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Production and Properties of Extracellular Endoxylanase from *Neurospora crassa*†

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***Neurospora crassa* 870 produced 14 and 0.025 U of extracellular xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1,4-β-xylan xylohydrolase; EC 3.2.1.37) per ml, respectively, in 4 days when commercial xylan was used as a carbon source. The effects of pH and carbon sources on xylanase production by *N. crassa* are discussed. Two xylanases (I and II) were purified and had pI values of 4.8 and 4.5 and molecular weights of 33,000 and 30,000. The maximum degree of hydrolysis of xylan by the extracellular culture broth was 66% in 4 h. The end products of xylan hydrolysis by xylanase I and II showed the presence of xylose, xylobiose, xylotriose, xylotetraose, xylopentose, and arabinose, indicating that they are endoxylanases capable of hydrolyzing 1,3-α-L-arabinofuranosyl branch points. Both xylanases showed activity toward carboxymethyl cellulose but no activity toward *para*-nitrophenyl-β-D-xyloside or laminarin. Xylanase I showed appreciable activity toward *para*-nitrophenyl-β-D-glucoside, whereas xylanase II was inactive.**

Hemicellulose is one of the major components of lignocellulosic materials, comprising 15 to 35% of agricultural and forestry residues (11). For the maximum utilization of biomass for alcohol production, the hydrolysis of hemicellulose and fermentation of derived sugars are important. Economic evaluations are mostly based on the assumption that both glucose and xylose are fermentable to ethanol (13). The primary component of hemicellulose is xylan, a polymer of xylose. Xylan can be hydrolyzed by microbial enzymes to the pentose sugar xylose. The production and properties of xylanases from many fungal cultures have been reported (4).

Previously we have reported the production of cellulases by *Neurospora crassa* 870 and their ability to ferment D-glucose, D-xylose, and treated cellulosic substrates to ethanol (9). Recent work also indicated the ability of *N. crassa* 870 to directly ferment xylan and the carbohydrate component of bagasse to produce alcohol with a conversion of 58 and 90%, respectively (V. Deshpande, S. Keskar, C. Mishra, and M. Rao, VII International Biotechnology Symposium, IIT, New Delhi). Since *N. crassa* directly fermented xylan to ethanol, it was of interest to study the production and properties of xylanases. The process of enzymatic hydrolysis of hemicellulose and cellulose would be greatly enhanced if the hydrolysis could be carried out by one enzyme mixture. The present paper reports the production of extracellular xylanase by *N. crassa* and hydrolysis of xylan by this enzyme. The purification and properties of the xylanases are also discussed.

MATERIALS AND METHODS

Chemicals. The following chemicals were used: cellulose powder CP 100 (Cellulose Products India Ltd., Ahmedabad); yeast extract (Difco Laboratories, Detroit, Mich.); malt extract (SDS Laboratory Chemical Industries, Bombay, India); peptone (Biochemical Unit, New Delhi, India); xylan (Fluka AG, Buchs, Switzerland); sodium salt of carboxymethyl cellulose (CM-cellulose; low viscosity), *p*-nitrophenyl-β-D-xyloside (PNPX), *p*-nitrophenyl-β-D-glucoside (PNPG), xylose, arabinose, laminarin, sodium dodecyl sulfate (SDS) molecular weight determination kit, and Ampholines (Sigma Chemical Co., St. Louis, Mo.); Tris, TEMED (*N,N,N',N'*-tetramethylethylenediamine), acrylamide, and Coomassie blue (Koch-Light Laboratories, United Kingdom); UM-10 ultrafiltration membranes (Amicon Corp., Lexington, Mass.); collodion membrane (Sartorius Membrane Filter GmbH, Federal Republic of Germany). Authentic xylobiose and xylosaccharides (xylotriose to xylopentose) were obtained from A. H. Lachke of this laboratory. All other chemicals were of analytical reagent grade.

Microorganism. *N. crassa* 870 was obtained from the National Collection of Industrial Microorganisms, India (9).

Media and cultivation conditions. Enzyme production was studied in 500-ml Erlenmeyer flasks containing 100 ml of medium. The composition of the medium was as follows (grams per liter of distilled water): KH_2PO_4, 2.0; $MgSO_4$, 0.3; $CaCl_2$, 0.3; peptone, 5.0; yeast extract, 3.0; malt extract, 3.0. The medium also contained the following trace elements (milligrams per liter): $FeSO_4 \cdot 7H_2O$, 5.0; $ZnSO_4 \cdot 7H_2O$, 1.4; $MnSO_4 \cdot 4H_2O$, 1.6; $CoCl_2$, 2.0. The carbon sources used were 2% glucose, 2% xylan, or 2.5% cellulose powder. The pH of the medium was adjusted to 5. Cellulose powder was also used in combination with 1% wheat bran, bagasse, or mesta wood (*Hibiscus cannabinus*). Alkali-pretreated cellulose powder (10 g of cellulose powder was autoclaved with 50 ml of 1 N NaOH at 16 lb/20 min and washed free of alkali) was also used as a carbon source at 1%. The media were inoculated with heavy spore suspensions of *N. crassa* culture grown for 7 days in a malt extract yeast extract medium (9). The flasks were incubated at 28°C on a rotary shaker at 220 rpm. Samples were withdrawn periodically for checking the enzyme activity. The pH was maintained at 5.0 throughout the fermentation. The effect of pH on xylanase production was studied by adjusting and maintaining the desired pH by adding sterile NaOH or HCl at an interval of 8 h.

Enzyme assays. Activities toward CM-cellulose, xylan, Walseth cellulose, and laminarin were determined by incubating a 1-ml reaction mixture containing 0.5% substrate and suitably diluted enzyme in 50 mM acetate buffer (pH 4.8) for 30 min at 50°C. The reducing sugar formed was determined by the dinitrosalicylic acid method (7) by using D-glucose or D-xylose as standard. Activity toward PNPX or PNPG was assayed by incubating 1 ml of 0.1% PNPX or 0.3% PNPG

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TABLE 1. Production of cellulase and xylanase by *N. crassa*

Carbon source	CM-cellulase production (U/ml) at day:				Xylanase production (U/ml) at day:			
	2	4	7	10	2	4	7	10
Glucose (2%)	0.15	0.2	0.25	0.25	0.25	0.6	0.7	0.7
Xylan (2%)	0.8	1.2	1.5	1.4	7.6	14	14.5	14
Treated cellulose powder (1%)	1.0	2.2	5.0	5.0	0.6	2.4	7	7
Cellulose powder (2.5%)	0.8	3.0	5.0	4.8	1.2	5.6	10	9.8
Cellulose powder (2.5%) + wheatbran (1%)	1.8	4.0	10	10	5.2	7	12	11
Cellulose powder (2.5%) + mesta wood (1%)	1.8	5.6	10	10	0.6	6	7	7
Cellulose powder (2.5%) + bagasse (1%)	1.0	3	8	7.5	0.6	6	6	6

with 0.1 ml of suitably diluted enzyme in 50 mM acetate buffer (pH 4.8) for 30 min at 40°C. The reaction was stopped by the addition of 2 ml of 1 M Na_2CO_3. The liberated *p*-nitrophenyl was measured at 400 nm.

Estimation of mycelium-bound xylanase and β-xylosidase. *N. crassa* was grown for 6 days with 1% xylan as carbon source as described above. The culture broth (100 ml) was centrifuged, and the mycelium was homogenized with glass powder by using a glass homogenizer. The mycelium was suspended in 100 ml of 0.05 M acetate buffer (pH 4.8), and the activities of the mycelial suspension were estimated as described above.

Unit of activity. One unit of enzyme activity was defined as that amount of enzyme which produces 1 μmol of reducing sugar in 1 min under the assay conditions. Protein was determined by the spectrophotometric method of Warburg and Christian as modified by Jagannathan et al. (6).

Hydrolysis of xylan. The hydrolysis was carried out in stoppered tubes with different amounts of substrate in 2 ml of reaction mixture containing suitably diluted enzyme in 50 mM acetate buffer (pH 4.8) at 50°C. The reducing sugar formed was estimated by the dinitrosalicylic acid method, using xylose as standard.

Purification of xylanases. The enzyme for purification studies was produced in a medium containing 2% alkali-pretreated cellulose powder as carbon source. The clarified culture broth (65 ml) was concentrated by ultrafiltration with a UM-10 membrane to a residual volume of 4 ml. The concentrated broth was dialyzed against 0.05 M acetate buffer (pH 4.8).

Preparative isoelectric focusing. Preparative isoelectric focusing was carried out as described by LKB Produkter AB, Stockholm, Sweden (application note 198). Isoelectric focusing was performed by using polyurethane strips in place of Ultrodex (1). Isoelectric focusing of proteins was carried out in the LKB Multiphore apparatus with the LKB Multiphore tray (24.5 by 11 by 0.5 cm) and Ampholines in the pH range 3.5 to 5. Ultrodex was substituted with 24 strips of foam (5 by 1 by 9.5 cm each). The strips were soaked in 7 ml of 2% (wt/vol) Ampholine in water and placed horizontally on the tray. The anodic strip was soaked in 1 M H_3PO_4, and the cathodic strip was soaked in 1 N NaOH. Two strips were removed, squeezed, dipped in the solution containing enzyme sample and replaced on the tray. The electrofocusing was run with a constant current of 12 mA and a constant power of 8 W for 18 h at 10°C. After termination of the run, foam strips were squeezed, and the material was collected in different test tubes and assayed for the protein and activity. The pH was measured with the help of a surface electrode.

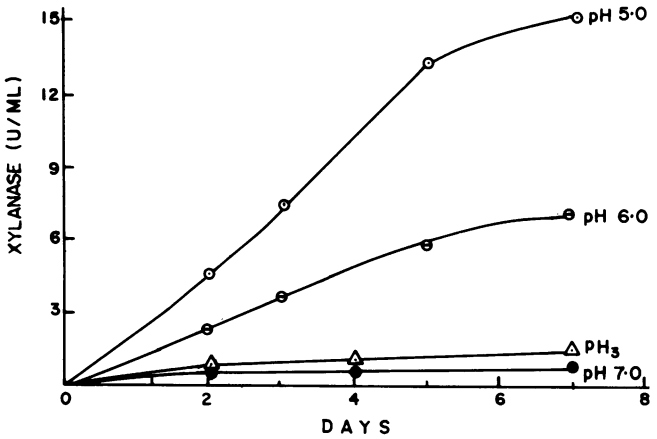

FIG. 1. Production of xylanase at different pHs. The carbon source was 2% xylan. Cultural conditions are described in the text.

SDS gel electrophoresis. Electrophoresis in slab gel of polyacrylamide containing SDS was performed by the method of Weber and Osborn (15) by using lysozyme (14,000 molecular weight), trypsin (24,000), pepsin (34,700), ovalbumin (45,000), bovine serum albumin (68,000), and transferrin (90,000) as standard proteins. Electrophoretic mobilities of purified xylanases and of the reference proteins on SDS-polyacrylamide were plotted versus their molecular weights.

Analytical isoelectric focusing. Isoelectric focusing in thin polyacrylamide gel was carried out by the method of Vesterberg (14). The Ampholine range used was pH 3.5 to 5.

Disc gel electrophoresis. Polyacrylamide gel electrophoresis was carried out in 7% acrylamide gel with Tris glycine buffer (8).

Determination of K_m and V_{max}. Suitably diluted xylanase I or II was incubated with different amounts (2 to 10 mg) of

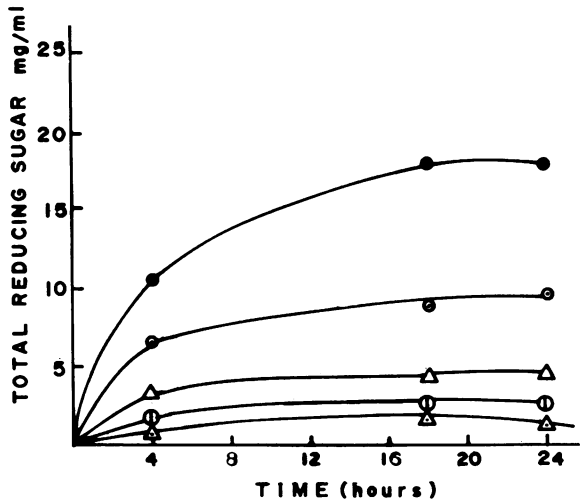

FIG. 2. Hydrolysis of different amounts of xylan by *N. crassa* xylanase. Xylanase (3 U) was incubated with various amounts of xylan at pH 4.8 and 50°C. Xylan (mg/ml): △, 2.5; ⊕, 5; △, 10; ⊙, 25; ●, 50.

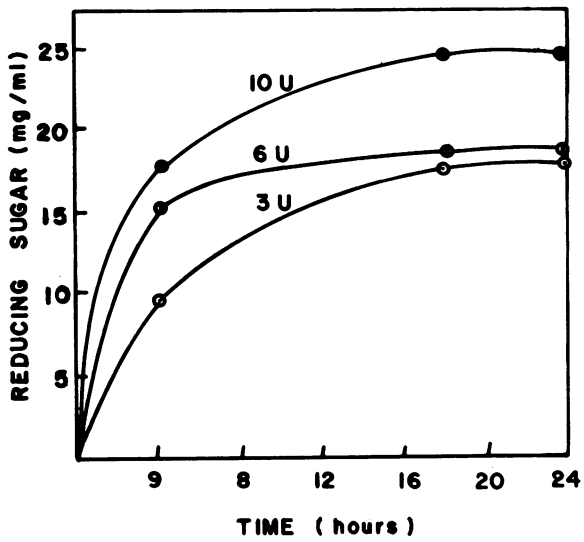

FIG. 3. Hydrolysis of xylan with different amounts of enzyme. Substrate (100 mg) was incubated with 3, 6, and 10 U of xylanase at pH 4.8 at 50°C.

xylan under the assay conditions described. K_m and V_{max} values were determined from Lineweaver-Burk plots.

Determination of K_i. Inhibition of xylanase activity of xylanase I and II by xylose was studied, and the K_i values were determined with a 0.5% solution of xylan as substrate. The enzyme activity was determined in the presence of xylose at final concentrations of 2, 5, and 10 mM. Inhibition constants (K_i) were determined by the method of Deshpande et al. (5).

Effect of pH and temperature on purified xylanases. Suitably diluted enzyme was incubated with 0.5 ml of 1% xylan at pH 3, 4, 5, 6, and 7 in a reaction mixture volume of 1 ml containing 50 mM of the respective buffer at 50°C for 30 min. Enzyme activity toward xylan was determined at different temperatures (27, 37, 50, 60, and 70°C) under the same conditions.

TABLE 2. Purification of xylanase I and II

Fraction	Total vol (ml)	Total units	Total protein (mg)	Sp act (U/mg)	Purification
Culture broth	65	390	260	1.5	1
Ultrafiltered dialyzed broth	4	350	80	4.3	2.8
Isoelectric focusing					
Xylanase I (fraction 8)	0.5	11	0.6	18.3	4.2
Xylanase II (fraction 11)	0.5	20	1.2	16.6	4.0

Determination of xylan degradation products. Samples (150 μg) of xylanase I and II were incubated with xylan (10 mg) in the presence of 50 mM acetate buffer (pH 4.8) for 24 h. The end products formed were analyzed by paper chromatography in the solvent system butanol-acetic acid-water (3:1:1). The paper chromatograms were sprayed by the method of Trevelyn et al. (12).

RESULTS

Production of xylanase and cellulase. The production of extracellular cellulase and xylanase by *N. crassa* 870 with different carbon sources is shown in Table 1. The extracellular activities were very poor when the fermentation was carried out with glucose. The xylanase was produced in significant amounts even when only cellulose powder was used as a carbon source. The maximum production of xylanase (14 U/ml) was observed in 4 days when the

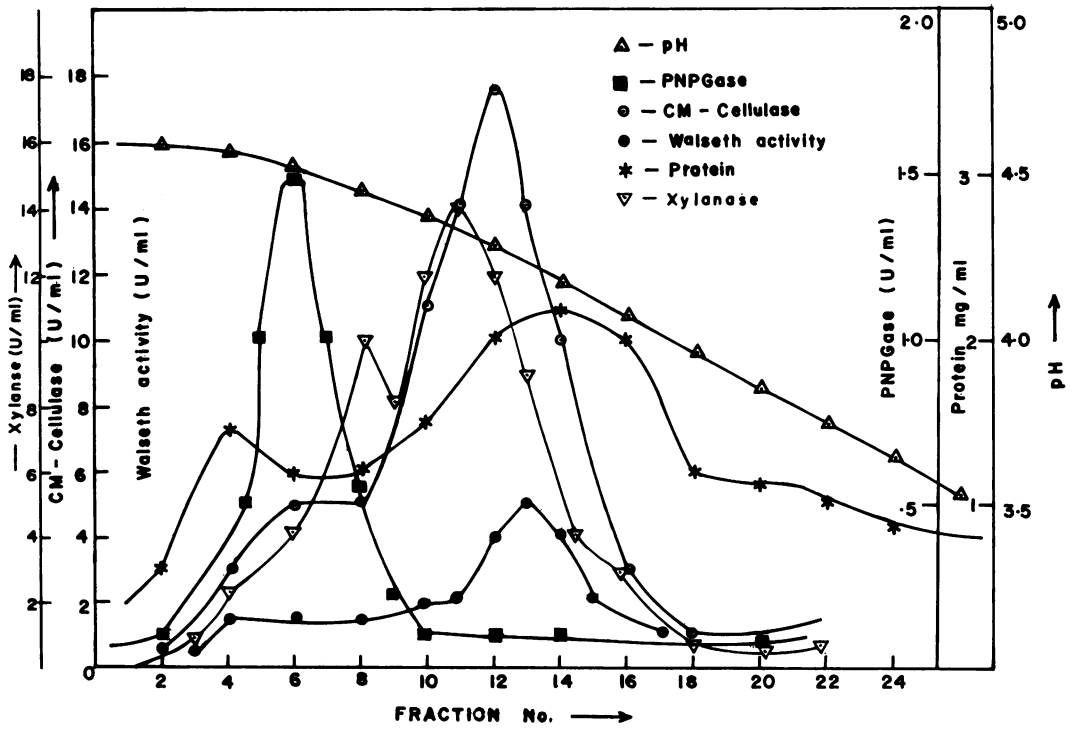

FIG. 4. Purification of xylanase I and II by preparative slab gel isoelectric focusing, pH 3.5 to 5.

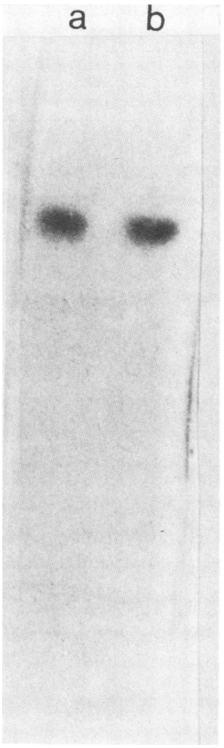

FIG. 5. Analytical isoelectric focusing (pH 3.5 to 5) of (a) xylanase I and (b) xylanase II; 25 μg of protein was loaded and stained with Coomassie blue.

organism was grown on commercial xylan. The cellulase activity was highest when cellulose powder in combination with wheat bran or wood was used as carbon source.

Effect of pH on xylanase production was studied with 2% xylan as carbon source. The optimum pH was 5, and pH 3, 6, and 7 were found to be unfavorable (Fig. 1). The β-xylosidase activity (0.025 U/ml) was detected in the 6-day-old culture broth with xylan as carbon source.

Intracellular xylanase and β-xylosidase. *N. crassa* mycelium grown with xylan as a carbon source showed 1 and 0.03 U of xylanase and β-xylosidase per ml, respectively, in 6 days. The intracellular xylanase activity was only 7% of the extracellular xylanase, but the β-xylosidase activity was almost equally distributed between the cell-free and cell-bound fractions.

Hydrolysis of xylan. A maximum hydrolysis of 66% of the xylan was observed in 4 h when 5 mg of substrate was used. Increasing the substrate concentration gave only 36% hydrolysis (Fig. 2). Increasing the enzyme concentration gave 50% hydrolysis in 18 h (Fig. 3). Even though the increased enzyme concentration resulted in a slightly higher percentage of hydrolysis, the extent of increase in the hydrolysis of xylan by the addition of large amounts of enzyme did not result in higher conversion yields.

Purification of xylanases. The purification of xylanase is summarized in Table 2. The concentrated and dialyzed broth was subjected to preparative flat-bed isoelectric focusing over a pH range of 3.5 to 5. The eluates were analyzed for xylanase and cellulase activity (Fig. 4). Fractions 8 and 11, which showed high xylanase activity, were dialyzed and concentrated through collodion membrane. These two fractions were designated as xylanase I and xylanase II.

Characterization of xylanases. Xylanases I and II showed single bands on a 7% polyacrylamide gel and analytical isoelectric focusing (Fig. 5) with pI values of 4.5 and 4.8.

Both xylanases had optimal activity at 50°C and pH 4.8. Molecular weights of xylanase I and II determined by SDS-polyacrylamide gel electrophoresis were 33,000 and 30,000, respectively. On the basis of Lineweaver-Burk plots (Fig. 6). K_m values calculated for xylanase I and II were 4 and 5 mg/ml, and V_{max} values were 14.4 and 8.8 mol/min per mg, respectively. Xylanase I and II showed K_i values of 2.4 and 1.0 mM, respectively. Xylanase I and II both yielded xylose, arabinose, and a mixture of xylo-oligosaccharides (2 to 5 saccharide units in length) after xylan hydrolysis (Fig. 7).

Substrate specificity. Both of the purified xylanases showed high activity toward xylan. They also showed activity toward CM-cellulose but no activity toward PNPX and laminarin. Xylan I showed activity towards PNPG, but xylanase I did not (Table 3).

DISCUSSION

Previous work has indicated that *N. crassa* 870 produces an extracellular cellulase complex and has the capacity to directly ferment cellulose to ethanol. The present studies

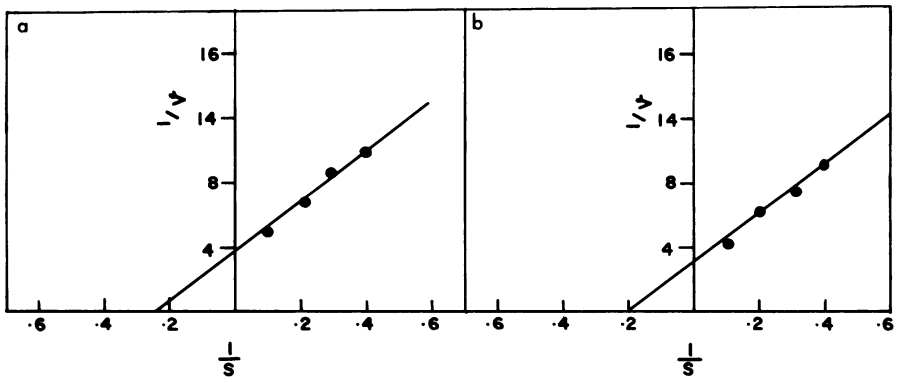

FIG. 6. Kinetics of the hydrolysis of xylan by xylanase I (a) and xylanase II (b) as expressed in a Lineweaver-Burk reciprocal plot.

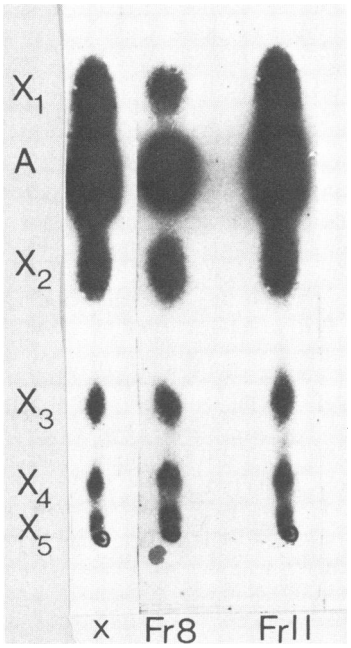

FIG. 7. Paper chromatogram of hydrolysis products of xylan by xylanases. Lane X, A mixture of 10 μg of xylose (X_1), 20 μg of xylobiose (X_2), 50 μg of xylotriose (X_3), 50 μg of xylotetraose (X_4), 50 μg of xylopentose (X_5), and 10 μg of arabinose (A). Fr 8 and 11 represent the xylan hydrolysis products of xylanase I and II, respectively.

show that *N. crassa* also produces extracellular xylanase. The maximum xylanase production was observed when the fungus was grown on commercial xylan. However, appreciable amounts of xylanase were produced when cellulose powder was used as a carbon source. Several other workers have also demonstrated that many fungal species produce xylanase when cultured on cellulose (4). This may be due to cellulases having multisubstrate activity. Cellulases have also been reported as adaptive enzymes, and the multiplicity of such enzymes may imply that pseudo-xylanase activity is produced when the fungi are grown on cellulose (2). The maximum degree of hydrolysis of xylan by the *N. crassa* xylanase was 63%. The incomplete hydrolysis may be due to the poor amounts of xylosidase produced by *N. crassa*. Dekkar (3) has shown the increased rate of hydrolysis of xylan by *Trichoderma reesei* xylanases when the exogenous β-xylosidase from *Aspergillus niger* was added. The leveling off of xylan hydrolysis beyond 18 h was probably due to enzyme stability and end product inhibition.

The purified xylanases appear to have very similar isoelectric points and molecular weights. They differ in their kinetic properties and their action toward PNPG. The end product analysis of xylan hydrolysates showed that they are endoxylanases and are capable of hydrolyzing the 1,3-α-L-arabinofuranosyl branch points. Several other purified xylanase preparations have also been shown to liberate arabinose (4, 10) from xylan. The purified xylanases show appreciable activity toward CM-cellulose, resembling the purified endoxylanases from other cellulolytic cultures, *T. reesei* and *Irpex lacteus* (4).

TABLE 3. Activity of xylanase I and II toward different substrates[a]

Substrate	Activity (U/mg)	
	Xylanase I	Xylanase II
Xylan	20	16
CM-cellulose	14	8
PNPX	0	0
PNPG	2	0
Laminarin	0	0

[a] Xylanase I or II (10 μg) was incubated with different substrates. The assay conditions are described in the text.

ACKNOWLEDGMENT

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