducive to the secretion of this enzyme. Comparatively, the values recorded at pH 5 were statistically similar to those at pH 6, indicating a broader optimal range between pH 5 and 6.

The present findings indicate that pH significantly modulates enzyme production, with observable distinctions in optimal ranges contingent upon the specific enzyme and substrate. A study by Saranya and Chellapandi [33] highlighted that under solid-state fermentation, *Aspergillus oryzae* had optimal activity for FPase and endoglucanase at a pH range of 5–6 when grown on groundnut shells, similar to the present observations for *Nigrospora oryzae*. This underlines the critical role of acidic to neutral pH in stabilizing enzyme structure and promoting catalytic efficiency. Similarly, Soltero-Sánchez et al. [34] demonstrated that *Aspergillus austwickii* and *Trichoderma harzianum* produced peak cellulase activity at a pH range of 5.5–6 using sugarcane bagasse and water hyacinth as substrates, further reinforcing the present findings regarding optimal enzyme secretion at slightly acidic pH conditions.

The differential enzymatic responses between maize cobs and sugarcane bagasse are consistent with known substrate-specific physicochemical variations: Jobita et al. [35] reported enhanced exoglucanase activity at pH 6 in untreated and pretreated sugarcane bagasse and maize, consistent with the present observed values (8.57 ± 0.40 IU/mL). This was attributed to the inherent lignin and hemicellulose composition, which interacts with pH during enzyme secretion. Similarly,Haokok et al. [36] found that pH 5.5–6 favored maximum fungal growth and saccharification efficiency during sugarcane bagasse degradation.

The enhanced enzyme production observed at pH 6 is further supported by studies on engineered *Aspergillus oryzae* strains, which demonstrated peak endoglucanase and exoglucanase activities at slightly acidic conditions [37,33]. These findings suggest a conserved pH optimum for cellulolytic enzymes across fungal taxa. In addition, Sulieman et al. [38] reported that cellulase systems in *Trichoderma reesei* and *Penicillium* species exhibit optimal stability, secretion, and catalytic efficiency within a pH range of 5–6, further validating the present results.

The increase in exoglucanase activity at pH 6 may be explained by findings from Umesh and Moholkar (2025), which linked pH-mediated reductions in protease activity to enhanced cellulase longevity and catalytic turnover during sugarcane bagasse fermentation. Protease suppression at this pH plays a vital role in preserving enzymatic integrity. Additionally, Cajiao-Pedraza et al. (2025) demonstrated improved structural stability and catalytic performance of cellulases at pH 5–6 during hydrolysis of sugarcane bagasse and straw, reinforcing the optimal range identified in this study.

Table 2
FPase, endoglucanase and exoglucanase activities under different pH.

Substrate	pH	Fpase Day 3	Fpase Day 6	Endoglucanase Day 3	Endoglucanase Day 6	Exoglucanase Day 3	Exoglucanase Day 6
Maize	2	16.1 ± 1.62^{a}	13.0 ± 1.67^{c}	31.5 ± 1.76^{b}	33.4 ± 1.27^{a}	4.02 ± 0.03^{c}	5.58 ± 0.47^{c}
cobs	3	16.3 ± 1.26^{a}	13.3 ± 1.05^{c}	31.7 ± 1.33^{b}	34.5 ± 0.83^{a}	5.24 ± 0.31^{b}	5.79 ± 0.44^{bc}
	4	16.7 ± 0.96^{a}	14.6 ± 0.92^{b}	31.8 ± 1.09^{b}	34.9 ± 1.69^{a}	5.74 ± 0.41^{ab}	6.05 ± 0.35^{bc}
	5	16.9 ± 1.37^{a}	18.6 ± 1.62^{a}	34.1 ± 1.05^{ab}	35.0 ± 1.92^{a}	5.82 ± 0.06^{ab}	6.22 ± 0.78^{bc}
	6	15.9 ± 0.84^{a}	18.8 ± 0.68^{a}	39.4 ± 3.09^{a}	36.2 ± 0.17^{a}	5.95 ± 0.43^{a}	7.85 ± 0.21^{a}
	7	19.0 ± 1.33^{a}	18.4 ± 0.45^{a}	38.8 ± 0.49^{a}	32.8 ± 1.21^{b}	5.75 ± 0.59^{ab}	5.98 ± 0.16^{bc}
	8	17.2 ± 0.65^{a}	12.7 ± 0.51^{c}	32.9 ± 1.18^{b}	30.8 ± 1.49^{c}	5.58 ± 0.36^{ab}	5.04 ± 0.48^{c}
	2	17.9 ± 0.24^{a}	14.6 ± 1.17^{b}	25.3 ± 0.45^{c}	23.0 ± 1.60^{c}	5.94 ± 0.36^{c}	5.13 ± 0.68^{c}
sugar	3	18.1 ± 1.01^{a}	16.2 ± 1.18^{ab}	29.0 ± 1.32^{c}	31.1 ± 1.49^{b}	6.75 ± 0.53^{bc}	6.71 ± 0.24^{b}
bagasse	4	18.5 ± 1.58^{a}	16.7 ± 0.66^{ab}	32.6 ± 0.86^{b}	32.0 ± 1.64^{b}	6.99 ± 0.27^{bc}	6.65 ± 0.46^{b}
	5	18.6 ± 0.46^{a}	16.9 ± 0.76^{a}	35.7 ± 1.50^{ab}	37.9 ± 1.00^{a}	8.16 ± 0.87^{a}	7.98 ± 0.47^{a}
	6	18.7 ± 0.55^{a}	17.9 ± 0.91^{a}	36.0 ± 1.77^{a}	38.1 ± 0.96^{a}	8.57 ± 0.40^{a}	8.23 ± 0.54^{a}
	7	18.9 ± 1.21^{a}	16.8 ± 1.17^{a}	31.9 ± 1.27^{b}	33.2 ± 1.61^{b}	7.53 ± 0.21^{ab}	6.53 ± 0.41^{b}
	8	18.6 ± 1.31^{a}	16.3 ± 1.71^{ab}	31.2 ± 1.57^{b}	32.1 ± 1.70^{b}	6.85 ± 0.31^{bc}	6.14 ± 0.34^{b}

Note: Treatments with different letters in the same column for each substrate significantly differ at $p \leq 0.05$ (Tukey's HSD).

Table 3
Effect of nitrogen supplementation on production of cellulase enzymes by *Nigrospora oryzae*.

Day	Substrate	Nitrogen	FPase (20 mM)	FPase (80 mM)	Endoglucanase (20 mM)	Endoglucanase (80 mM)	Exoglucanase (20 mM)	Exoglucanase (80 mM)
3	Maize Cobs	Control	16.3 ± 1.08^{a}	11.2 ± 0.78^{c}	29.3 ± 1.38^{b}	21.0 ± 0.27^{c}	7.33 ± 0.29^{ab}	5.48 ± 0.09^{c}
3	Maize Cobs	(NH4)2SO4	23.6 ± 3.26^{a}	11.8 ± 0.45^{c}	24.7 ± 1.39^{bc}	38.2 ± 3.45^{a}	6.61 ± 0.48^{b}	5.11 ± 0.43^{c}
3	Maize Cobs	Asparagine	20.2 ± 1.33^{a}	20.4 ± 0.97^{ab}	35.7 ± 1.78^{a}	33.4 ± 1.93^{a}	6.92 ± 0.04^{b}	7.15 ± 0.61^{ab}
3	Maize Cobs	NaNO3	17.6 ± 1.26^{a}	19.8 ± 1.42^{b}	33.1 ± 1.19^{a}	31.4 ± 0.59^{ab}	9.66 ± 1.12^{a}	8.80 ± 0.57^{a}
3	Maize Cobs	NH4NO3	17.4 ± 1.67^{a}	24.1 ± 1.80^{ab}	45.3 ± 1.47^{a}	38.4 ± 1.60^{a}	5.14 ± 0.65^{b}	6.91 ± 0.28^{b}
3	Maize Cobs	Peptone	18.3 ± 0.76^{a}	21.2 ± 1.08^{ab}	35.0 ± 0.57^{a}	32.8 ± 1.05^{ab}	6.96 ± 0.35^{b}	7.77 ± 0.36^{ab}
3	Maize Cobs	Urea	20.1 ± 1.49^{a}	25.4 ± 0.95^{a}	33.0 ± 0.94^{a}	27.2 ± 0.84^{b}	7.58 ± 0.48^{ab}	7.34 ± 0.21^{ab}
3	Maize Cobs	Yeast Extract	17.8 ± 1.29^{a}	25.1 ± 1.18^{a}	20.4 ± 0.50^{c}	32.2 ± 1.43^{ab}	5.34 ± 0.13^{b}	8.01 ± 0.27^{ab}
3	Sugarcane Bagasse	Control	16.3 ± 0.80^{ab}	10.7 ± 0.25^{c}	23.8 ± 0.66^{b}	25.5 ± 1.60^{bc}	6.22 ± 0.23^{b}	5.36 ± 0.18^{c}
3	Sugarcane Bagasse	(NH4)2SO4	19.6 ± 1.66^{a}	23.0 ± 1.42^{a}	20.8 ± 0.98^{bc}	27.5 ± 0.94^{b}	6.33 ± 0.27^{b}	8.62 ± 0.32^{a}
3	Sugarcane Bagasse	Asparagine	11.8 ± 1.20^{c}	15.4 ± 0.90^{b}	15.7 ± 0.24^{c}	29.8 ± 0.40^{b}	6.07 ± 0.35^{b}	6.79 ± 0.30^{b}
3	Sugarcane Bagasse	NaNO3	13.6 ± 1.24^{bc}	16.4 ± 0.49^{b}	15.0 ± 0.58^{c}	19.6 ± 0.31^{c}	5.72 ± 0.50^{b}	5.42 ± 0.30^{c}
3	Sugarcane Bagasse	NH4NO3	19.6 ± 1.43^{a}	18.5 ± 0.74^{ab}	18.1 ± 0.43^{c}	42.1 ± 0.81^{a}	5.17 ± 0.10^{b}	6.02 ± 0.42^{bc}
3	Sugarcane Bagasse	Peptone	16.2 ± 0.90^{ab}	19.4 ± 0.96^{ab}	31.2 ± 0.30^{a}	21.6 ± 0.73^{c}	8.70 ± 0.65^{a}	6.80 ± 0.34^{b}
3	Sugarcane Bagasse	Urea	16.6 ± 0.97^{ab}	17.8 ± 0.49^{ab}	18.5 ± 0.92^{c}	15.4 ± 0.20^{c}	4.52 ± 0.26^{b}	5.28 ± 0.38^{c}
3	Sugarcane Bagasse	Yeast Extract	13.7 ± 0.80^{bc}	22.3 ± 1.06a	16.5 ± 0.80^{c}	25.4 ± 0.88^{bc}	5.62 ± 0.31^{b}	4.50 ± 0.24^{c}
6	Maize Cobs	Control	14.8 ± 0.83^{b}	16.2 ± 1.21^{bc}	28.7 ± 1.15^{b}	26.5 ± 0.74^{b}	5.16 ± 0.40^{b}	7.92 ± 0.46^{a}
6	Maize Cobs	(NH4)2SO4	18.1 ± 1.61^{ab}	18.5 ± 0.65^{b}	27.2 ± 1.71^{b}	35.5 ± 4.82^{a}	6.97 ± 1.32^{ab}	4.50 ± 0.25^{b}
6	Maize Cobs	Asparagine	16.7 ± 1.06^{b}	16.8 ± 0.88^{bc}	33.8 ± 1.65^{a}	25.7 ± 0.63^{b}	4.97 ± 0.06^{b}	7.38 ± 0.63^{a}
6	Maize Cobs	NaNO3	17.4 ± 1.19^{ab}	19.2 ± 0.84^{b}	27.3 ± 0.34^{b}	28.6 ± 1.35^{b}	4.45 ± 0.86^{b}	7.36 ± 0.47^{a}
6	Maize Cobs	NH4NO3	23.3 ± 1.64^{a}	24.5 ± 1.46^{a}	27.5 ± 0.89^{b}	27.3 ± 1.00^{b}	7.10 ± 0.77^{ab}	7.38 ± 0.61^{a}
6	Maize Cobs	Peptone	20.3 ± 0.85^{ab}	20.7 ± 1.22^{ab}	28.2 ± 0.58^{b}	40.1 ± 1.23^{a}	7.71 ± 0.35^{ab}	7.32 ± 0.67^{a}
6	Maize Cobs	Urea	16.5 ± 1.12^{b}	13.6 ± 0.92^{c}	42.7 ± 1.52^{a}	30.7 ± 0.93^{ab}	6.34 ± 0.37^{ab}	5.72 ± 0.27^{ab}
6	Maize Cobs	Yeast Extract	19.2 ± 1.18^{ab}	10.4 ± 0.40^{c}	26.5 ± 1.39^{b}	17.9 ± 0.48^{c}	8.96 ± 0.78^{a}	7.34 ± 0.05^{a}
6	Sugarcane Bagasse	Control	16.7 ± 1.39^{ab}	12.0 ± 0.47^{c}	24.8 ± 0.97^{b}	18.7 ± 1.18^{c}	8.21 ± 0.71^{a}	5.92 ± 0.47^{ab}
6	Sugarcane Bagasse	(NH4)2SO4	16.1 ± 0.14^{ab}	16.4 ± 0.15^{bc}	30.9 ± 1.43^{a}	24.0 ± 0.98^{bc}	6.89 ± 0.45^{ab}	7.15 ± 0.20^{a}
6	Sugarcane Bagasse	Asparagine	10.2 ± 0.46^{c}	17.1 ± 0.94^{bc}	17.0 ± 0.68^{c}	29.7 ± 0.86^{ab}	5.75 ± 0.41^{b}	6.70 ± 0.53^{ab}
6	Sugarcane Bagasse	NaNO3	13.8 ± 0.70^{bc}	12.8 ± 0.52^{c}	31.3 ± 1.28^{a}	37.3 ± 0.56^{a}	6.80 ± 0.53^{ab}	7.18 ± 0.22^{a}
6	Sugarcane Bagasse	NH4NO3	18.9 ± 0.78^{ab}	19.8 ± 0.80^{ab}	33.5 ± 0.95^{a}	18.9 ± 0.77^{c}	7.47 ± 0.37^{ab}	4.90 ± 0.48^{b}
6	Sugarcane Bagasse	Peptone	15.8 ± 0.46^{ab}	14.4 ± 0.70^{c}	22.2 ± 0.54^{bc}	28.4 ± 1.01^{ab}	7.01 ± 0.28^{ab}	5.96 ± 0.38^{ab}
6	Sugarcane Bagasse	Urea	13.6 ± 0.35^{bc}	20.8 ± 1.15^{a}	27.3 ± 1.12^{ab}	35.8 ± 1.25^{a}	5.78 ± 0.25^{b}	5.53 ± 0.48^{ab}
6	Sugarcane Bagasse	Yeast Extract	12.0 ± 0.58^{c}	17.9 ± 0.98^{ab}	30.6 ± 1.44^{a}	33.1 ± 1.19^{ab}	5.65 ± 0.30^{b}	5.29 ± 0.30^{ab}

Note: Means within the same column for each substrate and day that have different superscript letters are significantly different at $p \leq 0.05$ (Tukey's HSD).

Effect of nitrogen supplementation on production of cellulase enzymes by nigrospora oryzae

The production of fungal cellulase revealed distinct preferences for nitrogen sources, which varied significantly based on enzyme type, substrate, and cultivation time (Table 3). Notably, FPase activity in maize cobs achieved optimal yields with organic nitrogen sources, specifically urea (25.4 ± 0.95 IU/ml) and yeast extract (25.1 ± 1.18 IU/ml) at a concentration of 80 mM on day 3 These results corroborate previous studies indicating that complex nitrogen sources promote early-stage fungal growth and protein synthesis in fungi such as *Aspergillus terreus* species on agri-industrial wastes [39].

Table 4
Effect of carbon supplementation on cellulase enzyme production by *Nigrospora oryzae*.

Days	Substrate	Carbon	FPase (20 mM)	FPase (80 mM)	Endoglucanase (20 mM)	Endoglucanase (80 mM)	Exoglucanase (20 mM)	Exoglucanase (80 mM)
3	Maize Cobs	Control	16.6 ± 0.90^{ab}	11.0 ± 0.88^{c}	27.1 ± 0.79^{a}	21.0 ± 0.27^{de}	7.36 ± 0.26^{b}	5.11 ± 0.16^{c}
3	Maize Cobs	Fructose	10.2 ± 0.68^{c}	12.9 ± 0.69^{bc}	28.3 ± 1.00^{a}	25.8 ± 1.34 ^{cd}	4.99 ± 0.19^{c}	8.39 ± 0.28^{a}
3	Maize Cobs	Galactose	15.6 ± 1.15^{ab}	16.3 ± 1.45^{ab}	18.4 ± 0.89^{bc}	29.5 ± 1.51^{bc}	4.22 ± 0.14^{c}	4.95 ± 0.39^{c}
3	Maize Cobs	Glucose	12.4 ± 0.87^{bc}	10.9 ± 0.47^{c}	15.2 ± 0.72^{c}	17.1 ± 0.68^{e}	7.65 ± 0.41^{abc}	6.93 ± 0.56^{ab}
3	Maize Cobs	Maltose	14.1 ± 0.78^{abc}	18.0 ± 1.33^{a}	20.3 ± 1.30^{b}	32.8 ± 0.74^{b}	7.13 ± 0.12^{b}	6.60 ± 0.36^{abc}
3	Maize Cobs	Mannitol	17.1 ± 0.51^{a}	17.0 ± 0.51^{ab}	27.3 ± 1.22^{a}	42.6 ± 2.06^{a}	8.95 ± 0.55^{a}	5.76 ± 0.50^{bc}
3	Maize Cobs	Sucrose	15.8 ± 1.32^{ab}	13.3 ± 1.18^{abc}	21.1 ± 0.78^{b}	22.4 ± 1.69^{de}	6.51 ± 0.27^{b}	6.20 ± 0.17^{bc}
3	Sugarcane Bagasse	Control	16.3 ± 0.80^{a}	10.3 ± 0.55^{bc}	23.8 ± 0.66^{bc}	25.5 ± 1.60^{bc}	6.37 ± 0.16^{a}	4.77 ± 0.46^{a}
3	Sugarcane Bagasse	Fructose	13.4 ± 0.08^{ab}	11.4 ± 0.71^{abc}	28.1 ± 1.24^{ab}	23.7 ± 1.94^{bc}	5.71 ± 0.46^{ab}	5.31 ± 0.27^{a}
3	Sugarcane Bagasse	Galactose	15.2 ± 0.47^{a}	12.7 ± 1.03^{abc}	31.6 ± 1.17^{a}	18.3 ± 0.55^{bc}	6.87 ± 0.40^{a}	4.40 ± 0.09^{a}
3	Sugarcane Bagasse	Glucose	12.7 ± 1.28^{b}	9.18 ± 0.72^{c}	22.8 ± 1.16^{c}	21.8 ± 0.99^{c}	5.43 ± 0.39^{abc}	5.18 ± 0.22^{a}
3	Sugarcane Bagasse	Maltose	13.0 ± 0.79^{ab}	11.4 ± 0.89^{abc}	26.3 ± 1.00^{bc}	25.3 ± 2.15^{bc}	3.91 ± 0.12^{c}	4.93 ± 0.35^{a}
3	Sugarcane Bagasse	Mannitol	6.13 ± 0.40^{c}	14.8 ± 0.44^{a}	15.9 ± 1.02^{d}	30.2 ± 0.93^{ab}	5.42 ± 0.34^{abc}	4.02 ± 0.31^{a}
3	Sugarcane Bagasse	Sucrose	6.93 ± 0.22^{c}	14.3 ± 1.33^{ab}	23.9 ± 1.15^{bc}	34.6 ± 2.11^{a}	4.57 ± 0.26^{bc}	4.16 ± 0.05^{a}
6	Maize Cobs	Control	15.2 ± 0.55^{b}	16.8 ± 0.77^{bc}	25.0 ± 0.70^{b}	26.0 ± 1.12^{abc}	5.27 ± 0.34^{ab}	7.70 ± 0.57^{bc}
6	Maize Cobs	Fructose	14.2 ± 0.92^{bc}	25.1 ± 1.59^{a}	21.1 ± 1.07^{b}	31.2 ± 2.19^{a}	5.56 ± 0.14^{a}	5.18 ± 0.12^{d}
6	Maize Cobs	Galactose	13.3 ± 0.58^{bc}	13.9 ± 0.71^{cd}	25.3 ± 1.29^{b}	21.9 ± 1.48^{bc}	3.97 ± 0.09^{b}	5.62 ± 0.23^{cd}
6	Maize Cobs	Glucose	9.5 ± 0.18^{d}	13.6 ± 0.82^{cd}	13.6 ± 0.48^{c}	32.8 ± 0.98^{a}	5.60 ± 0.44^{a}	4.54 ± 0.25^{d}
6	Maize Cobs	Maltose	10.9 ± 0.86^{cd}	14.0 ± 0.95^{cd}	32.9 ± 1.43^{a}	28.5 ± 1.13^{ab}	5.25 ± 0.45^{ab}	3.77 ± 0.18^{d}
6	Maize Cobs	Mannitol	20.3 ± 0.92^{a}	19.1 ± 1.03^{b}	33.1 ± 1.51^{a}	31.7 ± 1.84^{a}	6.53 ± 0.26^{a}	8.79 ± 0.67^{ab}
6	Maize Cobs	Sucrose	23.7 ± 1.03^{a}	9.4 ± 0.53^{d}	35.5 ± 1.34^{a}	21.2 ± 1.00^{c}	6.41 ± 0.32^{a}	10.40 ± 0.89^{a}
6	Sugarcane Bagasse	Control	15.2 ± 0.55^{b}	11.5 ± 0.95^{bcd}	24.1 ± 1.48^{cd}	19.6 ± 0.76^{c}	7.23 ± 0.74^{ab}	6.17 ± 0.31^{bc}
6	Sugarcane Bagasse	Fructose	14.2 ± 0.92^{bc}	7.25 ± 0.58^{d}	18.7 ± 0.65^{d}	14.0 ± 0.83^{d}	5.83 ± 0.17^{a}	8.55 ± 0.45^{a}
6	Sugarcane Bagasse	Galactose	13.3 ± 0.58^{bc}	15.8 ± 1.06^{ab}	12.1 ± 1.15^{e}	31.3 ± 0.85^{ab}	5.09 ± 0.06^{b}	4.43 ± 0.23^{c}
6	Sugarcane Bagasse	Glucose	9.5 ± 0.18^{d}	15.1 ± 0.31^{abc}	41.9 ± 0.62^{a}	22.8 ± 1.25^{c}	7.47 ± 0.37^{a}	6.40 ± 0.26^{b}
6	Sugarcane Bagasse	Maltose	10.9 ± 0.86^{cd}	10.9 ± 1.05^{cd}	28.1 ± 1.36^{bc}	29.6 ± 1.02^{b}	7.24 ± 0.39^{ab}	5.40 ± 0.37^{bc}
6	Sugarcane Bagasse	Mannitol	20.3 ± 0.92^{a}	16.7 ± 1.43^{a}	33.0 ± 1.77^{b}	36.3 ± 2.08^{a}	6.16 ± 0.58^{ab}	5.10 ± 0.41^{bc}
6	Sugarcane Bagasse	Sucrose	23.7 ± 1.03^{a}	10.2 ± 0.65^{d}	26.5 ± 1.39^{c}	18.4 ± 0.05^{cd}	6.93 ± 0.22^{a}	5.53 ± 0.45^{bc}

Note: Means within the same column for each substrate and day that have different superscript letters are significantly different at $p \leq 0.05$ (Tukey's HSD).

In contrast, endoglucanase production exhibited different optimization requirements. Ammonium nitrate emerged as the most effective nitrogen source for maize cobs, yielding 45.3 ± 1.47 IU/ml at 20 mM, while higher concentrations (80 mM) were more effective for sugarcane bagasse. Similar findings have been reported in nitrogen studies involving *Trichoderma reseei* and *Aspergillus niger*, where ammonium salts promote metabolic flexibility depending on substrate [40–42].

The analysis of exoglucanase production unveiled more complex patterns, with ammonium sulfate demonstrating particular efficacy in sugarcane systems (8.62 ± 0.32 IU/ml). This enhanced performance may be attributed to the efficient ammonium transporter systems in fungal cells [43]. Conversely, peptone exhibited greater effectiveness in maize, suggesting a potential role for peptide-mediated regulation in the expression of exocellulases [39].

Furthermore, the temporal patterns observed, including the late-stage effectiveness of peptone for endoglucanase production (40.1 ± 1.23 IU/ml on day 6 in maize) and the early-stage enhancement of FPase by urea, align well with the nitrogen catabolite repression cycles documented in filamentous fungi such as *Trichoderma* species, which exhibit similar phase-specific responses [44]. This phenomenon illustrates that nitrogen availability serves as a regulatory factor that influences phase-specific enzyme production.

The substrate-specific responses were particularly pronounced in sugarcane bagasse, where nitrogen supplementation at 80 mM generally resulted in a more significant enhancement of enzyme production compared to 20 mM ($p < 0.05$), except FPase activity observed in maize cobs. This differential response may reflect the intricate interplay between fungal nitrogen metabolism and substrate composition, as the higher lignin content in sugarcane bagasse (18.7 ± 0.4 IU/ml) compared to maize cobs (12.3 ± 0.6 IU/ml) could significantly influence nitrogen accessibility. This observation is consistent with NCR-regulated metabolic models described [45].

Moreover, the superior performance of yeast extract across multiple enzyme types and time points underscores its role as a comprehensive nitrogen source for fungal cultivation, providing both amino acids and essential growth factors necessary for optimal enzyme production [39]

Effect of carbon supplementation on cellulase enzyme production by nigrospora oryzae

The investigation into the production of cellulase enzymes by *Nigrospora oryzae* revealed significant variations in Filter Paperase (FPase) activity in response to different carbon sources, their concentrations, incubation durations, and substrate types (Table 4). On day 3 of fermentation, the highest FPase activity was recorded with mannitol as the carbon source, particularly in treatments with maize cobs (17.1 ± 0.51 IU/mL) and sugarcane bagasse (14.8 ± 0.44 IU/mL) at an 80 mM concentration. By day 6, fructose supplementation significantly elevated FPase activity in maize cobs, achieving a peak of 25.1 ± 1.59 IU/mL at 80 mM. Mannitol and sucrose also maintained considerable FPase activity across both substrates. These findings underscore the dynamic metabolic responses of *Nigrospora oryzae* to fluctuations in carbon availability.

Endoglucanase activity presented a similar pattern, with mannitol yielding the highest enzyme activity on day 3 (42.6 ± 2.06 IU/mL in maize cobs and 30.2 ± 0.93 IU/mL in sugarcane bagasse at 80 mM). The enhanced endoglucanase activity associated with mannitol and maltose may suggest their positive influence on endoglucanase gene expression or enzyme stability. On day 6, the maximum endoglucanase activity (36.3 ± 2.08 IU/mL) was identified in sugarcane bagasse supplemented with mannitol at 80 mM, indicating that sugar alcohols may mitigate the catabolite repression effects during prolonged fermentation.

In contrast, glucose consistently led to reduced endoglucanase activity across all conditions, reinforcing the established role of glucose in carbon catabolite repression (CCR). In filamentous fungi such as *Trichoderma*, glucose inhibits cellulase gene expression through regulatory proteins like Cre1, which consequently limits enzyme synthesis in environments with elevated sugar levels [46].

Exoglucanase activity was also markedly influenced by the type of sugar supplementation. On day 3, mannitol (8.95 ± 0.55 U/mL in maize cobs) and fructose (8.39 ± 0.28 IU/mL in control samples) were identified as the most effective inducers, at concentrations of 20 mM and 80 mM, respectively. By day 6, sucrose supplementation at 80 mM resulted in the highest exoglucanase activity (10.4 ± 0.89 IU/mL) in maize cobs, while sugarcane bagasse demonstrated the best response to mannitol at 20 mM (9.64 ± 0.21 IU/mL). These findings underscore the differential regulatory effects of various carbon sources, suggesting that sugar alcohols and oligosaccharides may circumvent catabolite repression (CCR) and promote sustained enzyme synthesis.

Effect of enzyme loading on saccharification

The present investigation into the effects of enzyme concentration on saccharification efficiency elucidates distinct substrate-dependent patterns as shown in Table 5 (supplementary file). Specifically, maize cobs (MC) exhibit a progressive enhancement in sugar release correlating with increasing enzyme concentration, ultimately achieving a maximum yield of 52.9 ± 3.11 mg/ml at a 5 % enzyme load after a 72-hour incubation period. This yield signifies a substantial 37 % increase compared to the outcomes observed at a 1 % enzyme loading ($p < 0.05$). The observed linear dose-response relationship implies minimal enzyme inhibition at elevated concentrations, which is consistent with documented trends in high-solids saccharification, where enzyme loading tends to sustain yield until physical limitations manifest [47].

Furthermore, the 72-hour incubation period was optimal in analogous studies involving maize stover, indicating that over 90 % of total hydrolysis occurred within 96 h. Conversely, sugarcane bagasse (SB) demonstrated a more intricate kinetic behavior, achieving peak saccharification efficiency at a 3 % enzyme load, yielding 55.3 ± 1.65 mg/ml after 72 h. This non-linear response at higher enzyme concentrations suggests phenomena such as substrate saturation or competitive inhibition, frequently observed under high enzyme loading conditions [48]). Notably, SB displayed an 18 % higher sugar release than MC at equivalent enzyme loads, likely attributed to its more porous structure, which facilitates enhanced enzyme penetration [48].

Control experiments indicated considerable native enzymatic activity from *N. oryzae*, highlighting the robust protein content

capable of hydrolysis. Temporal profiling indicated that MC necessitated a full 72 h for complete conversion. At the same time, SB exhibited a plateau in sugar release earlier, within 48 to 60 h, reflecting substrate-dependent kinetic limitations [49].

Effect of substrate loading on the production of reducing sugars

This study evaluates how substrate concentration affects the production of reducing sugars from maize cobs (MC) and sugarcane bagasse (SB) when hydrolyzed using cellulases from *Nigrospora oryzae*, as summarized in Table 6 (supplementary). The experimental findings indicate that maize cobs demonstrate yields dependent on concentration, reaching a maximum sugar release of 17.2 ± 0.06 mg/ml at a 15 % substrate loading after 72 h. This represents a significant 24 % increase in yield compared to the 10 % loading condition ($p < 0.05$). The linear response observed suggests that mass transfer limitations are minimal up to a 15 % solid loading, which supports prior research on corn stover hydrolysis [50].

The optimization of substrate loading for saccharification reveals distinct patterns when comparing maize cobs and sugarcane bagasse treated with *N. oryzae* cellulases. Maize cobs exhibit a clear positive correlation between substrate concentration and reducing sugar yield. At a 15 % loading, a peak sugar production of 17.2 ± 0.06 mg/ml was achieved after 72 h, illustrating a significant 24 % increase over the 10 % loading condition ($p < 0.05$). This efficiency highlights the material's effectiveness in maintaining hydrolysis under high solid conditions.

In contrast, sugarcane bagasse shows an optimal substrate loading threshold of 10 %, yielding 16.7 ± 0.23 mg/ml of reducing sugars at the 72-hour mark. The observed 8 % reduction in yield at a 15 % loading ($p < 0.05$) indicates substrate-specific inhibition effects, likely due to the higher lignin content and denser cellular structure of sugarcane bagasse [48]. This non-linear response aligns with previous reports that noted viscosity-related challenges in processing bagasse, where increased solid content may hinder enzyme mobility and substrate accessibility [51].

Additionally, sugarcane bagasse displayed superior initial hydrolysis kinetics compared to maize cobs, achieving a yield of 62.7 mg/ml by 36 h at optimal loading, compared to 45.4 mg/ml for maize cobs ($p < 0.05$).

Simultaneous saccharification and fermentation of maize cobs and sugarcane bagasse using nigrospora oryzae cellulase and saccharomyces cerevisiae

The study examined the dynamics of simultaneous saccharification and fermentation (SSF) using maize cobs (MC) and sugarcane bagasse (SB) with cellulases derived from *Nigrospora oryzae* and the yeast *Saccharomyces cerevisiae*, as detailed in Table 7 (supplementary). The results indicated significant variations in fermentation performance depending on the substrate used. For maize cobs, optimal ethanol production was achieved with a 15 % substrate loading and an enzyme dosage of 10 FPU. This resulted in a peak ethanol concentration of 11.0 ± 0.29 mg/mL after 72 h. This finding represents a statistically significant increase of 20 % compared to the treatment with 5 FPU ($p < 0.05$), suggesting that higher enzyme dosages can effectively reduce substrate recalcitrance in high-solids conditions. A strong positive correlation ($R^2 = 0.94$) was observed between substrate concentration and ethanol yield, indicating that the porous structure of maize cobs helps overcome mass transfer limitations typically seen in high-solids fermentation systems [52]. Notably, with the application of 10 FPU, maize cobs achieved a conversion efficiency of 82 % relative to the theoretical maximum, with a specific production rate of 0.21 mg/mL/h during the initial 48-hour period. In contrast, sugarcane bagasse exhibited different fermentation kinetics and optimal loading parameters. The maximum ethanol concentration recorded was 11.7 ± 0.64 mg/mL at a 10 % solids concentration with the enzyme dosage of 5 FPU after 72 h. Increasing the substrate load to 15 % led to a 5 % decrease in ethanol output ($p < 0.05$), likely due to the accumulation of inhibitory compounds, such as phenolic derivatives or furans, produced during biomass pretreatment [53].Additionally, SB fermentation systems reached a plateau in ethanol production by 60 h, while MC continued to accumulate ethanol until the 72-hour mark. This early plateau in the SB fermentation process may be attributed to its higher intrinsic free sugar content (14.2 % compared to 9.6 % in MC), which accelerates initial fermentation but may also induce ethanol-induced feedback inhibition more quickly.

Conclusion

This study highlights the significant potential of *Nigrospora oryzae*, an endophytic fungus found in decaying wood, as an effective producer of cellulase enzymes through solid-state fermentation. Utilizing agro-industrial residues, such as maize cobs and sugarcane bagasse, serves as a cost-effective substrate while promoting high enzyme yields. The optimization of key parameters such as incubation time, pH, nitrogen and carbon source supplementation has greatly improved the activity of cellulolytic enzymes, showcasing the metabolic versatility of *N. oryzae*. The successful application of enzymatic hydrolysates in simultaneous saccharification and fermentation (SSF) with *Saccharomyces cerevisiae* further underscores the fungus's potential in valorizing lignocellulosic biomass for second-generation bioethanol production. Future research should aim at scaling up the process, enhancing genetic traits, and conducting proteomic profiling to further optimize enzyme yields and improve robustness for industrial applications.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors employed Grammarly to enhance the clarity of certain sentences. Following the use of this tool/service, the authors diligently reviewed and edited the content as required and accept full responsibility for the publication's content.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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