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Newly Isolated *Penicillium ramulosum* N1 Is Excellent for Producing Protease-Resistant Acidophilic Xylanase

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Key Words
Xylanase · Protease resistance · *Penicillium ramulosum* · Cellulase

Abstract
Penicillium ramulosum N1 was isolated from decaying wood. This strain produces extracellular xylanases and cellulases. The highest activities of xylanases (250 U/ml) and carboxymethyl cellulose (CMCase; 6.5 U/ml) were produced when 1% barley straw was added as a carbon source. The optimum temperature and pH for xylanase activity was 55 and 3.0°C, respectively. The xylanases exhibited strong protease resistance. CMCase revealed maximum activities at pH 3.0 and in the range of 60–70°C. Filter paper activity was optimally active at pH 5.0 and 55°C. The zymograms produced by the SDS-PAGE resolution of the crude enzymes indicated that there are four bands of protein with xylanase activity and three bands of proteins with endoglucanase. The results revealed that *P. ramulosum* N1 is a promising acidophilic and protease-resistant xylanase-producing microorganism that has great potential to be used in animal feed and food industry applications.

Introduction

Xylan is the major component of hemicellulose in plant cell walls and is the second most abundant polysaccharide in nature [Collins et al., 2005]. Xylanases can cleave the β-1,4-xylosidic linkages in xylan to generate some value-added products. Thus, xylanases have potential applications in various industrial processes, such as animal feed, xylitol production, the food industry and pulp biobleaching [Polizeli et al., 2005].

Many microorganisms, including bacteria, fungi and yeasts, have been reported to produce xylanases. However, most of the xylanases characterized show optimal activity at slightly acidic pH values (pH 5.0–6.0) and at temperatures between 40 and 70°C [Dutta et al., 2007]. Specific applications of xylanases usually require one or more significant enzyme properties, such as acidophilic xylanases for animal feed [Knob and Carmona, 2010] and thermoalkaliphilic xylanase for the paper and pulp industry [Collins et al., 2005]. To meet the needs of the industry, more attention has been focused on enzyme stability under extreme conditions.

In the present paper, the *Penicillium ramulosum* N1 was isolated from decaying wood. This organism produc-

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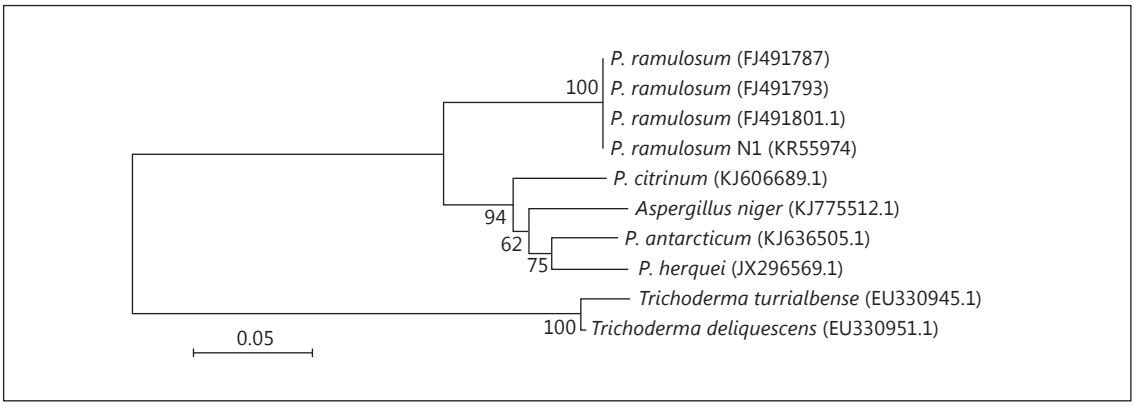

Fig. 1. The phylogenetic tree of ITS rRNA sequences of *P. ramulosum* N1 and related species based on a neighbor-joining analysis. Bootstrap values are expressed as percentages of 1,000 replications. The scale bar represents 0.05 substitutions per nucleotide position. The numbers that follow the names of the strains are the accession numbers of published sequences [Bailey et al., 1992; Knob and Carmona, 2010; Peterson et al., 2011; Polizeli et al., 2005].

es acidophilic and protease-resistant xylanases. The xylanolytic enzymes produced by *P. ramulosum* N1 were characterized and are suggested to be quite promising for industrial applications.

Results

Identification of the N1 Strain

The fungal strain N1, which was isolated from decaying wood, was primarily identified to be a xylanolytic enzyme-producing fungus on xylan medium. Identification of the strain was made on the basis of the sequence variation present in the internal transcribing spacer (ITS) region. The ITS of N1 was amplified and sequenced. Sequence data were aligned and analyzed to identify the closest homologs with a submitted sequence in the NCBI database. The sequences, which shared over 98% similarity with currently available sequences, were considered to be the same species. According to the phylogenetic analysis of the ITS rRNA sequence, the N1 strain was identified as *P. ramulosum* and designated as *P. ramulosum* N1 (fig. 1). The ITS sequence has been deposited in the NCBI GenBank with the accession number KR55974.

Effect of Different Carbon Sources on the Production of Xylanolytic Enzymes

The time course of xylanase production with different carbon sources is shown in figure 2a. The xylanase activities of barley straw increased gradually after 2 days of culture and reached a maximum (250 U/ml) on days 6 and 7. With wheat bran, the activities of xylanases increased by day 2 and reached a maximum on day 3. Thereafter they gradually diminished, probably because the xylanases were degraded by proteolytic enzymes after 3 days. Negligible xylanase activities were achieved in agave and pine sawdust.

The results for carboxymethyl cellulose (CMCase) activity in this experiment are shown in figure 2b. It was found that the medium had endoglucanase activities when supplemented with wheat bran and barley straw. Peaks of endoglucanase activities were observed on the fifth day in barley straw (6.5 U/ml) and on the sixth day for wheat bran (6.0 U/ml). Negligible CMCase activities were achieved in agave and pine sawdust. Similarly, the highest activity was obtained for the third day of wheat bran media (0.75 U/ml) and the fourth day for barley straw (0.74 U/ml; fig. 2c). Negligible filter paper activity (FPase) was achieved in agave and pine sawdust media.

SDS-PAGE and Zymogram Analysis of the Expression Multiplicity of Xylanases

The zymogram of the crude enzyme samples from culture containing barley straw revealed four bands of proteins with xylanase activity. The band with greatest enzyme activity was approximately 25 kDa (fig. 3a). The proteins with endoglucanase activity from the supernatant of the barley straw culture media had one band with the highest endoglucanase activity at approximately 25 kDa and another two bands between 55 and 70 kDa (fig. 3b).

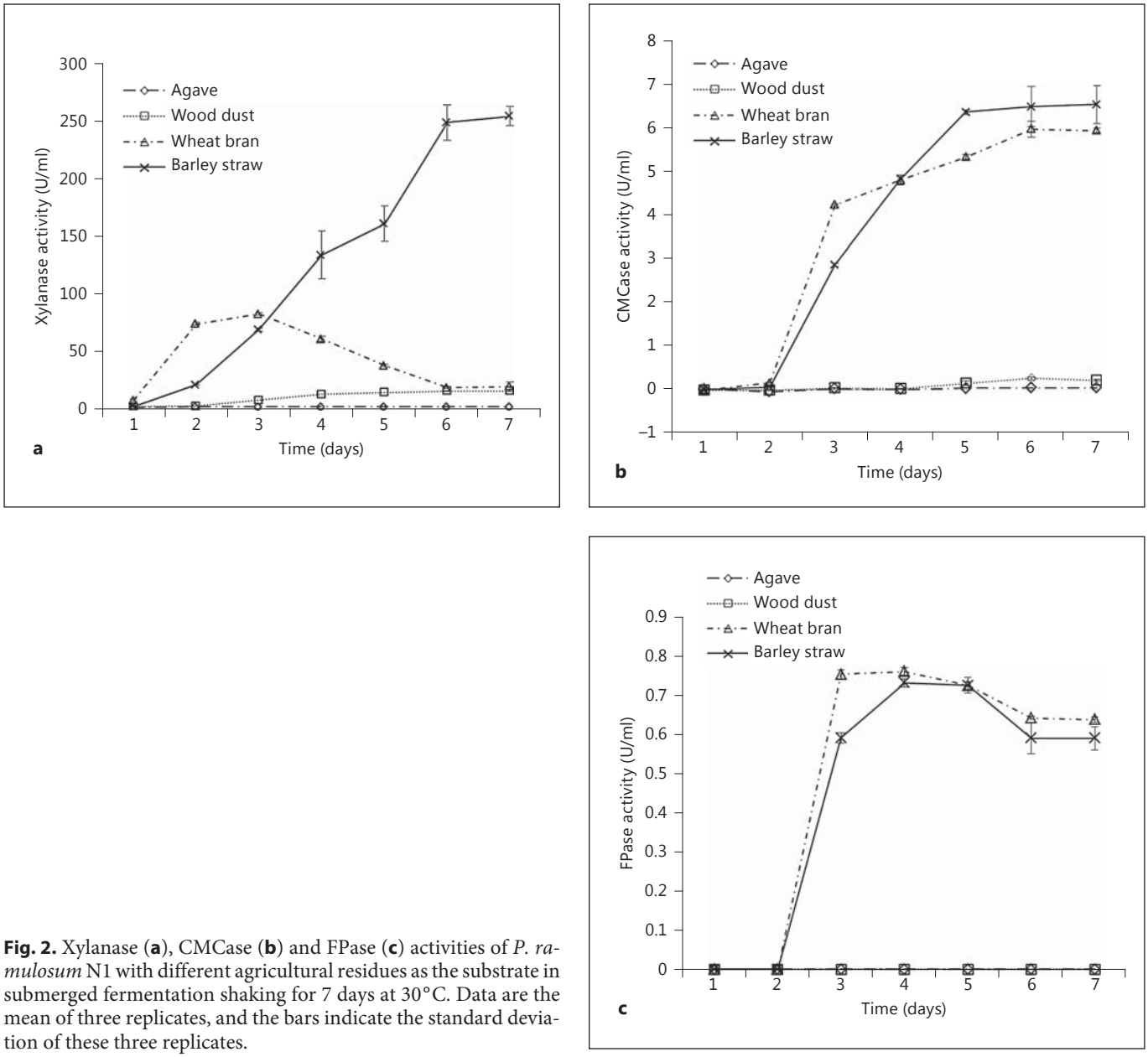

Fig. 2. Xylanase (**a**), CMCase (**b**) and FPase (**c**) activities of *P. ramulosum* N1 with different agricultural residues as the substrate in submerged fermentation shaking for 7 days at 30°C. Data are the mean of three replicates, and the bars indicate the standard deviation of these three replicates.

Effect of Temperature and pH on Enzyme Activity

It was observed that the optimum temperature was 55°C for the xylanase and FPase activity. At 60°C, xylanase activities rapidly decreased, and at 70°C xylanases were almost denatured; however, FPase can maintain 51% of its activity even at 75°C. The optimum temperature for CMCase activity was in the range of 60–70°C, mostly because there were several different endoglucanases present in the crude enzyme (fig. 4).

The pH optimal profile showed that the optimum pH for xylanase and CMCase activity was 3.0. After pH 3.0, xylanase and CMCase activity decreased rapidly, and at pH 6.0, more than 90% of the activity of CMCase and xylanase was lost. In contrast, the optimal pH for FPase activity was 5.0, and it had 33 and 37% of the maximum activity at pH 3.0 and 9.0, respectively (fig. 5).

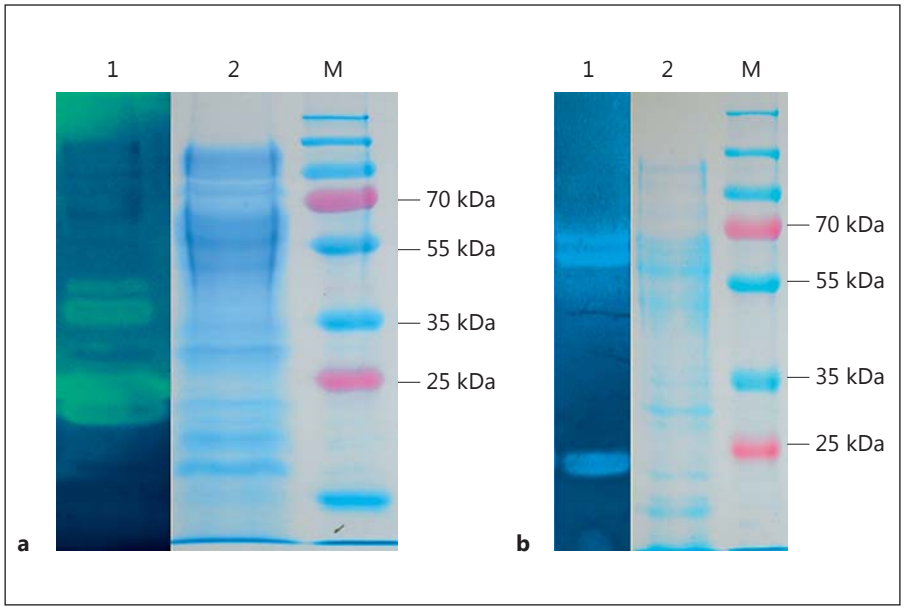

Fig. 3. SDS-PAGE and zymogram of the supernatant expression of *P. ramulosum* N1. **a** Xylanase zymogram. 1 = Lane 1, Congo red staining of the SDS-PAGE gel containing 0.1% beechwood xylan; 2 = lane 2, Coomassie blue staining of the SDS-PAGE gel containing 0.1% CMC-Na; M = standard protein molecular weight markers. **b** Endoglucanase zymogram. 1 = Lane 1, Congo red staining of the SDS-PAGE gel; 2 = lane 2, Coomassie blue staining of the SDS-PAGE gel; M = standard protein molecular weight markers.

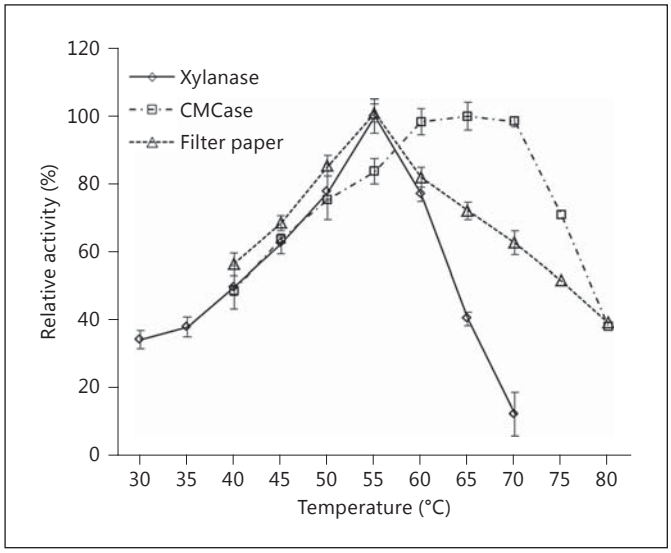

Fig. 4. Effect of temperature on enzyme activity. The optimal temperature for enzyme activity was determined by measurement of its activity at temperatures ranging from 30 to 80°C. Data are the means of three replicates, and the bars indicate the standard deviation of these three replicates.

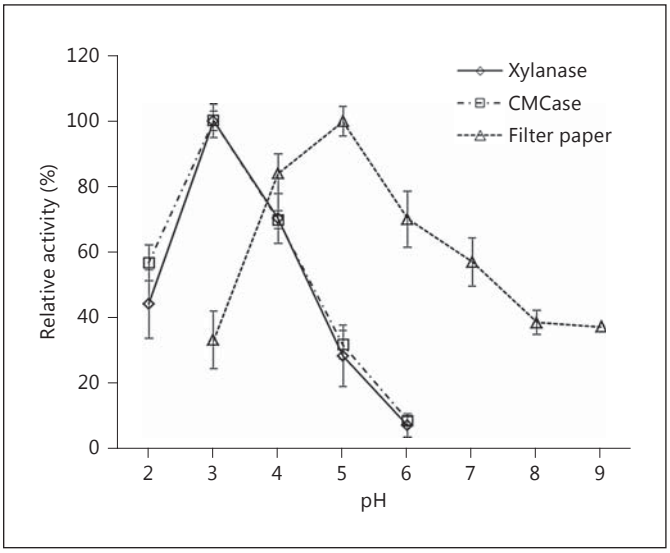

Fig. 5. Effect of pH on enzyme activity. The optimal pH for enzyme activity in the crude enzyme sample was determined at a range from 2.0 to 9.0. The 0.05-M glycine-HCl buffer was used for pH 2.0, 0.05-M citric acid-Na_2HPO_4 buffer for pH 3.0–7.0, 0.05-M sodium phosphate buffer for pH 8.0 and 0.05-M glycine-NaOH buffer for pH 9.0. Data are the means of three replicates, and the bars indicate the standard deviation of these three replicates.

Effect of Proteinase on Xylanases

To investigate the proteolytic stability of xylanase, the crude xylanases were incubated with pepsin, trypsin, collagenase A and proteinase K. The residual activities were detected after being treated at 37°C for 1 h. The crude xylanases were resistant to pepsin, trypsin and collagenase A. The xylanases retained more than 90% of their initial activity after incubation in pepsin, trypsin or collagenase A. However, they lost more than 70% of their activity after incubation in 50 μg/ml of proteinase K (table 1).

Table 1. Effect of protease on xylanase stability

Protease	Relative activity, %		
	500 μg/ml	100 μg/ml	50 μg/ml
Pepsin	104.8±4.8	104.6±6.2	103.2±4.2
Trypsin	128.1±6.7	101.4±1.7	104.4±3.8
Proteinase K	8.5±1.2	21.2±1.9	29.2±2.8
Collagenase A	92.3±6.8	98.3±4.7	99.6±8.9

Values are means ± standard deviation (n = 3) relative to the untreated control samples.

Discussion

P. ramulosum N1 was isolated from decaying wood and tested for its ability to produce xylanase using agro-industrial residues as a substrate. The choice of an appropriate substrate is very helpful for enzymatic expression. The substrate not only serves as a carbon source but also provides some compounds to induce the expression of the enzymes [Haltrich et al., 1996]. It was reported that xylanase can be effectively induced by soluble xylooligosaccharides, such as beechwood xylan. However, the use of commercial xylan as a carbon source is uneconomical for the large-scale production of xylanase [Dwivedi et al., 2009]. In the current study, low-cost, natural residues that have different hemicellulose contents were employed as the sole carbon source. Among these natural residues, agave and pine sawdust are composed of high amounts of cellulose (~45–50%) but low percentages of hemicellulose (~25%) [McGinnis et al., 1983; Mylsamy and Rajendran, 2010], and the content of hemicellulose in wheat bran and barley straw is around 30 and 37%, respectively [Brillouet and Mercier, 1981; Sun et al., 2011].

Among our four substrates, barley straw was the most effective at causing *P. ramulosum* N1 to produce xylanase and cellulase. *P. ramulosum* N1 can grow well in the media when barley straw or wheat bran is used as a carbon source; however, in pine sawdust or agave media, *P. ramulosum* N1 cannot grow well. It seems, therefore, that *P. ramulosum* N1 cannot use pine sawdust or agave as a carbon source. A number of studies have revealed that rice straw and wheat straw are good substrates for penicillium to produce xylanolytic enzymes. These are more easily digested to release soluble oligosaccharides to induce penicillium to express more hydrolysis enzymes. Hydrolysis enzymes accelerate in degrading the cell wall and release more monosaccharides to promote penicillium growth [Liao et al., 2012]. Similar to wheat straw, barley straw also has a high hemicellulose content and is more easily degraded. *P. ramulosum* N1 can degrade barley straw and releases monosaccharides to promote *P. ramulosum* N1 growth. However, barley straw has received little attention as a low-cost agricultural residue, and its use is still limited. It was traditionally regarded as an agricultural waste product and its application as a carbon source in xylanolytic enzyme production has been seldom reported. Barley is grown in many regions of the world. Worldwide, it is the fourth most produced cereal grain in terms of quantity. Using barley straw for xylanolytic enzyme production not only provides an alternative substrate but also helps to reduce environmental issues.

In the wheat bran media, *P. ramulosum* N1 can grow fast and produce enzymes quickly, but the xylanase activities are much lower than those observed for barley straw, probably because wheat bran contains too much starch. Starch is easily degraded and supplies sufficient carbohydrates for penicillium growth. It is not necessary for *P. ramulosum* N1 to get more monosaccharides by producing xylanolytic enzyme to digest hemicellulose. As suggested in the report by Sun et al. [2008], increased starch content has been correlated with a decrease in the expression level of xylanases. Similarly, Liao et al. [2012] reported that wheat bran was not a good substrate for xylanase production.

The study of the hemicellulolytic enzymes of *P. ramulosum* N1 showed that this fungus was much more xylanolytic than cellulolytic. Xylanases of fungal origin are more active at a pH between 5.0 and 7.0, and the majority of them showed high activity under slightly acid conditions [Knob and Carmona, 2010]. However, the xylanases from *P. ramulosum* N1 were found to be active over a broad pH range of 2.0–5.0, showing maximum activity at pH 3.0. This characterization makes it a good candidate for its potential application in the animal feed industry.

The xylanases from *P. ramulosum* N1 also showed strong resistance to pepsin, trypsin and collagenase A. Several protease-resistant xylanases have been cloned from fungi. Zhou et al. [2010, 2011] cloned two protease-resistant xylanases from *Streptomyces* sp. TN119. Both xylanases showed high resistance to trypsin, but they only exhibited high activity under slightly acidic conditions. Xyl11B from the acidophilic fungus *Bispora* sp. MEY-1 was highly resistant to both pepsin and trypsin, and it exhibited maximum activity at around pH 3.0 [Luo et al., 2009]. The characterization of Xyl11B is similar to xylanases from *P. ramulosum* N1. It indicates that the xylanases produced by *P. ramulosum* N1 may have a similar sequence or structure to Xyl11B.

In conclusion, a new strain of fungus was isolated and identified as *P. ramulosum* N1. It grew well and produced high levels of xylanases when barley straw was utilized as its sole carbon source. The production of xylanase may be improved by a further optimization of the media and culture conditions. Characterization of the crude enzyme clearly showed that xylanases from *P. ramulosum* N1 have optimal activity in extremely acidic conditions and are protease resistant. Therefore, this fungus meets the needs of the animal feed and food industry.

Methods

Isolation and Identification of P. ramulosum N1

P. ramulosum N1 was isolated from decaying wood (Thunder Bay, Ont., Canada). Five grams of decaying wood were added to 50 ml of sterile water; the mixture was shaken for 30 min at 120 rpm at room temperature, and 0.1 ml of the suspension was spread on xylan medium (5 g of beech xylan powder, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 g of KH_2PO_4, 1.3 g of $K_2PO_4 \cdot 2H_2O$, 1 g of NH_4NO_3, 50 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2$ and 15 g of agar in 1 liter of water). Colonies showing good growth and halo zones around the colonies were selected and purified. The N1 strain was selected for further study because it had the biggest halo zone and the highest xylanase activity. The strain was maintained on PDA (potato dextrose agar) medium. To identify N1, its genomic DNA was extracted using a genomic extraction kit (Bio Basic, Markham, Ont., Canada). The ITS region was amplified using the universal primer set ITS 1(TCCGTAGGTGAACCTGCGG) and ITS 4(TCCTCCGCTTATTGATATG). The PCR products were sequenced and aligned using BLAST analysis. Multiple alignments were performed using Clustal X1.83, and a phylogenetic tree was constructed using MEGA 6.0 [Liao et al., 2012]. The accession number of the ITS sequence is KR559743.

Preparation of Agricultural Residues

A conidial suspension of strain N1 was harvested from a 6-day-old PDA plate incubated at 30°C and suspended in distilled 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 $MgSO_4 \cdot 7H_2O$, 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 1 liter of water) was supplemented with 1% of the agricultural residues (pine sawdust, agave, wheat bran or barley straw) in 250-ml flasks. Fresh conidia suspensions of strain N1 were inoculated at a ratio of 1:50 in the medium and incubated at 30°C for 7 days.

Enzyme Assay

During submerged fermentation, 1.8-ml samples were collected at 24-hour intervals, and the supernatants were obtained as crude enzyme solutions after centrifugation at 15,000 *g* for 5 min. The xylanase activity was determined using the same method as described by Bailey et al. [1992], and the released reducing sugars from beechwood xylan (1%, w/v) were measured using the DNS (3,5-dinitrosalicylic acid) method with some modifications [Miller, 1959]. Briefly, a 200-µl reaction mixture containing 10 µl of diluted crude enzyme and 190 µl of a 1% (w/v) suspension of beechwood xylan in a 50-mM citrate buffer (pH 3.0) was incubated at 55°C for 10 min. Next, 300 µl of DNS reagent was mixed with the enzyme reaction mixture and boiled for 5 min. One unit of activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute under these conditions.

SDS-PAGE and Zymogram Analysis

SDS-PAGE was performed using a 10% (w/v) polyacrylamide gel with an 8% stacking gel and the Mini-Protean II system (Bio-Rad) according to the manufacturer's instruction manual with some modifications. The proteins were visualized by staining with Coomassie brilliant blue R-250. The zymogram analysis of the xylanase enzymes was performed according to Peterson et al. [2011] with some modifications. The crude enzyme samples were mixed with a loading buffer and boiled at 100°C for 40 s. After the separation of the enzyme samples by SDS-PAGE containing 0.1% beechwood xylan or sodium carboxyl methyl cellulose (CMC-Na), the gel was divided into two parts. One part, which contained the samples and molecular markers, was stained with Coomassie brilliant blue R-250. The other part of the gel was soaked for 30 min in 1% Triton X-100 to remove the SDS and allow refolding of the proteins in the gel. For xylanase activity, the gel was incubated at 55°C for 20 min for xylanase activity. The CMC-Na gel was incubated at 55°C for 1 h in a 50-mM acetate buffer (pH 4.8) to detect cellulase activity. The gel was submerged in 0.1% (w/v) Congo red solution for 30 min and destained with 1 M NaCl until pale-red hydrolysis zones appeared against a red background. Dipping the gel in a 4% acetic acid solution stopped the reaction.

Influence of Temperature and pH on the Activity of Xylanase Enzymes

The xylanase activity of the crude extract was assayed at different temperatures ranging from 30 to 70°C. The optimal pH for the activity in the crude enzyme sample was detected by incubating the crude enzyme mixture with 1% beechwood xylan 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).

The Effects of Proteases on the Crude Enzymes

To examine resistance to different proteases, the crude enzyme was incubated at 37°C for 1 h with pepsin (pH 2.0), trypsin (pH 7.0), collagenase A (pH 7.0) or proteinase K (pH 7.0), and the residual enzyme activity was measured under standard conditions [Zhou et al., 2011].

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