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Characterization of Xylanase and Cellulase Produced by a Newly Isolated *Aspergillus fumigatus* N2 and Its Efficient Saccharification of Barley Straw

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Abstract *Aspergillus fumigatus* N2 was isolated from decaying wood. This strain produces extracellular xylanases and cellulases. The highest xylanase (91.9 U/mL) and CMCase (5.61 U/mL) activity was produced when 1% barley straw was used as the carbon source. The optimum pH and temperature for xylanase activity were 6.0 and 65 °C, respectively. CMCase revealed maximum activity at pH 4.0 and in the range of 65 °C. The FPase was optimally active at pH 5.0 and 60 °C. The zymograms produced by the SDS-PAGE resolution of the crude enzymes indicated that multiple enzymes were secreted into the fermentation supernatant. Five bands of proteins with xylanase activity and four bands of proteins with endoglucanase were observed in the zymogram gel. The crude enzymes were used in the barley straw saccharification; an additive effect was observed when the commercial cellulase was added as supplement.

Keywords Xylanase · Cellulase · *Aspergillus fumigatus*

Introduction

Lignocellulosic biomass is an important renewable feedstock that has the potential to be converted into a number of different chemicals, fuels, or other value-added products [1].

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Enzymatic hydrolysis is an ecofriendly method of converting lignocellulosic biomass into desired end products. For efficient degradation of lignocellulosic biomass, cellulolytic and xylanolytic enzyme complexes are desired [2]. However, the high cost of commercial enzymes creates a bottleneck for bioconversion. Various strategies are used to decrease the cost of the hydrolysis process, such as using crude extract enzymes, improving enzyme activity, using enzyme cocktails, and maximizing enzyme production [3]. Using crude enzyme extracts can save purification costs and avoid the loss of enzyme activity during the concentration and purification processes. Numerous studies have utilized crude enzyme extracts or blends of crude enzyme extracts to increase enzyme synergy and improve hydrolysis efficiency [4]. To meet the requirements of the bioconversion, more attention has been focused on isolating new microorganisms that can produce compound enzymes to degrade the hemicellulose and cellulose of biomass.

The objective of this research was to isolate and characterize an *Aspergillus* fungus that is capable of producing extracellular xylanases and cellulases. The hydrolysis enzymes produced by *Aspergillus fumigatus* N2 were characterized. The low-cost natural residues were employed as the sole carbon source to produce cellulases and xylanases by *A. fumigatus* N2. The crude extract enzyme was used to evaluate the hydrolytic efficiency of untreated barley straw by performing saccharification assays and measuring the reducing sugar released from barley straw.

Methods

Isolation and Identification of *A. fumigatus* N2

A. fumigatus N2 was isolated from decaying wood (Thunder Bay, Ontario, Canada). Five grams of decaying wood was suspended in sterile water. After serial dilutions, the mixtures were spread on agar plates of medium containing beechwood xylan (5 g/L), $MgSO_4 \cdot 7\ H_2O$ (0.2 g/L), KH_2PO_4 (1 g/L), $K_2PO_4 \cdot 2H_2O$ (1.3 g/L), NH_4NO_3 (1 g/L), $FeSO_4 \cdot 7H_2O$ (50 mg/L), $CaCl_2$ (20 mg/L), and agar (15 g/L). The plates were incubated at 30 °C for 3–4 days. The colonies that grew well and showed a clear halo zone were selected and purified. After comparing the xylanase activity of each strain, the N2 strain was selected for further study. Strain N2 was maintained on potato dextrose agar (PDA) medium. N2 genomic DNA was extracted, and the internal transcribed spacer (ITS) region was amplified using the universal primer sets ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTG ATATG). The PCR products were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) comparison. The ITS sequences of *A. fumigatus* N2 have been deposited in GenBank under accession number KX611126. Multiple alignments were performed using Clustal X1.83, and a phylogenetic tree was constructed using MEGA 6.0 [5].

Preparation of Agricultural Residues

A conidial suspension of strain N2 was harvested from a 6-day-old PDA plate incubated at 30 °C and suspended in sterile water. A 10^7 conidia/mL suspension was used for inoculation. For submerged fermentation, 50 mL of a mineral salt medium (3.0 g of KH_2PO_4, 3.0 g of $NaNO_3$, 0.5 g of $CaCl_2$, 0.5 g of $MgSO_4 \cdot 7\ H_2O$, 7.5 mg of $FeSO_4 \cdot 7H_2O$, 2.5 mg of $MnSO_4 \cdot H_2O$, 2.0 mg of $ZnSO_4$, and 3.0 mg of $CoCl_2$ in 1000 mL of water) was supplemented

with 1% (*w*/*v*) of the agricultural residues (such as pine sawdust, agave, wheat bran, xylan, or barley straw collected from local farms) in 250-mL flasks. Fresh conidia suspensions of strain N2 were inoculated at a ratio of 1:50 in the medium and incubated at 30 °C for 7 days.

Enzyme Assay

After the culture had been harvested, the liquid media were centrifuged at 10,000 g for 10 min, and the supernatants were used as a crude enzyme for the analysis of xylanase and cellulase activity. Beechwood xylan, carboxymethyl cellulose, and Whatman No. 1 filter paper were purchased from Sigma-Aldrich. Xylanase activity was measured using a 1% (*w*/*v*) beechwood xylan solution as substrate. The released reducing sugar from beechwood xylan was measured as a xylose equivalent using the 3,5-dinitrosalicylic acid (DNS) method [6]. One unit of xylanase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute. The CMCase activity was determined according to Ghose [7], using a 2% (*w*/*v*) carboxymethyl cellulose (CMC-Na) solution in reaction buffer. Filter paper cellulase activity was measured according to IUPAC recommendations, and Whatman No. 1 filter paper was used as substrate [7]. The release of reducing sugars was measured as glucose equivalent using the DNS method. One unit of cellulase activity was defined as the amount of enzyme required to liberate 1 μmol of glucose per minute under the assay conditions. Relative activity is the percentage of the residual activity of the treated enzyme to the untreated enzyme.

SDS-PAGE and Zymogram Analysis

SDS-PAGE was performed using a 10% (*w*/*v*) polyacrylamide gel containing a 0.1% (*w*/*v*) substrate of target enzyme. Supernatants from the submerged fermentation were mixed with loading buffer and boiled at 100 °C for 40 s prior to electrophoresis. After the separation of the enzyme samples, the gel was divided into two parts. The part of the gel that contained the samples and molecular markers was stained in Coomassie Brilliant Blue R-250. The other part of the gel was soaked for 30 min in 1% (*v*/*v*) Triton X-100 to remove the SDS and allow refolding of the enzyme in the gel. The gel was incubated at 55 °C for 30 min to detect enzyme activity, then submerged in 0.1% (*w*/*v*) Congo red solution for 30 min and de-stained with 1 M of NaCl until pale-red hydrolysis zones appeared against a red background. Dipping the gel in a 4% (*v*/*v*) acetic acid solution stopped the reaction.

Influence of Temperature and pH on Crude Enzyme Activity

To determine the optimum temperature for the crude enzymes, the temperature range was varied from 40 to 80 °C. The optimal pH for the activity in the crude enzyme samples was detected by incubating the crude enzyme mixture with 1% (*w*/*v*) substrate dissolved in an appropriate buffer (0.05 M glycine-HCl buffer for pH 2.0; 0.05 M citric acid-Na_2HPO_4 buffer for pH 3.0–7.0; 0.05 M sodium phosphate buffer for pH 8.0; and 0.05 M glycine-NaOH buffer for pH 9.0).

Enzymatic Hydrolysis

Cellulase (Celluclast 1.5L, Novozymes, Franklington, USA) from *Trichoderma reesei* and β-glucosidase (Novozyme 188, Novozymes, Bagsvaerd, Denmark) from *Aspergillus niger* were

used. *A. fumigatus* N2 was cultivated under submerged fermentation using barley straw as the sole carbon source. The supernatants were used as crude enzymes after 4 days of fermentation.

Enzymatic hydrolysis was performed on 1% (*w*/*v*) barley straw in 50 mM of citrate buffer using 250-mL Erlenmeyer flasks, controlled at 50 °C and 200 rpm by a thermostat shaker. Barley straw without pretreatment was used as the substrate.

Three sets of hydrolysis were carried out. In one case, 200 μL of cellulase (Celluclast 1.5L) and 100 μL of β-glucosidase (Novozyme 188) were loaded into hydrolysis barley straw. The second treatment was hydrolysis barley straw with 2 mL of crude enzymes of *A. fumigatus* N2 and 100 μL of β-glucosidase (Novozyme 188). To investigate the synergism between cellulase and crude enzymes during barley straw hydrolysis, 2 mL of crude enzyme was supplemented to 200 μL of cellulase (Celluclast 1.5L) and 100 μL β-glucosidase (Novozyme 188). At intervals of 12 h, the supernatants were collected and the amount of reducing sugars was analyzed with the dinitrosalicylic acid method, using glucose as a standard.

Results

Identification of the N2 Strain

The fungal strain N2 was selected as the most active xylanase producer among the isolated strains. Its identification was made on the basis of the sequence variation present in the ITS region. The ITS of N2 was amplified and sequenced. The ITS sequence has been deposited in the NCBI GenBank with the accession number KX611126. The sequence was compared using BLAST analysis, and the closely related sequences were selected and analyzed with molecular evolutionary software MEGA 6.0. The phylogenetic tree was constructed, which confirmed that strain N2 exhibited 100% similarity with *A. fumigatus* (Fig. 1). The strain N2 was identified as *A. fumigatus* and designated as *A. fumigatus* N2.

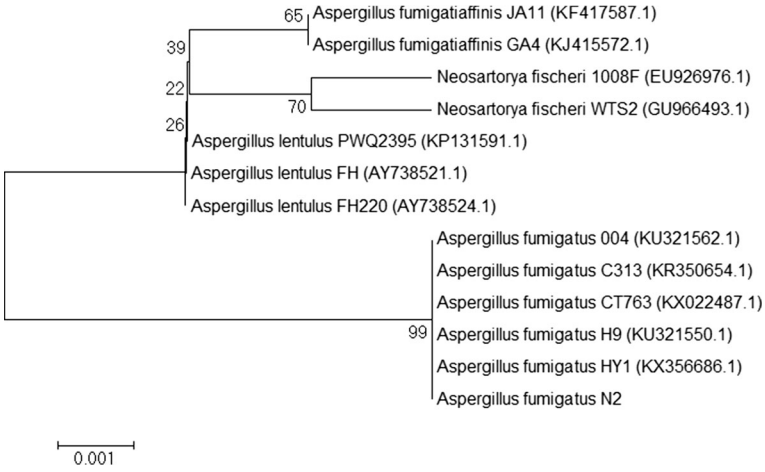

Fig. 1 The phylogenetic tree of ITS rRNA sequences of *Aspergillus fumigatus* N2 and related species based on a neighbor-joining analysis. Bootstrap values are expressed as percentages of 1000 replications. The *scale bars* represent 0.001 substitutions per nucleotide position. The numbers that follow the names of the strains are the accession numbers of published sequences

Effect of Different Carbon Sources on the Production of Hydrolysis Enzymes

The effects of different carbon sources on extracellular enzyme activity were tested. Wheat bran, barley straw, xylan, pine saw dust, and agave were used as sole carbon sources, and xylanase and cellulase activity was determined. The highest level of xylanase was observed with barley straw, reaching its maximum (91.9 U/mL) on day 4 and gradually diminishing thereafter, probably because the xylanases were degraded by proteolytic enzymes after 4 days. *A. fumigatus* N2 grown well in the media containing wheat bran, but the xylanase activity was lower than fermentation in the barley straw media. The maximum xylanase activity from xylan media was lower than the enzyme production from wheat bran and barley straw. However, the xylanase activity remained stable after reaching the highest activity in xylan media. Negligible xylanase activity was achieved in agave and pine saw dust media (Fig. 2).

The highest CMCase and filter paper activities were detected in medium containing barley straw. The peak endoglucanase activity was observed on the fourth day (5.61 U/mL) and the third day (0.77 U/mL) for filter paper activity in the barley straw medium. Lower CMCase and filter paper activities were detected when wheat bran or xylan was used as the carbon source. Negligible filter paper activity was achieved in the medium containing a carbon source of agave or pine saw dust. Barley straw was the best carbon source for *A. fumigatus* N2 for the production of xylanase and cellulase (Figs. 3 and 4).

SDS-PAGE and Zymogram Analysis of the Expression of Multiple Hydrolysis Enzymes

The zymogram of the crude enzyme samples from cultures containing barley straw revealed five protein bands with xylanase activity evident from *A. fumigatus* N2. The molecular weights of the three bright bands were approximately 20, 30, and 55 kDa (Fig. 5a).

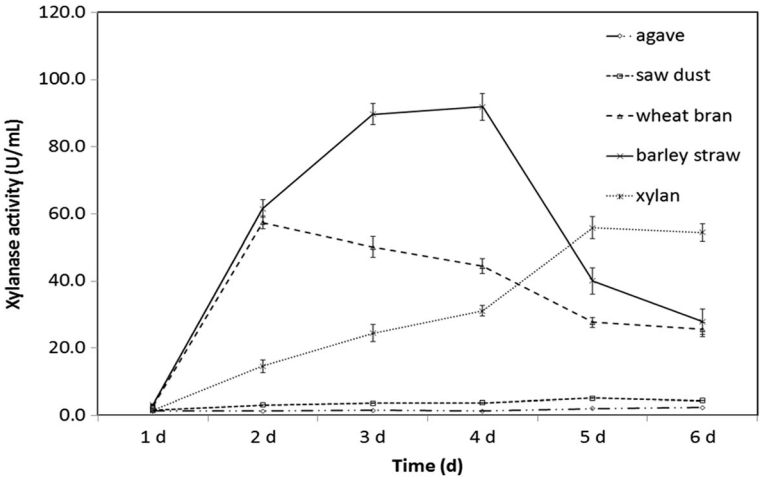

Fig. 2 Xylanase activity of *A. fumigatus* N2 with different agricultural residues as substrate in submerged fermentation with shaking for 6 days at 30 °C. Data are the mean of three replicates, and *bars* indicate the standard deviation of these three replicates

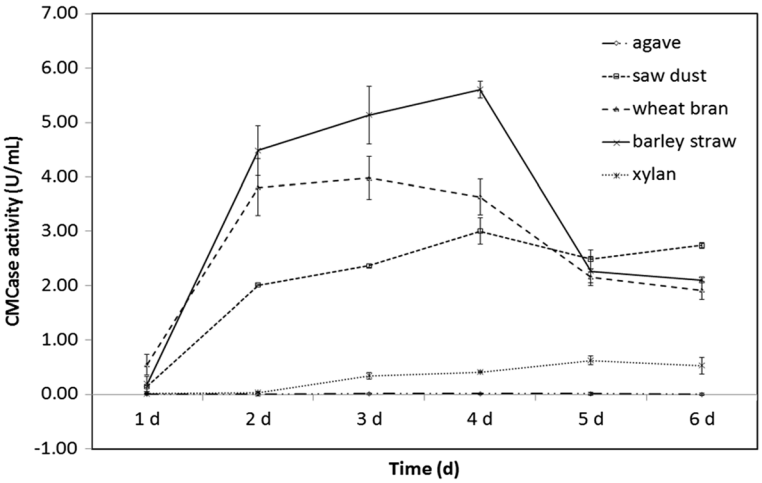

Fig. 3 CMCase activity of *A. fumigatus* N2 with different agricultural residues as the substrate in submerged fermentation with shaking for 6 days at 30 °C. Data are the mean of three replicates, and *bars* indicate the standard deviation of these three replicates

The boiled crude enzymes from the supernatant of the barley straw medium showed four protein bands with endoglucanase activity. The highest endoglucanase activity was observed at approximately 20 kDa, and the lower three bands were around 40, 45, and 100 kDa (Fig. 5b).

Effects of Temperature and pH on Enzyme Activity

It was observed that the optimum temperature was 65 °C for the xylanase. Xylanase activity rapidly decreased with increased temperature, and the xylanases were almost denatured at 80 °C. The optimum temperature for CMCase activity was in the range of 65 °C. The CMCase

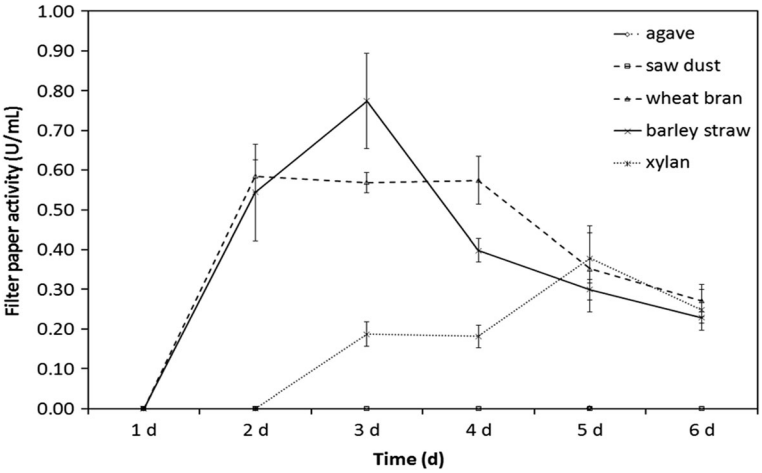

Fig. 4 FPase activity of *A. fumigatus* N2 with different agricultural residues as the substrate in submerged fermentation with shaking for 6 days at 30 °C. Data are the mean of three replicates, and *bars* indicate the standard deviation of these three replicates

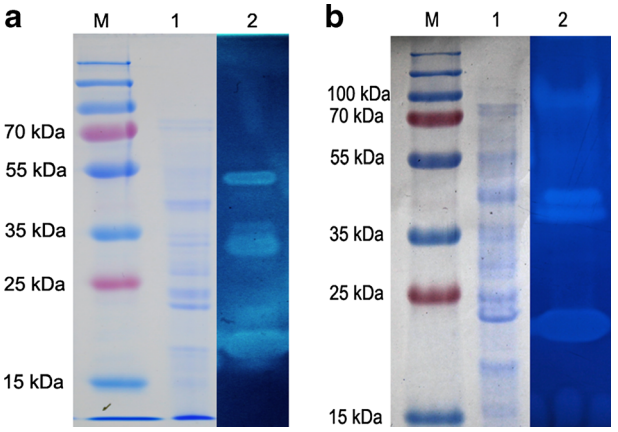

Fig. 5 SDS-PAGE and zymogram of the supernatant expression of *A. fumigatus* N2. **a** Xylanase zymogram. Coomassie blue staining of the SDS-PAGE gel (*lane 1*), Congo red staining of the SDS-PAGE gel containing 0.1% (*w/v*) beechwood xylan (*lane 2*), and standard protein molecular weight markers (*M*). **b** Endoglucanase zymogram. Coomassie blue staining of the SDS-PAGE gel (*lane 1*), Congo red staining of the SDS-PAGE gel (*lane 2*), and standard protein molecular weight markers (*M*)

activity was less than 50% at below 40 °C or over 75 °C. The maximum FPase activity in the crude enzymes was detected at 60 °C but lost more than 60% of activity at 70 °C (Fig. 6).

The crude enzymes exhibited the highest xylanase activity at pH 6.0, and significantly decreased enzyme activity was observed at pH <5.0 or pH >7.0. The optimum pH was 4.0 for the CMCase activity and 5.0 for the FPase activity (Fig. 7).

Effect of Crude Enzymes on the Enzymatic Hydrolysis of Barley Straw

In order to assess the hydrolysis efficiency of the crude enzymes from *A. fumigatus* N2, hydrolysis experiments were carried out to incubate the crude enzymes and commercial cellulase with barley straw, both alone and in combination. The incubations were both performed with supplementation of β-glucosidase.

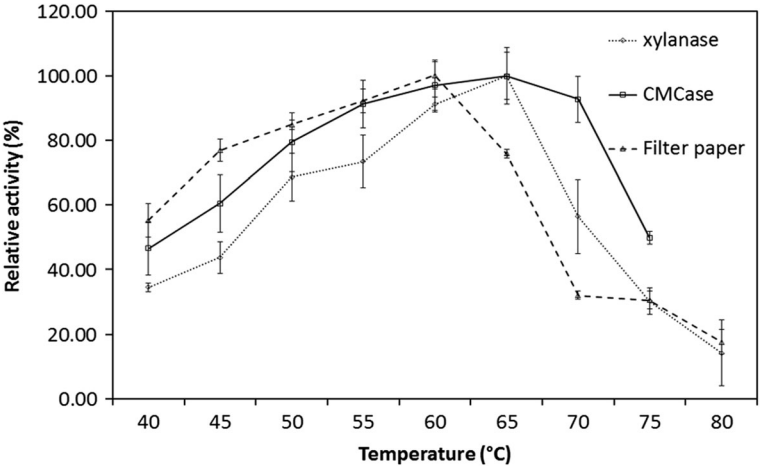

Fig. 6 Effect of temperature on enzyme activity. The optimal temperature for enzyme activity was determined by measurement of its activity at temperatures ranging from 40 to 80 °C. Data are the means of three replicates, and the *bars* indicate the standard deviation of these three replicates

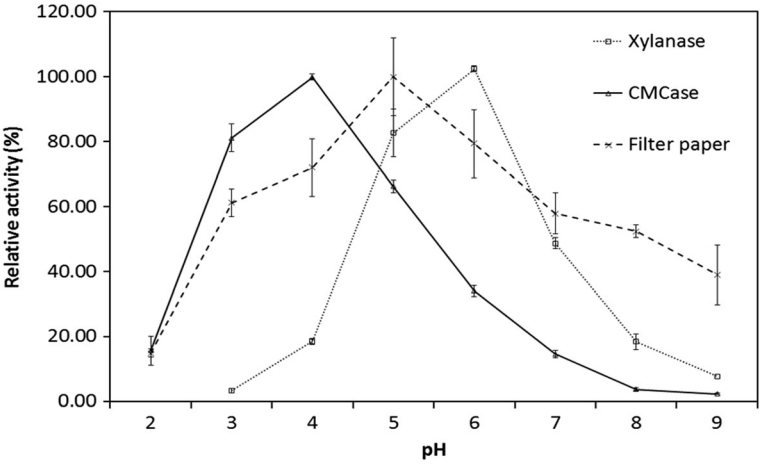

Fig. 7 Effect of pH on enzyme activity. The optimal pH for enzyme activity in the crude enzymes was determined at a pH range of 2.0 to 9.0. Data are the means of three replicates, and the *bars* indicate the standard deviation of these three replicates

The crude enzyme supplementation with commercial cellulase resulted in the highest production of reducing sugars. The lowest production of reducing sugars was from hydrolysis by crude enzymes (Fig. 8). It appears that the supplementation of the crude enzymes with commercial cellulases increased the yield of reducing sugars. The amount of reducing sugars from the combination of cellulase and the crude enzymes was similar to the sum of reducing sugars from the hydrolysis of individual enzymes. An additive effect was observed in the barley straw degradation experiment when the crude enzymes were supplemented by commercial cellulase.

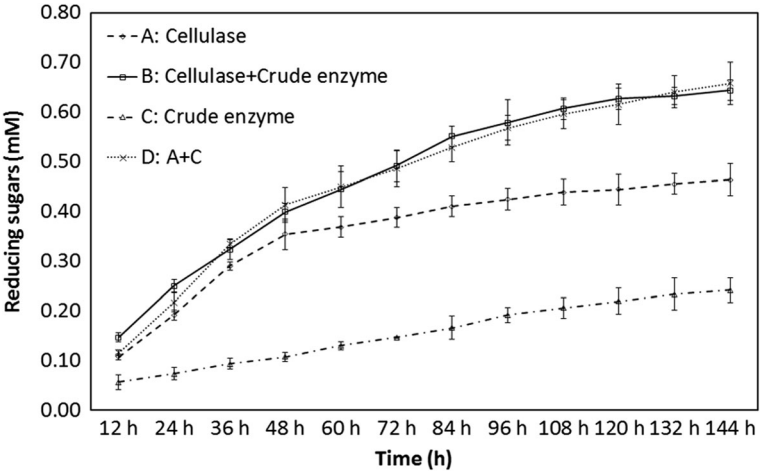

Fig. 8 Enzymatic hydrolysis of barley straw. **a** Hydrolysis with commercial cellulases and β-glucosidase; **b** hydrolysis with commercial cellulases, N2 crude enzyme, and β-glucosidase; **c** hydrolysis with N2 crude enzyme and β-glucosidase; and **d** the sum of **a** and **c**

Discussion

In this study, a lignocellulolytic enzyme-producing fungus was isolated from decaying wood and identified as *A. fumigatus* N2. Enzyme production experiments were carried out using agricultural residues as sole carbon sources. Maximal activity of xylanases and cellulases was obtained when barley straw was used as the sole carbon source. In wheat bran medium, *A. fumigatus* N2 could grow fast but failed to produce high enzyme activity. This is most likely because wheat bran contains a high content of starch and protein, thus serving as both a carbon and a nitrogen source. Starch from wheat bran is easier to degrade into usable carbohydrates for *A. fumigatus* N2 growth, compared to hemicellulose and cellulose. The enzyme titer was much lower compared to barley straw or wheat bran when beechwood xylan was used as the sole carbon source. The carbon source usually provides some compounds to induce the enzymes' expression in fermentation [8]. Barley straw has high hemicellulose content. It is easier to degrade, releasing monosaccharides, providing a carbon source for *A. fumigatus* N2, and inducing lignocellulolytic enzyme expression. Barley straw is regarded as a low-cost agricultural residue, and its use is still limited. This study indicates that barley straw can be applied as a low-cost carbon source in the lignocellulolytic enzyme production of *A. fumigatus* N2.

Many Aspergillus species were reported to produce cellulases and xylanases. The difference is that most strains of *A. fumigatus* produce more enzymes in wheat bran media than in wheat or rice straw media [9]. But *A. fumigatus* N2 might prefer barley straw as carbon source. The xylanase and cellulase activities obtained were higher than those produced by *A. fumigatus* NITDGPKA3 without optimizing the culture condition. After enhancing culture condition, enzyme production by *A. fumigatus* NITDGPKA3 dramatically increased and maximum xylanase increased to 193.58 U/mL in fermentation broth [9]. Cellulase and xylanase production by *A. fumigatus* N2 can be expected to greatly improve after optimizing fermentation condition, carbon source, and nitrogen source. Zymography analyses showed that there were four different CMCases and five xylanases in the fermentative supernate of *A. fumigatus* N2. Such multiple forms of cellulolytic and xylanolytic have also been reported in different fungi strains such as *Penicillium ramulosum* N1 [10], *Aspergillus* sp. *Clostridium*, and *Streptomyces* sp. [11]. Biomass-degrading microorganisms usually secrete multiple cellulolytic and xylanolytic enzymes to improve hydrolysis efficiency for complex lignocellulosic biomass. Those kinds of microorganisms have potential value to produce compound enzymes for hemicellulose and cellulose bioconversion. Similar results were found in lignocellulosic-degrading fungi, including *A. fumigatus* SK1 [12], *Penicillium brasilianum* [13], *P. ramulosum* N1 [10], and *Penicillium echinulatum* S1 M29 [14].

The saccharification of cellulose and hemicellulose in biomass, which results in sugar-rich liquid streams, is the key step of the bioconversion [15]. Xylanases can help to break down hemicellulose and provide greater access to cellulose. In the present study, saccharification of barley straw was performed to investigate the activities of the different enzymatic complexes. The highest yield of reducing sugars was obtained from the enzyme cocktail containing the crude enzymes, commercial cellulases, and β-glucosidase. The sums of reducing sugars from crude enzymes or commercial cellulase hydrolysis were similar to those from the enzyme cocktail (crude enzymes plus commercial cellulases). The additive effect was observed in barley saccharification with the enzyme cocktail. Xylanase supplementation can facilitate cellulose saccharification by improving cellulose accessibility. Synergistic action is usually found when xylanases are supplemented with cellulases during biomass saccharification [16]. The enzyme cocktail in our experiment showed no significant synergistic effect. This is mainly because the barley straw

application in the saccharification was without pretreatment. A pretreatment step can break down the lignin structure and disrupt the crystalline structure of cellulose for improving enzyme accessibility to the substrate [17]. Limited substrate accessibility causes inefficient hydrolysis. The reduction of pretreatment severity is helpful to reduce economic costs, but a low severity factor results in less sugar release, or more hydrolysis enzymes will be required. Efforts at cost reduction need to balance the costs of pretreatment and hydrolysis enzymes [18]. Future studies will investigate the saccharification efficiency of different pretreatments for barley straw with different enzymatic complexes of crude enzymes and cellulases.

Conclusions

The results of this study indicate that *A. fumigatus* N2 can produce multiple xylanases and cellulases. Barley straw was found to be a well-suited carbon source for submerged fermentation to produce high levels of lignocellulolytic enzymes. The saccharification of unpretreated barley straw was investigated with different enzymatic complexes. The synergistic effect was not showed when commercial enzymes were supplemented. Future research needs to investigate the relationship between pretreatment and saccharification efficiency.

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