Pure & Appl. Chem., Vol. 59, No. 12, pp. 1739–1752, 1987.
Printed in Great Britain.
© 1987 IUPAC

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY

APPLIED CHEMISTRY DIVISION

COMMISSION ON BIOTECHNOLOGY*

MEASUREMENT OF HEMICELLULASE ACTIVITIES PART 1: XYLANASES

Prepared for publication by

T. K. GHOSE and V. S. BISARIA

Biochemical Engineering Research Centre, Indian Institute of Technology, New Delhi–110016, India

* Membership of the Commission during the period 1981–1985 was as follows:

Chairman: 1981–83 H. Dellweg (FRG); 1983–85 C. L. Cooney USA); *Vice-Chairman*: 1981–83 C. L. Cooney (USA); 1983–85 M. Ringpfeil (GDR); *Secretary*: 1981–83 R. C. Righelato (UK); 1983–85 G. G. Stewart (Canada); *Titular and Associate Members*: H. T. Blachère (France; Titular 1981–83); V. K. Eroshin (USSR; Associate 1981–83); A. Fiechter (Switzerland; Associate 1981–83); T. K. Ghose (India; Titular 1981–85); P. P. Gray (Australia; Associate 1983–85); J. Holló (Hungary; Titular 1981–83); A. E. Humphrey (USA; Associate 1981–83); M. Linko (Finland; Associate 1983–85); R. C. Righelato (UK; Associate 1983–85); G. G. Stewart (Canada; Associate 1981–83); J. Takahashi (Japan; Titular 1981–83); J. E. Zajic (USA; Associate 1981–83); *National Representatives*: R. J. Ertola (Argentina; 1981–85); P. P. Gray (Australia; 1981–83); H. J. G. Wutzel (Austria; 1981–85); W. Borzani (Brazil; 1981–85); M. Moo-Young (Canada; 1983–85); B. Sikyta (Czechoslovakia; 1981–85); K. Von Meyenburg (Denmark; 1981–85); H. Dellweg (FRG; 1983–85); M. Linko (Finland; 1981–83); L. Penasse (France; 1983–85); M. Ringpfeil (GDR 1981–83); J. Holló (Hungary; 1983–85); V. Jagannathan (India; 1983–85); L. Goldstein (Israel; 1983–85); F. Parisi (Italy; 1983–85); S. Fukui (Japan; 1981–85); B. G. Yeoch (Malaysia; 1983–85); O. Ilnicka-Olejiniczak (Poland; 1981–83); E. Galas (Poland; 1983–85); A. Fiechter (Switzerland; 1983–85); V. Johanides (Yugoslavia; 1981–85).

Republication of this report is permitted without the need for formal IUPAC permission on condition that an acknowledgement, with full reference together with IUPAC copyright symbol (© 1987 IUPAC), is printed. Publication of a translation into another language is subject to the additional condition of prior approval from the relevant IUPAC National Adhering Organization.

Measurement of hemicellulase activities: Part I: Xylanases

The Commission on Biotechnology, International Union of Pure and Applied Chemistry at its meeting held on August 20-21, 1983 at Lyngby, Denmark decided to prepare a document incorporating recommendations on hemicellulase assay procedures and their evaluation. The decision was taken in light of the recently concluded global effort on preparation of a document on "Measurement of Cellulase Activities" which contains recommended cellulase assay procedures and their evaluation under process conditions. The cellulase document has since been adopted by the commission and published in February 1987 issue of Pure and Applied Chemistry.

We are aware that hemicellulases have been studied and characterized to a much lesser extent than cellulases. Further, hemicellulases are much more complex as they represent an array of enzymes such as xylanases, mannanases, arabinases (both endo and exo kinds) and their corresponding glycosidases.

The first step taken by us was to compile the information available on assay procedures being followed at various laboratories, which were contacted for this purpose. Those which responded are included in this report. Since most of them reported on xylanase assay procedures in response to our enquires, we propose to take up this assignment for xylanases only in the first phase. This is in view of the fact that xylanases are the most studied enzymes amongst hemicellulases. Other enzymes such as mannanases etc. may be taken up after completion of the first phase. The Commission may decide on the conduction of standard xylanase assays by those interested to participate on the basis of standard substrates, enzymes and methodologies as had been followed for cellulase assays.

CONTENTS

Preface
I. Introduction
II. Assay Procedures of Various Laboratories
III. Recommendations
References
International Collaborators

I. INTRODUCTION

Hemicellulases are a group of enzymes that are defined and classified according to their substrate hemicellulose. The hemicelluloses are polymers of xylose, galactose, mannose, arabinose, other sugars and their uronic acids. These are usually classified according to the sugar residue present. e.g., D-xylan, D-galactan, D-mannan, L-arabinan etc. However, they do not occur as homoglycans but rather as heteroglycans containing different types of sugar residues often as short appendages linked to the main backbone chain. Examples of these include L-arabino-D-xylan (wheat flour), L-arabino-D-glucurono-D-xylan (grass), D-glucurono-D-xylan (wood) etc. The hemicelluloses rank next to cellulose as the most abundant natural carbohydrate polymer in the biosphere. They are present in all layers of the plant cell wall but are concentrated mainly in the primary and secondary layers where they occur closely associated with cellulose and lignin. Unless the hemicelluloses can be properly utilized, biomass conversion based on cellulose utilization alone has little chance of becoming economically attractive due not only to the lost revenue from hemicelluloses but also to the added waste disposal costs.

The hemicellulases defined as glycan hydrolases (EC 3.2.1) attack the backbone chain of the hemicelluloses but are not responsible for cleavage of side-branch sugar appendages (mono-or oligosaccharides). The hemicellulases best characterized are 1,4-beta-D-xylanases and, in particular, those derived from fungal sources presumably because their substrates, xylans, constitute the largest proportion of hemicelluloses in pasture plants. The term "xylanase" refers to those enzymes which are capable of hydrolyzing the 1,4-beta-D-xylopyranosyl linkages of the 1,4-beta-D-xylans,namely, arabinoxylan, arabinoglucuronoxylan, arabino-4-0-methy-D-glucuronoxylan and glucuronoxylan. D-xylanases of this type have been assigned the Enzyme Commission numbers 3.2.1.8 (1,4-beta-D-xylan xylanohydrolase, endo-xylanase) and 3.2.1.37 (1,4-beta-D-xylan xylohydrolase, exo-xylanase).It may be mentioned here that the synergistic action of a multitude of different enzymes is required to hydrolyze a particular hemicellulose. For example,arabinoglucuronoxylans are degraded by the synergistic action of exo-glycosidases such as alpha-L-arabinosidases, alpha-D-glucuronidases and beta-D-xylosidases, and the exo-and endo-acting xylanases. (1)

Hemicelluloses from different plant sources have been used for assay of D-xylanase activity. The most commonly used substrates are arabinoxylan, arabinoglucuronoxylan, glucuronoxylan and xylan.

Soluble xylan derivatives that have been used include O- (carboxymethyl) -D- xylan and O-(hydroxyethyl)-D-xylan. Assay procedures usually involve measurement of the increase in reducing end groups by Somogyi- Nelson and Sumner reducing methods. Other assay procedures are based on the following methods: (i) loss in weight of recovered substrate after dialysis or following precipitation with ethanol, (ii) decrease in viscosity and (iii) measurement of clearance zones in hemicellulose agar e.g. the cup-plate assay.

D-xylanases are potentially of either the exo- or the endo-type. There are many claims in the literature on isolation of exo-xylanases, but such enzymes are difficult to distinguish from beta-D-xylosidases, as both are capable of degrading D-xylooligosaccharides of D.P. $\geqslant$ 2 but by definition only the former should attack xylan. The two enzymes can, however, be distinguished from each other by nuclear magnetic resonance spectroscopic analysis which can determine the configuration of the glycose residue released. Retention of configuration indicates a glycosidase and inversion of configuration, an exo-glycanase. Though exo-xylanases have been reported to be produced extracellularly by fungi such as Aspergillus & Coniophora,they have not been so extensively purified as endo-xylanases. Demonstration of exo-xylanases have been complicated due to the fact that the same results would be obtained if the enzyme preparation consisted of a mixture of beta-D-xylosidase and an endo-D-xylanase. Thus, unless exo-xylanases have been shown to be homogeneous, their properties and mode of action should be interpreted with caution.

Fungi have been the most common source of endo xylanases. The endo xylanases can be divided into two groups: (i) those liberating L-arabinose from enzymic hydrolysis of arabinoxylans and arabinoglucuronoxylans (i.e., arabinose-liberating xylanases) and (ii) those which do not liberate L-arabinose from these substrates (i.e.,the non arabinose-liberating xylanases). Both types are, however, capable of degrading glucuronoxylans and D-xylans.

Because of the involvement of different enzymes in the break down of various xylan polymers and in view of the applied nature of most hemicellulase work, it is understandable that investigators in different laboratories have each developed a series of assay procedures. While a common approach is shared, a situation nonetheless exists in which comparision of xylanase activities between laboratories in a quantitative manner is not readily possible.

II. ASSAY PROCEDURES OF VARIOUS LABORATORIES

Commonwealth Scientific & Industrial Research Organization, Clayton, Australia

Endo-1,4-beta-D-xylanases produced by the fungi Ceratocystis paradoxa, Cephalosporium sacchari, Sclerotium, rolfsii, a Monilia sp., Trichoderma reesei QM 9414 and C-30, Aspergillus niger, and Sporotrichum pulverulentum have been studied.

In assaying for xylanase activity it is quite difficult to find a suitable substrate (heteroxylan) which will show a linear relationship between the amount of reducing sugar produced and the time of hydrolysis. Many heteroxylans, depending on their source, are soluble in water and buffers of pH 4.0 - 6.0. Other xylan substrates are,however, sparingly soluble in water or buffers.For example, hemicellulose B is highly branched and likewise soluble in water (2). On the other hand, hemicellulose A is less branched and sparingly soluble in water. Whether the heteroxylan substrate is water soluble or not is not important provided the relationship between enzyme activity and hydrolysis time (at least up to 30 min) is linear. Such a relationship exists for a crude xylanase preparation from T.reesei QM 9414 and S.rolfsii when using hemicellulose A isolated from sugarcane bagasse as substrate (3). The relationship was linear over the first 20 min of hydrolysis. No linear relationship was observed for some other hemicellulosic xylans examined, e.g.,hemicellulose B (bagasse), and larchwood xylan (Sigma). When T.reesei QM 9414 beta-D-xylosidases were assayed, a linear relationship was observed for enzyme activity versus hydrolysis time of up to 25 min using o- and p-nitrophenyl-beta-D-xylopyranosides (Sigma) as substrates. Both enzyme assays were conducted at 50°C using 50mM citrate-phosphate buffers at pH 5.0 and 4.0 respectively.

A linear relationship between enzyme activity and enzyme concentration is also important. Such a relationship was observed for T.reesei QM 9414 beta-D-xylosidase in the range of 0- 0.3 IU/ml. A similar linear relationship could not be observed with T. reesei QM 9414 xylanase. The reason for this is believed to be due to the presence of inhibitory compounds in the enzyme preparation (probably phenolic in nature which are released from lignocellulose during growth of the fungus). Inhibition of xylanase activity can be alleviated to some extent by the removal of the inhibitors from the enzyme preparation by their adsorption on to polyvinylpyrrolidone, but even after this step the relationship was still not linear. Where inhibitory material is present in enzyme preparation, it is best to dilute the enzyme preparation such that the concentration of the inhibitor is low, so as not to interfere with enzyme activity and use the diluted preparation to assay for enzyme activity. Under these conditions, the initial velocity of reaction should be measured over as short an interval of time (e.g., 1-5 min) as possible.

Reducing sugars liberated during the course of hydrolysis of hemicellulose in the aforementioned experiments were measured by the method of Nelson (4). Absorbance measurements were made on a Pye Unicam SP-550 UV-Vis Spectrophotometer equipped with a flow through cell. The standard xylose curve was calibrated with D-xylose (BDH) within the range of 0-180 ug/ml.

The assay procedures used for T. reesei QM 9414 enzymes are as follows:

Xylanase: Incubate enzyme (0.1 ml of cell-free culture fluid or likewise suitably diluted) with hemicellulose A (from bagasse, 20 mg dry weight; or use 0.8 ml of a 2.5%, w/v, hemicellulose A solution in 50 mM citrate-phosphate buffer, pH 5.0) and citrate-phosphate buffer (pH 5.0) in a final volume of 1.0 ml at 50°C for 20 minutes. Remove an aliquot (0.1 ml), dilute with water if necessary, and determine the amount of reducing sugars by Nelson method.

Calculation:

$$\text{Xylanase Activity} = A_{520} \times DF \times (1/X) \times (1/Y) \times (1/t) \times (1/\text{slope})$$

where A_{520} is the absorbance at 520 nm of the total reducing sugars produced; X is the volume of enzyme used; Y is the volume of enzymic digest (hydrolyzate) used for assay of reducing sugars; t is the time of hydrolysis (20 min); 1/slope is determined from a standard curve of A_{520} versus xylose concentration (umoles/ml); and DF is the dilution factor (either for enzyme or hydrolyzate dilution).

Xylanase activity is expressed as the number of umoles of reducing sugars produced per minute of hydrolysis per ml of enzyme used; i.e.,the number of international units per ml.

Beta-Xylosidase: A typical assay consisted of incubating enzyme (0.1 ml cell-free culture fluid or a suitably diluted solution), p-nitrophenyl-beta-D-xyloside (Sigma, 0.5 ml of a 2.5 mM solution in 50 mM citrate-phosphate buffer, pH 4.0) and citrate-phosphate buffer (pH 4.0) in a final volume of 1.0 ml at 50°C for 20 minutes. The reaction was terminated by addition of 4 ml 0.25 M Na_2CO_3 solution and the absorbance measured at 400 nm.

Calculation:

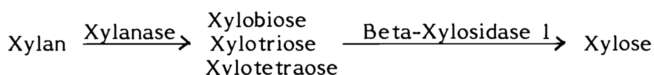
$$\text{Beta-xylosidase activity} = A_{400} \times DF \times (1/X) \times (1/t) \times (1/\text{slope})$$

where A_{400} is the absorbance at 400 nm of p-nitrophenol; X is the volume of enyzme used; t is the time of hydrolysis (20 min); 1/slope is determined from a standard curve of A_{400} versus p- nitrophenol concentration (umoles /ml); and DF is the dilution factor of enzyme solution.

Beta- Xylosidase activity is expressed as the number of umoles of p-niotrophenol liberated per minute of hydrolysis per ml of enzyme solution; i.e., number of international units per ml.

Osaka University–(I), Osaka, Japan

Degradation of xylan by Bacillus pumulus IPO takes place as follows:



Xylanase: The reaction mixture consisting of 1 ml of 1% xylan solution in 50 mM phosphate buffer, pH 6.5, and 0.5 ml of enzyme solution was incubated at 40° C for 10 min The reducing sugar liberated was measured by addition of 5 ml Somogyi´s reagent, and boiled for 10 min, cooled and titrated with 0.0025N $Na_2S_2O_3$ after addition of 1.0 ml of 5N H_2SO_4.

Preparation of xylan: Boil xylan (larch-wood, Sigma No. X-3875 Lot 128C- 03641) in 50 mM potassium phosphate buffer, pH 6.5, until well dissolved.

Xylosidase: The enzyme solution was prepared by disrupting the cell by sonication at 20 MHz for 3 min (1 min interval) at 0°C. The extract was then centrifuged at 10,000 g for 10 min and the supernatant was used for enzyme assay. The reaction mixture composed of 1 ml of 1 mg/ml p-nitrophenyl-beta-D-xyloside (Koch-Light) in 50 mM phosphate buffer, pH 7.0, and 1 ml enzyme was incubated at 37°C for 10 min. The reaction was stopped by adding 2.0 ml of 0.4 M Na_2CO_3 and the absorbance of p-nitrophenol released was measured at 405 nm. The enzyme activity was expressed as umoles of p-nitrophenol liberated per ml per minute.

Bakh Institute of Biochemistry, Moscow, USSR

Endoxylanase: The activity of endo-1,4-beta-D-xylanase was assayed by the viscosimetry method. Incubation mixture consisted of 3 ml of 1% carboxymethyxylan, 0.9 ml of 0.1 M sodium acetate buffer (pH 4.2) and 0.1 ml of the enzyme solution. Viscosity was assayed in a thermostat at 40°C at 2 min intervals. The time of the efflux of incubation mixture was measured. Activity of endo-

1,4-beta-D-xylanase was estimated by the velocity of relative viscosity:

$$1/n = To / (T-To)$$

Where To is the time of water efflux, T is the time of efflux of reaction mixture, n is the relative viscosity. The enzyme quantity providing the increase of the inverse value of relative viscosity by 1 over 1 min was assumed to be the activity unit.

Beta-Xylosidase: Assay of activity of exo-1,4-beta-D-xylosidase in relation to methyl-beta-D-xylopyranoside: 0.5 ml of 9 mM methyl-beta-D-xylopyranoside in 0.1 M acetate buffer (pH 4.2 was incubated for 30 min at 40°C in a thermostat. The reaction was stopped by adding 1 ml of the Somogyi reagent(5).The intensity of staining was measured in a Unicam spectrophotometer (Unicam SP-700) at 508 nm. The calibration curve was constructed for xylose in the concentration range of 0.3 - 2.0 mM.

Activity in relation to p- nitrophenyl-beta-D-xylopyranoside was determined in the following way. The reaction mixture, containing 0.5 ml of 2 mM p-nitrophenyl-beta-D-xylopyranoside in 0.1 M acetate buffer, pH 4.2, and 0.5 ml of the enzyme solution was incubated at 40°C for 30 min in a thermostat. The reaction was stopped by adding 3 ml of 0.13 M Na_2CO_3. The intensity of the staining was measured in Unicam SP-700 spectrophotometer at 400 nm. The calibration curve was constructed for p-nitrophenol in the concentration range of 0.02 - 0.1 mM.

Institute of Biotechnology, Julich, FRG

Endoxylanase (ex. Aspergillus niger):

Reagents : All chemicals (p.a) were from E. Merck (Germany)

Nelson A	25 g	Na_2CO_3 (anhydrous)
	25 g	K-Na-tartarate
	200 g	Na_2SO_4 (anyhdrous)
	20 g	$NaHCO_3$

dissolve in about 800 ml H_2O and dilute to 1000 ml. Store at room temperature.

Nelson B:	15 g	$CuSO_4.5H_2O$ in 100 ml water containing two drops of concentrated H_2SO_4
Nelson A/B:		Mix 25 ml Nelson A + 1 ml Nelson B
Nelson C.	25 g	$(NH_4)_6Mo_7O_{24}.4H_2O$/ 450 ml H_2O; add 21 ml concentrated H_2SO_4; mix and add 3 g $Na_2HAsO_4.7H_2O$/ 25 ml

keep at 37°C for 2 days and store in a brown bottle at room temperature.

Substrate: Homogeneous 2% suspensiion of ultrasonic treated xylan from oat husks (Roth, Karlsruhe, Germany) in 0.05 M acetate buffer, pH 4.6.

Enzyme assay:
1 ml 2% xylan suspension
+ 0.9 ml acetate buffer (0.05 M, pH 4.6)
+0.1 ml enzyme

Blank :
1 ml 2% xylan suspensiion
+1 ml buffer

incubate 20 min at 40°C; stop the reaction by adding 2 ml of Nelson A/B; centrifuge to remove the unhydrolyzed xylan.

Determination of reducing sugars: Boil the reaction mixture (0.4 ml) at 100°C for 20 minutes, add 0.2 ml Nelson C, mix and dilute to 5 ml with H_2O. Measure the extinction at 520 nm.(Standard: xylose). One unit of xylanase activity is defined as that amount of enzyme which will release one umole of reducing sugars (as xylose) per minute.

Exo-1,4-beta-D-xylosidase:

Reagents: p-Nitrophenyl-beta-D-xylopyranoside pure (PNXP) (Koch-Light); 0.05 M citrate-phosphate buffer, pH 3.0, and 10% Na_2CO_3.

Substrate: 5 mM solution of PNXP in 0.05 M citrate-phosphate buffer, pH 3.0.

Enzyme assay 1.9 ml 5 mM PNXP solution
+ 0.1 ml enzyme

Blank: 1.9 ml 5 mM PNXP solution
+ 0.1 ml buffer

Incubate at 40°C: stop the reaction by adding 2 ml of 10% Na_2CO_3 solution, mix and measure the extinction at 400 nm. (Standard: p-nitrophenol).

One unit of xylosidase activity will hydrolyze 1 umole of PNXP to p-nitrophenol and xylose per minute.

Standard deviation(s)

		Activity (U/ml)	
Enzyme	n	$\overline{X} \pm S$	S(%)
Beta-xylanase	5	806 ± 12	1.5
Beta-xylosidase	5	27 ± 0.2	0.7

University of New South Wales, Kensington, Australia

Bacterial enzyme assays are conducted at 37°C, pH 7.2. Fungal enzyme assays are conducted at 50°C, pH 4.8.

Xylanase

Principle: This assay is based on measurement, by the DNS reagent, of xylose equivalents (XE) liberated from xylan (actually an arbinoxylan) as a result of enzyme action for over 60 minutes. XE measured in a control are deducted from those found in the test before conversion of XE units to enzyme activity units.

Rickard and Laughlin (6) demonstrated that the error incurred due to lack of linearity between enzyme concentration and XE liberation could be minimized by comparing the XE liberated by an unknown preparation with those liberated by a standard enzyme preparation. For this purpose a standard activity curve of XE vs. time of action of the standard enzyme was prepared.

The unknown preparation is diluted to release between 100 and 800 ug (preferably between 300 and 800 ug) XE/60 min and incubated for 60 min; the XE are determined and the control value deducted. The time taken for the standard enzyme preparation to release the same XE value as the unknown is then read from the standard activity curve. The unit of enzyme activity in the unknown are calculated from a formula which accounts for:(a) differences in the times for the standard and unknown to liberate the same XE value, (b) the known activity of the standard; and (c) the dilution factor of the unknown.

When an enzyme preparation was diluted over a range of 1 to 20, values shown in the following table were recorded using the above procedure.

Conversion of Xylose Equivalents to Enzyme Activity			
Dilution Factor	Xylose Equivalents Released by Diluted Enzyme (ug/ml)	Activity of Undiluted Enzyme (munits/ml)	
1	780	192	
2	480	143	
5	315	181	Mean =179
10	210	217	
20	100	163	
	Standard deviation = 25		

Our enzyme preparations require dilutions of between 100 fold and 1000 fold to bring them into the range of the table. We find that two different dilutions , both within the range of a given preparation, yield values of enzyme activity which have a coefficient of variance of approximately 14%. In order to be doubly cautious, we consider variations between different preparations to be significant only if they vary by 30% or more.

Materials:

1. McIlvaine's buffer (pH 7.2) for bacterial enzyme assays (7)
   Mix 13.8 ml of 0.1 M citric acid (21 g $C_6H_8O_7.H_2O.H_2O/l$)with 86.2 ml of 0.2 M Na_2HPO_4(35.6g $Na_2HPO_4.2H_2O/l$). Use ten-fold diluted buffer to dilute bacterial enzyme preparations.

2. Citrate buffer (pH 4.8) for fungal enzyme assays
   prepare 0.05 M citrate buffer (pH 4.8)

3. DNS Reagent (8)
   Dissolve in about 600 ml distilled water in order of:

10 g	sodium hydroxide
192 g	potassium sodium tartarate (Rochelle salt)
10 g	dinitrosalicylic acid (add slowly while stirring)
2 g	phenol
0.5 g	sodium sulphite.

   Make up to 1 litre.

4. Xylan (arabinoxylan prepared from oats spetts (Fluka AG, Buchs SG, Switzerland). The sugar composition was found to be 73% xylose 12% arabinose and 15% glucose).
   1% (w/v) suspended in water. Maintain a uniform suspension by continuous stirring while aliquots are being taken.

5. Standard xylose (Aldrich Chemical Co.)
   1000 ug of xylose per ml of water (stored at 4°C over chloroform).

Methods:

Preincubate buffer, xylan and enzyme preparations separately for 10 min in water bath set at desired temperature. Prepare tests, controls and standards in duplicate.

Tests : Mix the following in an Erlenmeyer flask (25 ml capacity):

1 ml of buffer
1 ml of xylan
1 ml of enzyme preparation

Place in a water bath and shake (with orbital motion) for 60 min. Then immediately add 3 ml of DNS reagent, mix, transfer to test tubes and place in a boiling water bath for 15 min.*

Controls: Mix the following in an Erlenmeyer flask (25 ml capacity)
1 ml of buffer
1 ml of xylan

Place in a water bath and shake (with orbital motion) for 60 min. Then immediately add 3 ml of DNS reagent followed by 1 ml of enzyme preparation. Mix,transfer to test tubes and place in a boiling water bath for 15 min*.

Standards : Into six test tubes introduce the following:

Standard Xylose (ml)	Water (ml)	Buffer (ml)
1.0	1.0	1.0
0.8	1.2	1.0
0.6	1.4	1.0
0.4	1.6	1.0
0.2	1.8	1.0
0.0	2.0	1.0

To all six tubes add 3 ml of DNS reagent, mix and place in a boiling water bath for 15 min.*

NOTE: Tests, controls and standards should be treated with DNS and boiled at the same time.

Absorbance: Absorbance is read at 640 nm in a Spectronic 21(Bausch & Lomb spectrophotometer) of tests, controls and standards, using the standard blank (containing no xylose) for all readings. Use cuvettes rather than tubes. If cloudy, centrifuge first before reading.

Determine the mean absorbance for each pair of duplicates. Construct a standard curve and determine the reducing sugar concentration of the tests and controls. Deduct the reducing sugar of the control from that of the test and calculate the reducing sugar liberated per ml of enzyme preparation.

Conversion of reducing sugar units to xylanase activity:

For conversion to be accurate, enzyme should be diluted so that 1 ml liberates between 300 and 800 ug of reducing sugars (measured as xylose) in 60 min.

Definitions:

A_u = activity in milliunits per ml of the unknown (sample being tested)

A_s = activity of a standard enzyme (217 milliunits/ml= 217 nanomoles of xylose equivalents liberated /ml/min).

t_u = incubation time of the unknown, 60 min.

t_s = time required by 1 ml of standard enzyme to liberate the same number of ug of xylose equivalents as liberated by 1 ml of the unknown in 60 min; (obtained by reference to the standard xylanase activity curve). See Fig.1.

D_u = dilution factor of the unknown

V = total volume of enzyme preparation obtained from cellular fraction.

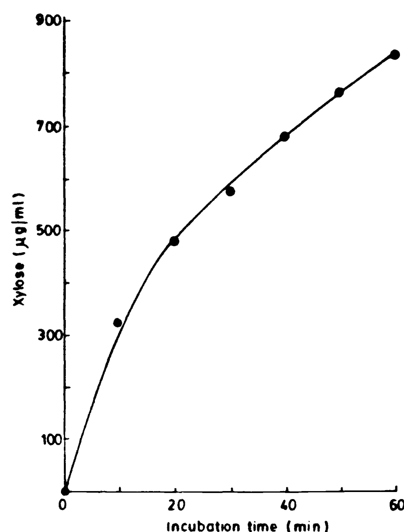


Fig.1 : STANDARD XYLANASE ACTIVITY CURVE

Formulae:

1. $$A_u = \frac{A_s \times t_s \times D_u}{t_u}$$

$$= \frac{217 \times t_s \times D_u}{60}$$

2. Activity/ ml of culture fluid

= A_u for extracellular enzyme

= A_u x V/200 for enzyme preparations obtained from cellular fraction (200 being total ml of culture fluid)

Limits of Sensitivity for xylanase activity are 14 munits/ml of culture fluid for the extracellular enzyme and (14 x V/200) munits/ml of culture fluid for the cellular enzyme. Values less than these should be expressed as less than 14 or "appropriate value", respectively.

Beta-Xylosidase (including other glycosidases) **:**

Materials :

1. Buffer - as described for xylanase
2. Nitrophenyl glycosides (NPG) : 0.1% (w/v) of p-nitrophenyl glycoside dissolved in water. This should be stored at 5° and discarded if hydrolysis has occured- yellow colour present. The glycosides listed in the following table are routinely used and obtained from Sigma.

Enzyme	Substrate
Alpha-L-arabinofuranosidase	p-nitrophenyl-alpha-L-arabinofuranoside
Alpha-galactosidase	p-nitrophenyl-alpha-D-galactopyranoside
Beta-galactosidase	p-nitrophenyl-beta-D-galactopyranoside
Exo-1,4-beta-xylosidase	p-nitrophenyl-beta-D-xylopyranoside
Alpha-glucosidase	p-nitrophenyl-alpha-D-glucopyranoside
Beta-glucosidase	p-nitrophenyl-beta-D-glucopyranoside
Beta-glucuronidase	p-nitrophenyl-beta-D-glucuronide
Alpha-mannosidase	p-nitrophenyl-alpha-D-mannopyranoside
Beta-mannosidase	p-nitrophenyl-beta-D-mannopyranoside

3. Standard Nitrophenol (NP) : p-nitrophenol (Hopkin and Williams, England) 30 ug/ml of water.

4. 1 M Na_2CO_3 : 10.6% (w/v) of Na_2CO_3 in water.

Methods :

Preincubate buffer, NPG and enzyme preparations as described for xylanase activity. Prepare tests, controls and standards in duplicate.

Tests : Mix the following in an Erlenmeyer flask (25 ml)

1 ml of buffer
1 ml of NPG
1 ml of enzyme preparation

Place in a water bath for 60 min (orbital motion shaking). Then immediately add 2 ml of 1M Na_2CO_3.

Controls: Mix the following in an Erlenmeyer flask (25 ml)

1 ml of buffer
1 ml of NPG

Place in a water bath for 60 min (orbital motion shaking).Then immediately add 2 ml of 1M Na_2CO_3 followed by 1 ml of enzyme preparation.

Standards : Into six test tubes introduce the following:

Standard(NP) (ml)	Water (ml)	Buffer (ml)
1.0	1.0	1.0
0.8	1.2	1.0
0.6	1.4	1.0
0.4	1.6	1.0
0.2	1.8	1.0
0.0	2.0	1.0

Incubate the six tubes at the required temperature for 10 min and then add 2 ml of 1M Na_2CO_3.

Absorbance: If solutions are cloudy, centrifuge first. Read absorbance at 400 nm as described for xylanase activity. Determine mean absorbances, prepare standard curve and determine product (in this case NP) liberated as described for xylanase activity.

Conversion of p-nitrophenol Units to Exo-glycosidase Activity:

Formulae :

1. A_u=activity in milliunits per ml(nanomoles of NP liberated/ml/min) of the unknown (sample being tested)

$$= \frac{P \times D_u}{t_u} \times \frac{10^3}{\text{mol. wt. of NP}}$$

$$= \frac{P \times D_u}{60} \times \frac{10^3}{139}$$

Where P = ug NP liberated by 1 ml of enzyme in 60 min.

2. Activity/ ml of culture fluid

= A_u for extracellular enzyme

= A_u x V/200 for enzyme preparation obtained from cellular fraction (200 being total volume of culture fluid)

See xylanase assay for definition of D_u, t_u and V.

Limits of sensitivity are 0.15 munits/ml of culture fluid for the extracellular enzyme and (0.15 x V/200)munits/ml of culture fluid for the cellular enzyme.Values less than these should be expressed as less than 0.15 or appropriate value, respectively.

Osaka University–(II), Osaka, Japan

Xylanase Activity : This method is based on the determination of reducing sugar released from xylan by xylanase action (9).

Reagents :

50mM acetate buffer, pH 5.0
10 mg/ml xylan from oat spetts (Sigma)
Somogyi- Nelson reagent
Partially purified xylanase

Method :

Place 1 ml of xylan solution in a test tube and incubate it in a water bath at 40°C. At zero time add 0.2 ml of a xylanase solution preincubated at 40°C. After 10 min add 2 ml of Somogyi solution to stop the reaction and heat the test tube in boiling water for 15 min. After cooling it in running water, add 2 ml of Nelson reagent and mix it thoroughly. After leaving for 10 min, add 20 ml of water and mix it. Measure it at 660 nm with 1 cm light path cuvette by spectrophotometer. Calculate the reducing sugar from the reference curve prepared by using xylose. The control experiment was carried out by adding Somogyi solution before the addition of enzyme and the reducing sugar was measured without incubation as described.

One unit of enzyme activity was defined as an amount of enzyme to release 1 umol of xylose-equivalent sugar in one minute. Usually an enzyme activity, E (units/ml) is calculated as follows.

$$E = \frac{A - B}{10} \times 0.2 \times d$$

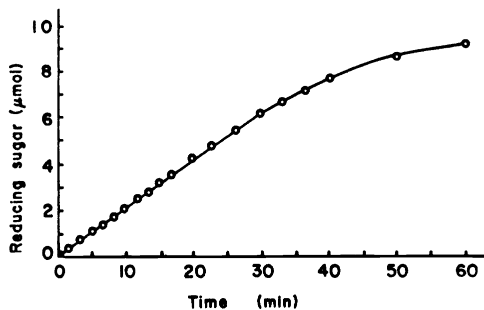
Where A (umol) and B (umol) are sugar concentrations of the enzyme reaction and the control experiment, respectively, d is dilution fold, 10 comes from 10 min reaction, and 0.2 comes from amount of enzyme used .

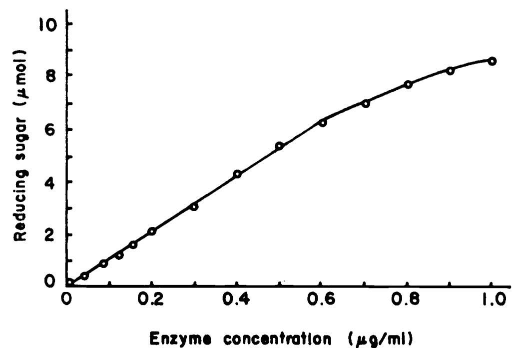
Experiments :

In Fig. 1, time course of enzyme reaction is shown. The reaction rate was constant until 30 min, that is, reducing sugar was released up to 6 umol at a constant rate.

In Fig. 2, effect of enzyme concentration on the reducing sugar formation is shown. The reducing sugar increased proportionally with the increase of the enzyme concentration up to 0.6 ug/ml. In this reaction mixture , 10 mg of xylan was present which meant that 75 umol of xylose unit were contained. So the hydrolysis of xylan proceeded linearly up to 8% .

In Fig. 3 effect of substrate concentration on the reducing sugar formation is shown . The reducing sugar formation increased up to 6 mg/ml of xylan and attained a plateau value at higher than 6 mg/ml. This meant that the substrate less than 6 mg/ml was not sufficient for the reaction and therefore 10 mg/ml of substrate was used in the enzyme reaction.





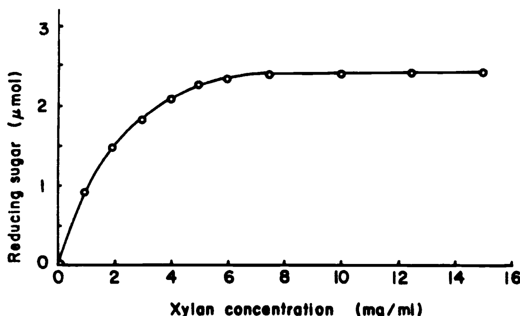


Fig.1 : Time course of xylanase reaction.

Xylanase was produced by cultivating <u>Humicola langinosa</u> in wheat bran,extracted with the acetate buffer, fractionated with ammonium sulfate of 40-80% saturation and dialyzed against the buffer. This enzyme preparation was free from cellulase activity. The enzyme reaction was carried out by using 0.2 ug/ml of enzyme.

Fig.2 : Effect of enzyme concentration on the reducing sugar formation.

Fig.3 : Effect of substrate concentration on the reducing sugar formation. The reaction was carried out by using 0.2 ug/ml of enzyme.

Following above experiments, the reaction conditions as described in Method were set up.

Iowa State University, Ames, IA, USA

Endoxylanase : The basic assay for the endoxylanases was conducted using the Somogyi-Nelson reducing sugar assay. Samples of 20-100 uL were mixed with 0.25 ml of approximately 1% soluble xylan (2% Koch-Light larchwood xylan dissolved in water at room temperature and the undissolved material centrifuged out) and 0.75 ml of 0.075 M sodium acetate at pH between 4.8 and 5.5. The mixture was incubated for 20 min at 40°C. One ml of Somogyi solution was added and the resulting mixture was boiled for 20 min. After cooling to room temperature, the mixture was treated with 1 ml Nelson solution. Its absorbance was measured at 500 nm in a 10 mm pathlength cuvette. The blank was measured on a mixture in which water replaced the enzyme.

One unit was defined as the amount of enzyme that released sugars equivalent to 1 umol of xylose in 1 min under the conditions of the assay.

When insoluble xylan was used as substrate, it was taken from the solid pellet that resulted from centrifugation of a 2% aqueous xylan mixture. The insoluble xylan was slurried to a concentration of 1% in water, and the assay was conducted as before. The final mixture was centrifuged if necessary before reading the absorbance.

Debranched soluble and insoluble xylan were produced by hydrolysis of the arabinosyl-initiated side chains with H_2SO_4 at pH 2 and 100°C for 90 min. The soluble fraction was neutralized with $Ba(OH)_2$, and the precipitate was centrifuged out. The insoluble fraction was neutralized with NaOH and washed with water. Because the released arabinose yielded a high blank, these substrates were employed at 0.1% concentration.

Beta-xylosidase : Beta-xylosidase was assayed by mixing 0.1 ml of the enzyme with 0.9 ml of 0.05 M sodium acetate buffer at pH 4.0 and with 1 ml of 4 mM of o- nitrophenyl-beta-D-xylopyranoside. After 15 minutes incubation at 40°C the reaction was stopped by adding 2ml of 20% Na_2CO_3.

The absorbance of the released o-nitrophenol in a 10 mm pathlength cuvette was measured at 400 nm. Net absorbance was that of the reaction sample minus that of a blank sample taken before the reaction commenced. Enzyme was diluted so that less than 10% of the substrate was hydrolysed. One unit was defined as the amount of enzyme that hydrolyzed 1 umol of substrate in 1 minute.

Indian Institute of Technology, Delhi, India

Xylanase Assay :

Reagents :

Solution A :	Na_2CO_3	2.5 g
	Potassium sodium-tartarate	2.5 g
	$NaHCO_3$	2.0 g
	Na_2SO_4	20.0 g

The volume of the solution was made upto 100 ml

Solution B :	$CuSO_4.5H_2O$	15 g
	H_2SO_4 (36N)	0.1 ml

The final volume of the solution was 100 ml.

Solution C : 25 parts solution A is mixed with 1 part solution B. This is prepared fresh.

Solution D : 5 g $(NH_4)_6Mo_7O_{24}.4H_2O$ is dissolved under constant (magnetic) stirring in 70 ml H_2O followed by mixing of 4.2 ml 36N H_2SO_4. To this 0.6 g $Na_2HAsO_4.7H_2O$ solution in 25 ml H_2O is added and mixed thoroughly. The solution is incubated for 24 h and 37°C(unshaken). The final reagent is preserved in a brown glass bottle with glass stopper at 4°C (shelf life: one month).

Citrate Buffer: 0.05 M sodium citrate buffer (pH 5.3) is prepared fresh from 1M stock solutions.

Preparation of xylan suspension : 1 g xylan (ex. larch saw dust, Sigma) is taken in a mortar and ground to finer particles. 10 ml of 0.05M NaOH is added and mixed vigorously with magnetic stirring for 15 min. The pH of the suspension is adjusted to 5.3 with glacial acetic acid and finally diluted to 100 ml by citrate buffer. The suspension is stable at 4°C for 15 days.

Standard of xylose solution: 1 mg/ml D(+) xylose (BDH,England) solution is prepared in 0.05M citrate buffer (pH 5.3).

Method :

To 1 ml of properly diluted enzyme solution in citrate buffer (0.05M, pH 5.3), 1 ml of 1% xylan is added. The mixture is incubated at 50°C for 30 min (unshaken). The reaction is stopped by addition of 1 ml Copper reagent (Solution C) and kept in boiling water for 10 min. The mixture is cooled at 30°C and 1 ml of arsenomolybdate reagent (Solution D) is added and mixed thoroughly. 15 ml water is then added. After proper mixing and centrifugation, absorbance of the mixture is taken at 500 nm. The resulting sugars are estimated as xylose.

Suitable enzyme and substrate blanks are included. The activity is expressed as umol of xylose produced per minute per ml of enzyme (IU/ml).

Nakamura Gakuen College, Fukuoka, Japan

Xylanase Assay: Substrate of xylanase was prepared from rice straw or corn cob by alkaline extraction.

Xylan from rice straw or corn cob was fractionated into water soluble part (sol.X) and insoluble part (insol.X). Xylan powder was also introduced to water soluble derivative, glycol xylan (G.X) with ethylene oxide.

Enzyme assay of xylanase was performed as follows.

Reducing power method :

The reducing sugar produced by enzyme action from substrate (G.X or Sol.X) was measured by Somogyi-Nelson method.

Turbidity method :

Enzyme activity against insol.X was determined by turbidity method. Enzyme solution was added to the substrate which was suspended in a buffer, and the decrease of turbidity was estimated by a spectrophotometer at 660 nm.

One turbidity unit was defined as the amount of enzyme which was able to decrease one optical density unit at 37°C in 3 min.

Viscosity method :

The decrease of viscosity of G.X. by enzyme was measured with Ostwald viscosimeter at 30°C.

One unit of viscometric activity was defined as the amount of enzyme to decrease one specific viscometric unit in 3 min incubation.

Products from sol.X or xylooligosaccharide, which appeared in the incubation mixture, were examined by paper chromatography.

US Army Natick Research & Development Laboratories, Natick, MA, USA

Xylanase :

The biggest problem in assay is in obtaining a suitable substrate. Many are not commercially available, and those, that are, may require purification. If contaminating polysaccharides are present, the activity which is intended to be measured may not actually be measured. Actual assay conditions are simple:

0.5 ml substrate (5 mg/ml)
+0.5 ml enzyme (appropriate dilution)
(both in buffer, usually 0.05 M citrate, pH 4.8 for fungal enzymes)

Incubate for 30-60 min at appropriate temperature, usually 50°C for fungal enzymes. Measure reducing sugar by DNS or other procedure. Calculate international units. Corrections must be made for appropriate blanks.

For purified enzymes, or fractions after separation (to distinguish endo- and exo-enzymes etc.) one may wish to examine products by HPLC.

Beta-D-xylopyranosidase :

The enzyme solution (0.5 ml) was added to the 0.5 ml methyl-beta-D-xylopyranoside (10 mg/ml in 0.05 M citrate buffer, pH 3.0). Incubate at 50°C for 60 min. Xylose was determined by the DNS method. Activity was expressed as equivalent of umol xylose produced/min/ml.

III. RECOMMENDATIONS

The principal objective of this document is to focus on the xylanase assays currently practised by many groups and how far these or their modified versions can be acceptable as standard methods for international usage. It is found that even though the results of these assays are expressed in some uniform international units, these are not strictly comparable because of the use of substrates of different chemical make-up by different laboratories. Furthermore, each assay method has some inherent advantages and disadvantages. With these in mind, an attempt has been made to recommend tentative basic methods for xylanase assays.

Endo-beta-D-xylanase :

Substrate

The biggest problem seems to be the non-availability of suitable substrate commercially which would show linear relationship between the amount of reducing sugars produced and the time of hydrolysis. The modified substrates may not be used as they tend to alter the native structure of the xylan. Keeping this in view, we suggest the use of either xylan (ex larch saw dust,Sigma) which is used by most workers or of arabinoxylan (ex oats, Fluka) used by Dr.P.A.D.Rickard of the University of New South Wales, Australia.

Enzyme

Availability of a standard enzyme preparation for comparison of the activity of unknown enzyme preparations (as followed by Dr. P.A.D. Rickard of University of New South Wales) will minimize the errors involved on account of absence of linearity between enzyme concentration of unknown samples and xylose liberation. In absence of a standard enzyme preparation, the conditions for linear relationship must be experimentally established before proceeding with an unknown enzyme sample for assay. It is likely that Dr. Rickard may be helpful in providing standard enzyme preparation for use by those who intend to conduct xylanase assays. Conditions of assay with respect to time, temperature, molarity of buffer and pH will be those pertaining to the standard enzyme.

Beta-D-Xylosidase :

Though most researchers are using either o-or p-nitrophenyl-beta-D-xyloside or methyl-beta-D-xyloside, xylobiose (or xylodextrins) should be the preferred substrate because these are the natural hydrolysis products of the xylan polymer. However, in view of the nonavailability of xylobiose (or the xylodextrins) commercially, p-nitrophenyl-beta-D-xyloside (PNPX) may be used as substrate for the time being. The assay using PNPX does not pose any problem.A standard enzyme preparation is also to be used for conduction of this assay by different laboratories.

Some researchers who could not send information on xylanase assay procedures followed in their laboratories but are actively involved in xylanase research may also be requested to participate in the conduction of standard xylanase assays.

The Commission is advised to entrust the job of supplying standard substrates, enzymes and assay procedures to one or two laboratories amongst several who participated in the preparation of this report. Following receipt of results from these laboratories on xylanase activity measurements, a workshop may be convened by the Commission to discuss and adopt the final recommendations. The procedures to be followed may be on the same lines as was done in the case of standard cellulase assays (10).

REFERENCES

1. R.F.H. Dekker, Biosynthesis and Biodegradation of Wood Components, T.Higuchi, ed., p. 505-531 Academic Press (1985)
2. T.E.Tinell, Methods in Carbohydrate Chemistry, 5, 135-141 (1965)
3. R.F.H. Dekker, Biotechnol. Bioeng. 25, 1127-1146 (1983)
4. N.Nelson, J. Biol. Chem., 153, 375-380 (1944)
5. M.Somogyi, J. Biol. Chem. 165, 19-28 (1952)
6. P.A.D. Rickard and T.A. Laughlin, Biotechnol. Lett., 2, 363-368 (1980)
7. Documenta Geigy Scientific Tables, 6th ed., Geigy Pub. Co., Manchester, p. 314-315
8. G.L. Miller, Anal. Chem., 31 (3), 426-428 (1959)
9. S. Kinoshita, T. Seki, M. Kishimoto, H. Taguchi, V. Kitpreechavanich and S. Nagai, Annual Reports of International Centre of Cooperative Research and Development in Microbial Engineering, Osaka University, Japan, 4, 408-410 (1981).
10. T.K. Ghose, Measurement of Cellulase Activities, Pure Appl. Chem., 59, 257-268 (1987).

INTERNATIONAL COLLABORATORS

Name and address	Abreviation used in the text
1. Dr. R.F.H. Dekker CSIRO Division of Chem. & Wood Technology Bayview Avenue, Clayton Vic 3168 Australia.	CSIRO, Clayton, Australia.
2. Prof. H. Okada Dept. of Fermentation Technology Osaka University, Suita-Shi, Osaka 565, Japan.	Osaka University-(I), Japan.
3. Prof. H. Sahm, Institute of Biotechnology KFA, Julich D 5170 FRG.	IBT, Julich, FRG.
4. Dr. N.A. Rodionova and Dr. I.M. Tavobilov, Bakh Institute of Biochemistry USSR Academy of Sciences Moscow, USSR.	BIB , Moscow, USSR.
5. Dr. P.A.D. Rickard, School of Biotechnology, University of New South Wales NSW- 2033 Australia.	University NSW, Australia.
6. Dr. S. Kinoshita Dept. of Fermentation Technology, Osaka University, Suita-Shi, Osaka 565, Japan.	Osaka University- (II), Japan.
7. Prof. P.J. Reilly, Dept.of Chemical Engineering Iowa State University, Ames, Iowa 50011, U.S.A.	Iowa State University, U.S.A.
8. Prof. T.K. Ghose and Dr. V.S. Bisaria Biochemical Engineering Research Centre Indian Institute of Technology - Delhi, New Delhi- 110 016, India.	IIT Delhi, India.
9. Dr. S. Hashimoto Dept. of Food & Nutrition Nakamura Gakuen College 5-7-1, Befu, Jonan-ku, Fukuoka- 814, Japan.	NGC, Fukuoka, Japan.
10. Dr. E.T. Reese & Dr. M. Mandels, U.S.Army Natick Res. & Dev. Laboratories Natick, Mass 01760, U.S.A.	USANL, Natick, USA .