

Production of Xylanases by *Verticillium albo-atrum*¹

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Abstract

A xylan extract from corncobs contained xylose and trace amounts of arabinose, glucose, and an unidentified uronic acid. The xylan was utilized as a carbon source for growth and production of xylanases by *Verticillium albo-atrum* in vitro and as a substrate for xylanase characterization.

An extracellular xylanase system was found in dialyzed, concentrated culture filtrates of *V. albo-atrum*. Optimum xylanase activity occurred in the range of pH 5.0 to 5.5 at 35 to 40 C. Chromatographic analysis of enzymatic hydrolysis products of xylan revealed the presence of xylose, xylobiose, xylotriose, xylotetraose, and trace amounts of glucose and a uronic acid. Heat inactivation of the xylanase system occurred between 61 and 70 C. Xylanase preparations heated to 50 C prior to enzyme-substrate incubation hydrolyzed xylan to xylose and xylobiose. Although reducing group liberation was observed in enzyme-substrate mixtures where the enzyme was heated to 60 C prior to incubation, no chromatographically detectable hydrolysis products were observed in the mixtures.

The results suggested that at least three xylanases were synthesized by *V. albo-atrum*. One appeared to liberate single xylcse units and a second appeared to hydrolyze xylan to xylobiose residues. Xylooligosaccharides in reaction mixtures suggested the presence of an endoxylanase.

Introduction

Verticillium albo-atrum Reinke and Berth. is the causal agent of *Verticillium* wilt of tomato and potato. The disease exhibits the three typical phases of development of a wilt syndrome (18). The pathogen penetrates the host roots and is confined to the xylem during early stages of the disease cycle. As the disease progresses, irreversible symptoms of stunt, chlorosis, epinasty, and wilt occur. As the host dies, the pathogen spreads throughout the tissue and eventually sporulates.

Hancock and Millar (8) suggested that the action of xylanases and other hemicellulases would allow a pathogen to spread throughout the host by the enzymatic removal of non-structural wall polymers associated with lignins and cellulosic materials. The products of degradation of such cell wall components might also serve as a readily accessible carbohydrate source, aiding the continued proliferation of the pathogen.

The chemical constitution of xylans indicates a wide variety of structures and molecular weights, and each plant species may have one or more characteristic xylans with molecular weights ranging from 7,000 to 30,000 (2, 5, 6, 11, 16). Studies of the enzymatic hydrolysis of xylans are limited by a lack of information concerning xylan and its

¹Journal Paper No. 6099, Purdue University Agricultural Experiment Station.

relative importance to the integrity of the cell wall. Extracellular xylanases are classified according to the manner of hydrolysis of xylan. The nomenclature is similar to that applied to enzymes that hydrolyze pectic substances (3). A xylanase that hydrolyzes the xylan chain by random cleavage is designated an endoxylanase. This term is also applied to xylanases that cleave xylooligosaccharides into smaller oligomers but not specifically to D-xylose. Exoxylanases remove single xylose units from xylan chains or oligomers. The objective of this research was to determine whether the wilt pathogen *V. albo-atrum* is capable of utilizing xylan as a carbon source and to partially characterize the xylanases produced.

Methods and Materials

Extraction of xylan.—Extraction and purification of xylan was by the method of Adams (1) using corncobs dried at 120 F and ground in a Wiley Mill (3 mm screen). The extract was acid hydrolyzed by a modification of the method of Sørensen (13). Ten ml of a 1% solution was mixed with 40 ml of 1.25 N H_2SO_4 and refluxed for 12 hours. The hydrolysate was cooled and the volume brought to 100 ml with deionized water.

A saturated solution of $\text{Ba}(\text{OH})_2$ was added until precipitation was complete. The precipitate was removed by centrifugation at 10,400 *g* for 20 minutes. The supernate was recovered and a solution of 5.0 percent ZnSO_4 was added to precipitate excess barium and reduce the quantity of barium salts of uronic acids. The precipitate was again removed by centrifugation, toluene was added (1 ml/1) and the supernate was placed in dialysis tubing and reduced to one-half volume by pervaporation. The concentrated hydrolysate was chromatographed on Whatman No. 4 paper in amounts of 50, 100, 150, 200 and 250 μl . Chromatograms were developed by descending chromatography. Solvent systems were ethyl acetate-acetic acid-water (9:2:2 v/v) or n-butanol-pyridine-water (10:3:3 v/v) (13).

After the solvent had traveled 47 cm, the chromatograms were dried and sprayed with a freshly prepared solution of equal volumes of 1.8% aniline in absolute ethanol and 1.8% oxalic acid in water and dried at 95-100 C for 10 minutes (10). By this procedure pentoses appeared red, hexoses brown, and uronic acids yellow.

Culture of Verticillium albo-atrum.—An isolate of *V. albo-atrum*, pathogenic to potatoes and tomatoes was maintained on potato-dextrose agar and grown in liquid shake culture in 500 ml flasks (100 rpm, 22 C) for induction of xylanase enzymes. The liquid medium contained 1% (w/v) xylan and the following salts per liter: 5.0 g NH_4NO_3 , 2.5 g KH_2PO_4 , 0.5 g MgSO_4 , 0.01 g ZnSO_4 , and 0.06 g FeCl_3 (final pH 5.0). After 6 days the contents of each flask were filtered through cheese-cloth, centrifuged for 20 minutes at 10,400 *g*, and the supernate was collected and filtered through a 0.22 μ Millipore filter (Millipore Filter Corp., Bedford, Mass.). Toluene (1 ml/1) was added to prevent contamination and the filtrate was dialyzed against deionized water for 12 hours. The filtrate was reduced to one-half the original volume with

Carbowax 4000, removed from dialysis tubing and checked for contamination by plating aliquots on potato dextrose and nutrient agar. Toluene (1 ml/1) was added and the preparation was frozen until used for enzyme assays.

Determination of xylanase activity:—Xylanase activity was measured by reducing group liberation from xylan. Reaction mixtures consisted of 3 ml culture filtrate and 11 ml 2% xylan in 0.1 M sodium acetate-acetic acid buffer. Reaction mixtures were buffered at pH 4.0, 4.5, 5.0, 5.5, 5.8 and 6.5 and incubated at 25, 30, 35, and 40 C for determination of pH and temperature optima.

At intervals throughout incubation, 1.0 ml aliquots of enzyme-substrate mixtures were assayed for reducing group liberation by the method of Somogyi (12) using Nelson's (9) arsenomolybdate color developer. Samples were filtered through an 8 μ Millipore filter and optical density was determined at 520 nm. Xylanase activity was expressed as μ M of xylose liberated/ml enzyme preparation. Reducing group measurements taken immediately after the addition of enzyme to substrate served as controls and were subtracted from all other readings to account for residual reducing groups.

Determination of inactivation temperature of the xylanase system:—The enzyme preparation was incubated for one hour at 50, 60, 70, or 80 C. After incubation, 1.5 ml aliquots were mixed with 5.5 ml xylan substrate buffered as previously described. Reaction mixtures were incubated at 35 C and at one, two and five hours after the start of incubation, 1.0 ml aliquots were removed and reducing capacities were determined. Controls were prepared similarly except that the enzyme preparation was inactivated by autoclaving for 30 minutes. Enzyme-substrate mixtures incubated for 20 hours were used for analysis of hydrolysis products by paper chromatography.

Xylanase assay by paper chromatography:—Xylanase activity was determined chromatographically by observing breakdown products of xylan at various pH and temperature values and at predetermined intervals during incubation. One hundred μ l aliquots were chromatographed on Whatman No. 4 paper. Chromatograms were developed at room temperature using a descending solvent of n-butanol-acetic acid-water (8:2:5 v/v) and were dried and sprayed with aniline-oxalic acid reagent for color development. The presence of xylooligosaccharides was determined by graphing R_m values [$\log (1/R-1)$] of successive spots against the number of xylose residues in a series of xylose oligomers (4).

Results

Xylan extract:—The presence of pentoses in the corncob extract was confirmed by a positive aniline acetate test for pentoses (15). Chromatographic analysis of the extract hydrolysate demonstrated the presence of two pentoses, a hexose and a uronic acid (Table 1). The pentoses had R_f values which corresponded to reference standards of xylose and arabinose and the hexose R_f corresponded to that of glucose. The uronic

TABLE 1. *Chromatographic analysis of products of acid hydrolysis of the corn cob xylan extract.*

Solvent : n-butanol-pyridine-water (10:3:3 v/v)		Solvent : ethyl acetate-acetic acid-water (9:2:2 v/v)	
Hydrolysis products	R _f	Hydrolysis products	R _f
pentose	0.570	pentose	0.702
pentose	0.462	hexose	0.622
hexose	0.338	uronic acid	0.505
Reference standards		Reference standards	
xylose	0.576	xylose	0.694
arabinose	0.465	arabinose	0.684
glucose	0.353	glucose	0.624

acid was not identified. Both the hexose and the uronic acid were only present in trace amounts.

Optimum temperature and pH for xylanase activity:—Optimum temperature and pH for xylanase activity was determined by reducing group assay of aliquots of each enzyme-substrate mixture sampled at 2, 4, 6, 8, 10, and 12 hours after enzyme-substrate incubation. After 12 hours incubation, the optimum activity for each pH was observed at 35 C except at pH 5.8 and 6.5 where optimum activity occurred at 40 C (Table 2). Maximum reducing group liberation was observed in enzyme-substrate mixtures incubated at 35 C and pH 5.5 and this combination was considered close to the optimum for the xylanase preparation.

Enzyme-substrate mixtures at pH 4.0 exhibited the least reducing group liberation at all temperatures studied (Table 2). The least activity was observed at 25 C for each pH studied. A marked decrease in enzyme activity of mixtures incubated at 35 C occurred when the pH was changed from 5.5 to 5.8 (Table 2, Fig. 1 and 2). However, activity of mixtures incubated at 40 C only decreased slightly from pH 5.5 to 5.8. The sharp decrease in activity from pH 5.5 to 5.8 observed in mixtures incubated at 35 C suggested inhibition of activity

TABLE 2. *Optimum temperature and pH for xylanase activity as determined by reducing group assay.*

pH	Temperature (centigrade)			
	25	30	35	40
4.0	4.43 ¹	3.08	4.63	2.08
4.5	7.75	9.96	12.52	10.96
5.0	7.80	9.64	13.46	13.46
5.5	8.09	9.96	14.08	12.94
5.8	6.84	10.20	10.05	12.44
6.5	5.91	6.61	6.50	7.16

¹Values presented are for μ M xylose equivalents liberated per ml enzyme after twelve hours incubation and represent the average of six replicates per treatment. Reducing capacity of samples taken immediately after the addition of enzyme to substrate served as controls and were subtracted from all other readings to account for residual reducing groups.

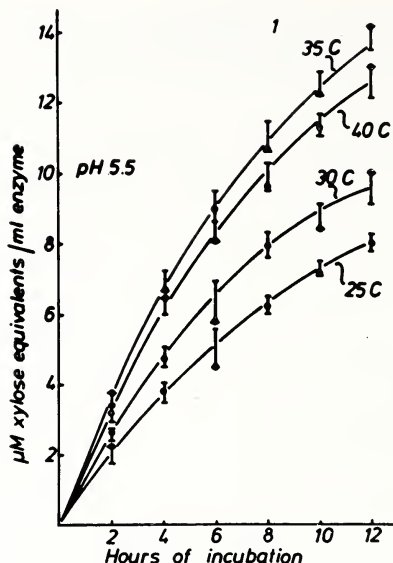


FIGURE 1. Effect of temperature on xylanase activity in reaction mixtures buffered at pH 5.5.

of an enzyme(s) sensitive to pH above 5.5. The same change in pH resulting in a decrease in activity of mixtures at 35 C could bring about an increase in activity of a second enzyme component. This would account for the observation of greater reducing activity of mixtures incubated at 40 C and pH 5.8 over those at 35 C and pH 5.8 (Fig. 2). *Chromatography of products of enzymatic hydrolysis of xylan:*—Enzyme-substrate mixtures used in determination of optimum temperature and pH were incubated for 36 hours at 25, 30, 35, or 40 C. After incubation, 100 μ l of each mixture were chromatographed and developed as previously described, using a descending solvent system of n-butanol-acetic acid-water (8:2:5 v/v).

Pentose oligomers, indicated by red spots, were the major products of hydrolysis. A linear relationship between the R_m values (4) and the number of repeating units in successive oligomers was observed (Fig. 3), indicating that the spots represented a series of xylooligosaccharides. By this method the R_f of xylose was calculated to be 0.441. The R_f values of xylose oligomers were: xylobiose, 0.337; xylotriose, 0.243; and xylotetraose, 0.168. Streaked areas of partially hydrolyzed substrate extended from the point of application of each enzyme-substrate mixture indicating the presence of oligomers of more than four xylose units. This suggested the presence of an endoxylanase that randomly hydrolyzed the xylan polymer.

Trace levels of a hexose and a uronic acid were observed on some chromatograms. The hexose was found between xylose and xylobiose and the uronic acid between xylotriose and xylotetraose. R_f values for the hexose and glucose were identical. The uronic acid was unidentified.

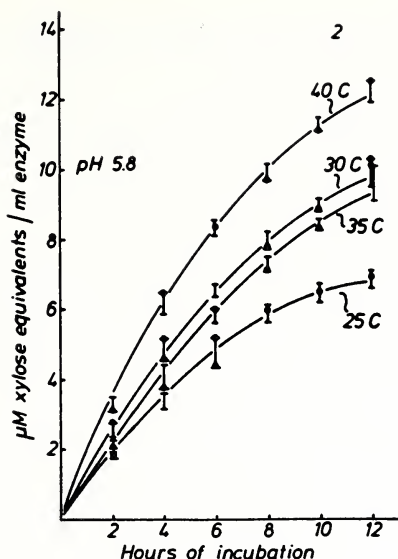


FIGURE 2. Effect of temperature on xylanase activity in reaction mixtures buffered at pH 5.8.

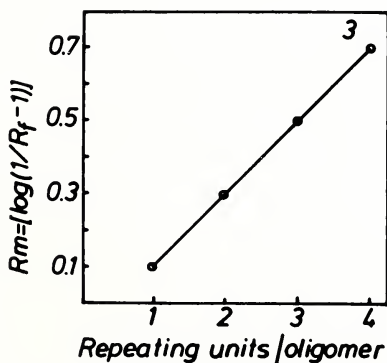


FIGURE 3. Relation of R_m value to the number of xylose units per oligomer.

The amount of each hydrolysis product appearing on chromatograms was estimated visually (Table 3). The greatest number of hydrolysis products was observed in mixtures incubated at 35 C and pH 5.0 to 5.5, suggesting that these conditions were optimum for hydrolytic activity of the xylanase system. The least amount of hydrolysis products was observed in mixtures at pH 4.0 at all temperatures.

The absence of xylose and the presence of xylose oligomers in mixtures incubated at 40 C and pH 6.5 suggested inactivation of a xylanase specific for the hydrolysis of single xylose units from xylose oligomers.

TABLE 3. *Chromatographic analysis of enzymatic hydrolysis products of xylan suspensions at six different pH values incubated at four temperatures for 36 hours.*

Conditions of hydrolysis		Hydrolysis products ¹					
Temperature	pH	xylose	xylo- biose	xylo- triose	xylo- tetraose	glu- cose	uronic acid
25 C	4.0	1	1	0	0	1	0
	4.5	2	2	1	0	1	1
	5.0	2	2	1	1	1	1
	5.5	3	2	2	1	1	1
	5.8	2	2	2	1	1	1
	6.5	1	2	1	0	1	0
30 C	4.0	1	1	1	0	1	0
	4.5	2	2	2	0	2	0
	5.0	2	3	2	1	2	1
	5.5	3	3	3	1	2	1
	5.8	3	3	3	1	1	1
	6.5	1	2	2	1	1	1
35 C	4.0	1	1	1	0	1	0
	4.5	3	2	1	1	2	0
	5.0	3	4	3	2	2	1
	5.5	3	4	3	2	2	1
	5.8	3	3	3	1	1	1
	6.5	1	2	3	1	1	0
40 C	4.0	1	1	0	0	1	0
	4.5	2	2	2	0	2	1
	5.0	3	2	2	1	2	0
	5.5	3	3	2	1	2	1
	5.8	3	3	2	1	1	1
	6.5	0	3	3	1	1	0

¹The quantity of each hydrolysis product was visually rated according to the following scale: 0=none, 1=trace, 2=slight, 3=moderate, 4=heavy.

Heat inactivation of the xylanase system:—Heating the enzyme preparation at 70 C for 1 hour resulted in complete loss of detectable enzyme activity as measured by reducing group liberation and chromatographic analysis. When the enzyme preparation was heated to 50 C an increase in reducing group liberation over the control series was readily apparent; however, only a slight increase in reducing groups was observed in enzyme-substrate mixtures where the enzyme was preheated to 60 C (Table 4). After five hours incubation, reducing activity was highest in enzyme-substrate mixtures buffered at pH 5.5 and lowest in mixtures buffered at pH 4.0 (Table 4).

Chromatographic analysis demonstrated faintly detectable hydrolysis products in mixtures incubated at 50 C. A pentose with an R_f identical to that of D-xylose was present in each mixture except those buffered at pH 6.5. A pentose with an R_f similar to that calculated for xylobiose was present only in enzyme-substrate mixtures buffered at pH 5.8 and 6.5. This suggested that an enzyme(s) capable of hydrolyzing xylan to xylose and xylobiose was present. Liberation of reducing groups by preparations heated to 60 C indicated that the enzyme preparation was not inactivated but the absence of chromatographically detectable hydrolysis products suggested either that hydrolysis products were

TABLE 4. Reducing group liberation from corn cob xylan by enzyme preparations preheated at 50, 60 and 70 C for 1-hour.

pH ¹	Heat Treatment		
	50 C	60 C	70 C
4.0	3.25 ²	3.00	0.00
5.5	5.15	3.80	0.00
5.8	4.45	3.50	0.00
6.5	4.30	3.30	0.00

¹pH of enzyme-substrate reaction mixture. Reaction mixtures were incubated at 35 C and consisted of 5.5 ml 2% xylan in 0.1 M acetate buffer and 1.5 ml enzyme preparation.

²Values are the average of six replications and are expressed as μ M D-xylose equivalents liberated/5 hr/ml enzyme preparation

present in undetectable concentrations or that they were not liberated from the xylan polymer.

Discussion

The xylan utilized in this study was extracted from corncobs by the method of Adams (1). Acid hydrolysis and chromatography of the xylan indicated the presence of xylose, arabinose, glucose, and an unidentified uronic acid. Arabinose, glucose and the uronic acid were present in relatively low levels as compared to xylose. Therefore, components found in the corn cob xylan of this study compared favorably with those previously reported (1, 2, 5, 16).

Extracellular xylanases of fungi previously investigated have exhibited optimum activity in the range of pH 3.4 to 7.5 and 30 to 50 C (7, 13, 14, 17). The xylanase system of *V. albo-atrum* exhibited optimum activity at 35 C and pH 5.5 for the temperature and pH combinations studied.

Inactivation of the xylanase system was measured by reducing group assay and paper chromatography. Reducing group liberation was not detected in enzyme-substrate mixtures where the enzyme preparation was heated for one hour at 70 C. However, enzyme preparations heated at 50 and 60 C retained some activity.

Chromatography of enzyme-substrate mixtures revealed that no products of hydrolysis could be detected in mixtures where the enzyme was heated to 60 C or above. However, an increase in reducing group liberation observed with preparations heated at 60 C indicated that some hydrolysis occurred. The hydrolysis products were either too large or too few for detection by chromatography.

Chromatography of enzyme-substrate mixtures where the enzyme was previously heated to 50 C revealed the presence of xylose in mixtures buffered at pH 5.0, 5.5, and 5.8. Xylobiose was present in mixtures buffered at pH 5.8 and 6.5. These results may indicate a suppression of xylanase activity at pH 6.5 assuming that the substrate was hydrolyzed to xylobiose prior to hydrolysis to xylose. However, these data may also indicate the presence of more than one xylanase in the enzyme preparation. The absence of xylose in mixtures buffered

at pH 6.5 may indicate the inactivation of an enzyme that liberated single xylose units from the substrate. The presence of xylobiose in mixtures buffered at pH 5.8 and 6.5 may indicate the presence of an enzyme that cleaved the substrate to xylobiose.

Chromatography of hydrolysis products of enzyme-substrate mixtures used in optimum temperature and pH studies also suggested the presence of more than one enzyme. Xylose and xylobiose were present in relatively large quantities in hydrolysates at optimum temperature and pH (35 C and pH 5.5, Table 3). However, at 35 C and pH 6.5 the amount of xylose present had decreased whereas xylobiose was still present in substantial quantity. The absence of xylose in mixtures incubated at 40 C and pH 6.5 and the increase of xylobiose over the levels in mixtures at the same pH and lower temperatures suggested that the absence of xylose in mixtures incubated at 40 C and pH 6.5 resulted from inactivation of a xylanase that liberated single xylose units from the substrate. The presence of xylotriose, xylotetraose, and larger xylose oligomers indicated the presence of an endoxylanase that randomly hydrolyzed the polymer.

Strobel (14) proposed that when corn cob xylan was utilized by *Diplodia viticola*, an enzyme was produced that released single unit branches of arabinose from the main chain. Sørensen (13) observed both arabinose and xylose-arabinose oligosaccharides in enzyme hydrolysates of xylan. Although arabinose was found to be present in the corn cob xylan used in this investigation, it was not observed as a product of enzymatic hydrolysis. This indicated that *V. albo-atrum* did not produce an arabinose specific enzyme under conditions of this study.

The presence of glucose in enzyme hydrolysates of this study suggests the possibility that *V. albo-atrum* produced an enzyme that released glucose from the main xylan chain or that glucose was removed by a xylanase that could not differentiate between the β -(1-4) glycosidic linkage of repeating xylose units and that attaching glucose to xylose.

Since xylans and other hemicelluloses are found associated with cell walls of higher plants (2, 6, 11), these constituents may be dissolved by extracellular hemicellulases of *V. albo-atrum*. Such enzymatic activity may be important to the final stage of the wilt disease syndrome since hydrolysis of xylans could facilitate passage of the fungus through the moribund plant and furnish the fungus with a carbon source.

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