

# Megazyme

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## *endo*-1,4- $\beta$ -XYLANASE ASSAY PROCEDURES

XYL 7/01

1. Xylazyme AX Tablets
2. Viscometric Assay
3. Reducing-Sugar Assay
4. Azo-Wheat Arabinoxylan



## GENERAL INTRODUCTION:

Xylanase (*endo*-1,4- $\beta$ -D-xylan xylanohydrolase; EC 3.2.1.8) is finding widespread application in chicken feed supplements, in bread improver mixtures and in research on the enzymic bleaching of wood pulp (Nissen et al. 1992)<sup>1</sup>. Historically, xylanase activity has been measured with the DNS reducing sugar assay (Bailey 1988)<sup>2</sup> with purified xylan from oat spelts, larchwood or birchwood as substrate. These assays are limited by their lack of sensitivity and linearity, and the fact that they cannot be used to assay activity in materials containing high levels of reducing sugars e.g. chicken feeds and bread improver mixtures.

In this technical booklet, alternative assay procedures for the measurement of xylanase will be detailed, which involve the use of soluble or insoluble dyed xylans and high viscosity wheat arabinoxylan. These substrates and assay procedures are:

1. **Xylazyme AX Test Tablets.** The active constituent in these tablets is dyed and crosslinked wheat arabinoxylan. This test is accurate and reliable, has a sensitivity equivalent to viscometric assays, and is simple to use.<sup>3,4</sup>
2. **Viscometric Assays using Wheat Arabinoxylan.** A highly purified and standardised wheat arabinoxylan is employed in a viscometric assay of xylanase. This assay is accurate and reliable, but very tedious and does not lend itself to the routine assay of numerous samples. However, since the parameter of interest in the industrial use of xylanases is usually viscosity decrease of wheat arabinoxylan, we consider that this assay should be established as a reference method.<sup>5</sup>
3. **Reducing Sugar Assay using Wheat Arabinoxylan.** In most cases it is desirable and necessary to standardise xylanase activity in terms of the rate of bond cleavage in the native substrate, wheat arabinoxylan. For this reason, our recommended reducing-sugar procedure, based on the Nelson/Somogyi<sup>6</sup> procedure is included. We prefer the Nelson/Somogyi procedure because, unlike many other procedures (such as the DNS method<sup>2</sup>), it gives stoichiometric colour response for the same molar concentration of oligosaccharides of varying degrees of polymerisation.
4. **Azo-Wheat Arabinoxylan.** This substrate is supplied as a powder or in a ready to use liquid form. The substrate in liquid form is chemically and physically stable i.e. blank absorbance values remain constant over several years and the substrate shows little tendency to settle from solution even on storage at

4°C for extended periods. Alternative soluble, chromogenic substrates are Azo-Xylan (oat spelts) and Azo-Xylan (birchwood). Each of these substrates have many of the favourable characteristics of Azo-Wheat Arabinoxylan, but Azo-Wheat Arabinoxylan is the substrate of choice.

Of the methods described in this booklet, we recommend both the Xylazyme AX and the Azo-Wheat Arabinoxylan procedures because of their convenience, reliability and accuracy. Xylazyme AX has greater sensitivity than Azo-Wheat Arabinoxylan, therefore it is the method of choice where the test material contains very low levels of activity. With the Azo-Wheat Arabinoxylan, reproducibility of the assay is slightly better than with Xylazyme AX, thus, this is the substrate of choice where the test material contains high levels of enzyme activity.

## REFERENCES:

1. Nissen, A.M., Anker, L., Munk, N. and Krebs Lange, N. (1992). "Xylanases for the pulp and paper industry". In "Xylans and Xylanases" (Visser, J., Beldman, G., Kusters-van Someren, M.A. and Voragen, A.G.J. Eds) Elsevier Science Publishers, Amsterdam, pp.325-338.
2. Bailey, M.J. (1988). "A note on the use of dinitrosalicylic acid for determining the products of enzymatic reactions". *Applied Microbiology and Biotechnology*, **29**, 494-496.
3. McCleary, B.V. (1995) "Measurement of polysaccharide-degrading enzymes in plants using chromogenic and colorimetric substrates". In *New Diagnostics in Crop Sciences* (J.H. Skerritt and R. Appels, Eds) CAB International, pp 277-301.
4. McCleary, B.V. (1992). "Measurement of endo-1,4- $\beta$ -D-xylanase". In "Xylans and Xylanases" (Visser, J., Beldman, G., Kusters-van Someren, M.A. and Voragen, A.G.J. Eds) Elsevier Science Publishers, Amsterdam, pp. 161-170.
5. Buckee, G.K. and Baker, C.D. (1988). "Collaborative trial on the determination of  $\beta$ -glucanases in malt by viscometric and dye-labelled methods". *Journal of the Institute of Brewing*, **96**, 387-390.
6. Somogyi, M. (1960). "Modifications of two methods for the assay of amylase". *Clinical Chemistry*, **6**, 23-35.
7. McCleary, B.V. and Monaghan, D. (1999) "New developments in the measurement of  $\alpha$ -amylase, endo-protease,  $\beta$ -glucanase and  $\beta$ -xylanase. In "Proceedings of the Second European Symposium on enzymes in grain processing" (M. Tenkanen Ed.) VTT Information Service, pp 31-38.

# XYLAZYME AX TEST TABLET PROCEDURE

## SUBSTRATE:

The substrate employed is Azurine-crosslinked wheat arabinoxylan. This substrate is prepared by dyeing and crosslinking highly purified wheat arabinoxylan to produce a material which hydrates in water but is water insoluble. Hydrolysis by *endo*-(1-4)- $\beta$ -D-xylanase (xylanase) produces water soluble dyed fragments, and the rate of release of these (increase in absorbance at 590 nm) can be related directly to enzyme activity. The substrate is supplied commercially in a ready-to-use tablet form as **Xylazyme AX** tablets.

## BUFFERS:

### (A) Concentrated Acetate Buffer

[Sodium acetate (1 M, pH 4.7)]

Glacial acetic acid (60.0 g, 1.05 g/mL) is added to 800 mL of distilled water. This solution is adjusted by pH 4.7 by the addition of 4 M (16 g/100 mL) sodium hydroxide solution. The volume is then adjusted to one litre. Store at room temperature.

### (B) Acetate Extraction/Dilution Buffer

[Sodium acetate (25 mM, pH 4.7) containing sodium azide (0.02%)]

An aliquot (25 mL) of Concentrated Acetate Buffer is added to 950 mL of distilled water and sodium azide (0.2 g) is added and dissolved. The solution is adjusted to pH 4.7 by dropwise addition of 2 M hydrochloric acid solution, and the volume is adjusted to one litre. Store at room temperature.

### (C) Concentrated Phosphate Buffer

[Sodium Phosphate (0.5 M, pH 6.0).]

Add sodium dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ; 156 g) to 900 mL of demineralised water. Adjust the pH to 6.0 with 4 M NaOH. Adjust the volume to 2 litres and add 0.2 g of sodium azide. Store at 4°C.

### (D) Phosphate Extraction/Dilution Buffer

[Sodium Phosphate (25 mM, pH 6.0)]

Add an aliquot (50 mL) of Concentrated Phosphate Buffer (C) to 940 mL of distilled water and add sodium azide (0.2 g). Adjust the pH to 6.0 with 1 M HCl or NaOH and adjust the volume to one litre. Store at 4°C.

## ENZYME STANDARDS

Purified *A. niger* and *Trichoderma longibrachiatum* xylanases are supplied in the "Xylanase Assay Kit". Dilute these 1:100 in the appropriate buffer before use [*A. niger* in 25 mM sodium acetate (pH 4.7) and *T. longibrachiatum* in 25 mM sodium phosphate (pH 6.0)]. To improve stability, add bovine serum albumin (Sigma C-7880) at 0.5 mg/mL and sodium azide (0.02%). These solutions are stable to repeated freezing and thawing cycles.

### IMPORTANT NOTE:

Do not add the sodium azide until the pH is adjusted. Acidification of sodium azide releases a poisonous gas. Sodium azide is a poisonous chemical and is added solely as a preservative against microbial infection. This chemical can be deleted from the buffers, but their long-term stability will be reduced.

### NOTES:

1. Xylazyme AX tablets contain AZCL-Wheat Arabinoxylan i.e. it is based on the native substrate of interest, i.e. wheat arabinoxylan.
2. In the development of Xylazyme AX standard curves, the xylanase activity was first standardised in reducing sugar Units using wheat arabinoxylan and the Somogyi reducing-sugar method, or in Inverse Reciprocal Viscosity Units (IRVU) using wheat arabinoxylan as substrate (see Method 2, this booklet).
3. In the assay format described here, a single blank is required for each set of determinations and this is used to zero the spectrophotometer. The absorbance of the reaction solutions are measured against this blank.
4. The standard curve relating xylanase activity to absorbance (590 nm) is not perfectly linear (refer to Figs. 1, 2 and 3), but over a broad range of absorbance values (i.e. 0.2-2.0) it is reasonably linear, allowing a regression equation to be used.
5. Stirring of the test tube on addition of the Xylazyme AX tablet to the enzyme solution gives just a slight (about 5%) increase in absorbance value, but it also gives less reproducible results. Consequently, tubes are not stirred after addition of the substrate tablet.
6. AZCL-Wheat arabinoxylan (the active ingredient in Xylazyme AX) has limited stability in solutions of high pH. Consequently, we recommend the use of Trizma base stopping solution (pH~9). However, since some xylanase enzymes still have some activity at pH 9-10 (e.g. *Humicola xylanase* in Novo Biofeed Plus), it is then advisable to use 2% tri-Na phosphate (pH 11) as the stopping reagent. In these cases we recommend filtration of the stopped reaction within 5 min of terminating the reaction.

## ENZYME EXTRACTION AND DILUTION:

1. Using a positive displacement dispenser, add 1.0 mL of liquid enzyme sample to 99 mL of extraction/dilution buffer B (pH 4.7) or D (pH 6.0) and mix thoroughly. Alternatively, for powder enzyme samples, add 1.0 g to 100 mL of extraction/dilution buffer and stir and extract the slurry over 15 min.
2. Dilute an aliquot (0.5 mL) of the solution obtained in step 1 10-fold by addition to 4.5 mL of dilution buffer B or D. This process of dilution is repeated until a suitable dilution for assay is achieved. For example, for the industrial enzyme preparations Avizyme 1310 and BioFeed Plus, a dilution of the **original preparation** of 10,000 is required.
3. For chicken feeds, extraction of enzyme is complicated by binding of enzyme to feed components (such as cellulose). Consequently, extraction conditions may vary depending on the nature of the feed mixture and the particular xylanase employed. Please refer to separate Data Sheets (Xylanase in animal Feeds; XYLAXFD 11/99) for information on the measurement of xylanase in these materials.

## STOPPING REAGENTS:

### A. Trizma Base Solution (2%; pH ~9)

Trizma base (Sigma cat. no. T-1503, 20 g) is dissolved in 1 litre of distilled water. The solution is stored at room temperature.

### B. tri-Sodium Phosphate [(Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O); 2% w/v, pH 11]

tri-Sodium Phosphate (20 g) is dissolved in 900 ml of distilled water. pH is adjusted to 11.0 with 4M HCl and volume is adjusted to 1 litre. The solution is stored at room temperature.

## EQUIPMENT (RECOMMENDED):

1. Glass test tubes (16 x 120 mm, ~16 mL capacity, round bottomed).
2. Micro-pipettors, 0.50 mL (e.g. Gilson Pipetman<sup>®</sup>).
3. Positive displacement pipettor e.g. Eppendorf Multipette<sup>®</sup> with 5.0mL Combitip<sup>®</sup> (to dispense 0.50 mL aliquots of enzyme concentrate).
4. Adjustable volume dispenser set at 10.0 mL (for Trizma base or tri-sodium phosphate).
5. Bench centrifuge (required speed 3,000 rpm).
6. Analytical and top-pan balances.

7. Vortex mixer (e.g. Thermolyne Maxi Mix 11).
8. Thermostatted water bath set at 40.0°C (e.g. Julabo PC).
9. Stop clock.
10. Whatman No. 1 (9 cm) filter circles and filter funnels.
11. Spectrophotometer set at 590 nm.

### ASSAY PROCEDURE:

1. Add aliquots (0.50 mL) of suitably diluted enzyme preparation [in sodium acetate buffer (25 mM, pH 4.7) or sodium phosphate buffer (25 mM, pH 6.0)] to glass test tubes (16 x 120 mm) and equilibrate at 40°C for 5 min.
2. Add a Xylazyme AX test tablet to initiate the reaction. The tablet rapidly hydrates. The suspension **should not** be stirred.
3. After **exactly 10 min** (from the time of addition of the tablet) **terminate** the reaction by adding 10.0 mL of Trizma base solution (2% w/v, pH ~8.5); or 10.0 mL of tri-sodium phosphate solution (2% w/v, pH 11.0; refer to Note 6) with vigorous stirring on a vortex mixer.
4. Leave the tubes at **room temperature** for about 5 min and stir the slurry again. Filter the slurry through a Whatman No. 1 (9 cm) filter circle.
5. Measure the absorbance of the filtrate at 590 nm against a **substrate/enzyme** blank. The **substrate/enzyme** blank is prepared by adding Trizma base (or 2% tri-sodium phosphate) to the enzyme solution before the addition of the Xylazyme AX tablet. The slurry must be left at **room temperature**.

### STANDARDISATION:

Standard curves relating the activity of pure xylanases from *Aspergillus niger*, *Trichoderma longibrachiatum*, and *Humicola insolens* on Xylazyme AX tablets and on Wheat Arabinoxylan are shown in Figs 1-3. Details of the Somogyi reducing-sugar assay are given later in this booklet.

In all cases, **One Unit** of activity of enzyme activity is the amount of enzyme required to release one micromole or reducing sugar equivalents (as xylose by the Somogyi reducing-sugar method) from arabinoxylan per minute under standard assay conditions (40°C and pH 4.7 or 6.0).

## CALCULATION OF ACTIVITY:

Xylanase activity is determined by reference to the appropriate standard curve to convert absorbance (590 nm) to milli-Somogyi Units of activity per assay, and then calculated as follows:

### Units/mL or gram of original preparation:

$$= \text{milli-Units (per assay i.e. per 0.5 mL)} \times \frac{1}{1000} \times 100 \times 2 \times \text{Dilution}$$

where:

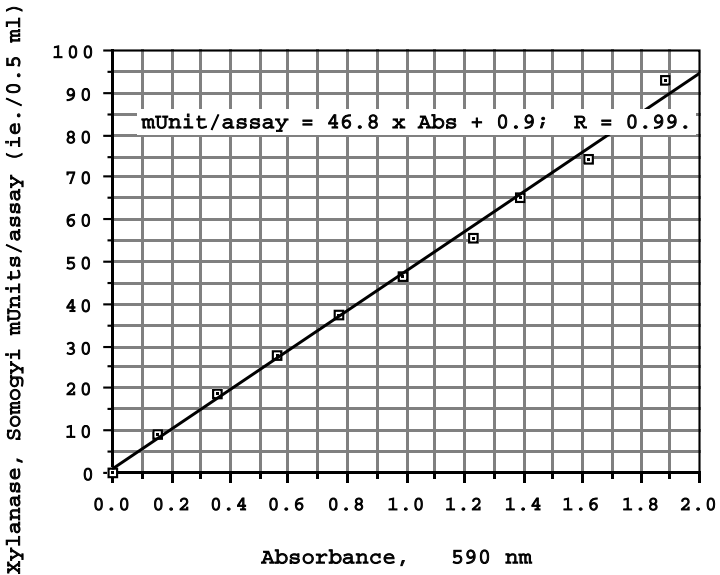
$\frac{1}{1000}$  = conversion from milli-Units to Units.

100 = initial extraction volume (i.e. 100 mL per g of solid).

2 = conversion from volume assayed (0.5 mL) to 1 mL of extract.

Dilution = further dilution of the initial extraction solution.

**NOTE:** The regression equations shown in Figs. 1-3 should only be used over the absorbance range 0.2-2.0.



**Figure 1.** Pure *Aspergillus niger* xylanase standard curve on Xylazyme AX (Lot 40602) at pH 4.7. Reaction stopped with Trizma base.

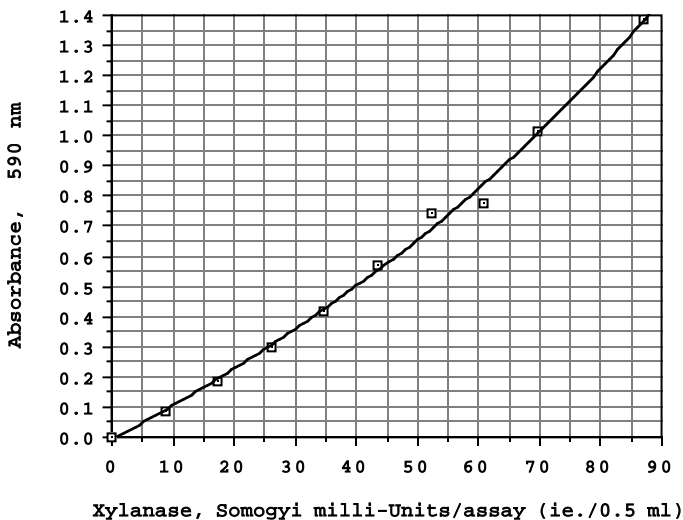


Figure 2. *Trichoderma longibrachiatum* xylanase (pl 9.0 form) standard curve on Xylazyme AX (Lot 40602) at pH 6.0. Reaction stopped with Trizma base.

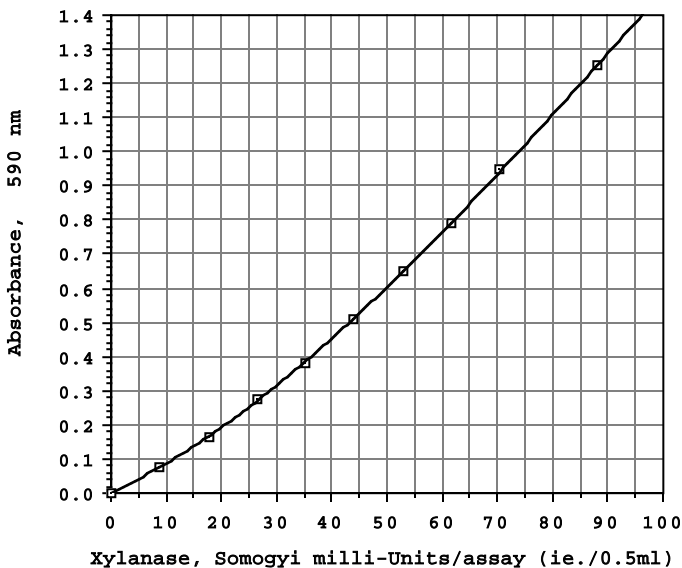


Figure 3. Pure *Humicola insolens* xylanase standard curve on Xylazyme AX (Lot 40602) at pH 6.0. Reaction stopped with Trizma base.

# VISCOMETRIC ASSAY OF $\beta$ -XYLANASE USING WHEAT ARABINOXYLAN

## INTRODUCTION:

This method describes a viscometric assay for the measurement of *endo*-xylanase (xylanase) in microbial preparations, animal feeds and bread improver mixtures.

## PRINCIPLE:

Activity is measured by incubating suitably diluted enzyme extract with a solution of wheat flour arabinoxylan (1% w/v, 20-25 cSt) in appropriate buffer (Na acetate or Na phosphate, 100 mM) and measuring the rate of decrease in viscosity at 40°C.

## UNIT DEFINITION:

One Inverse Reciprocal Viscosity Unit (IRVU) is the increase in reciprocal viscosity per hour per 1 mL of diluted enzyme (or enzyme extract), under standard assay conditions.

## ARABINOXYLAN SUBSTRATE:

Typical specifications of medium viscosity wheat arabinoxylan (P-WAXYM) from Megazyme are as follows:

Viscosity: ~20 cSt (1% w/v; Ostwald C-Type viscometer, 30°C)

Sugar composition: arabinose, 41%; xylose, 59%.

**Purity:** >98%

**Starch content:** <0.1%

**$\beta$ -Glucan content:** <0.1%

**Protein:** ~1.0%

**Moisture:** <5%

**Description:** White, odourless powder.

## PREPARATION OF ARABINOXYLAN SUBSTRATE:

Wheat arabinoxylan (1 gram) is accurately weighed into a 120 mL dry pyrex beaker. The sample is wet with 6 mL of 95% ethanol and then with 80 mL of cold water. A magnetic stirrer bar is added to the beaker and the beaker is placed on a magnetic stirrer-hotplate and heated at a setting of 120°C with vigorous stirring. The beaker is loosely covered with aluminium foil and stirred and heated for about 15 min. The arabinoxylan should completely dissolve. However, if dissolution is not complete, continue stirring for a further 30 min with the heating turned off. Add 10 mL of acetate buffer (1 M, pH 4.7) or 20 mL of phosphate buffer (0.5 M, pH 6.0) and adjust the pH to the desired value. The solution is adjusted to 100 mL, and is stored in a well sealed glass bottle. Microbial contamination is prevented by overlaying the solution with a few drops of toluene. This solution can be stored at room temperature for several weeks.

The solution is viscous and may be very slightly turbid.

## APPARATUS:

1. Grinding mill, Fritsch centrifugal with 12-tooth rotor and 0.5 mm sieve, or similar (e.g. Retsch centrifugal ZM 10 mill).
2. Balance: accuracy  $\pm 0.005$ .
3. Bench centrifuge (required speed 3,000 rpm).
4. Filter funnels using Whatman No. 1 paper.
5. Magnetic stirrer/hotplate.
6. Glass or acrylic water bath equilibrated at 40°C  $\pm 0.1^\circ\text{C}$ .
7. Ostwald viscometer: Standard U-tube viscometer type C.
8. Stop clocks.

## REAGENTS:

1. Wheat arabinoxylan (1% w/v, buffered, 100 mM).
2. Deionised water.
3. Sodium acetate buffer (1 M, pH 4.7) or sodium phosphate buffer (0.5 M, pH 6.0). For use in the preparation of buffered substrate.
4. Sodium acetate buffer (100 mM, pH 4.7) containing bovine serum albumin (BSA; 0.5 mg/mL) or sodium phosphate buffer (100 mM, pH 6.0) containing BSA (0.5 mg/mL) for enzyme extraction and dilution.
5. Ethanol (95% v/v).

## ENZYME EXTRACTION AND DILUTION:

1. Dry samples are milled in a centrifugal mill (apparatus 1).
2. Weigh exactly 1.0 g of flour or powdered enzyme preparation into a 250 mL Erlenmeyer flask and add 100 mL of appropriate extraction buffer, stir and extract over 15 min at room temperature.
3. Filter an aliquot of the slurry through a Whatman No. 1 filter circle, or centrifuge at 3,000 rpm for 10 min. Store the filtrate in an ice bath or, for longer periods of time, at -20°C in polypropylene tubes.
4. Dilute the filtrate (or supernatant) with the appropriate buffer (by sequential dilution of 1 mL to 10 mL with dilution buffer) to give an enzyme concentration suitable for assay (i.e. a slope value "A" of 0.06 to 0.60).
5. Liquid enzyme samples are prepared by dilution of 1.0 mL to 10 mL with appropriate buffer. This solution is further diluted as for extracts of dry samples.

## ASSAY OF ENZYME ACTIVITY (VISCOMETRY):

1. Pre-equilibrate enzyme preparation at 40°C for 5 min.
2. Pipette 12 mL of buffered wheat arabinoxylan solution (1% w/v) into the C-type viscometer (in a water bath at 40°C) and allow to equilibrate for 5 min.
3. Add 1 mL of the enzyme solution to the viscometer and mix the contents by blowing air into the viscometer tube. Immediately start a stop clock and leave this running throughout the entire assay to record incubation time (in min).
4. Using a second stop clock, take five falling time readings (in seconds) over a period of approximately 30 min. Take the time of each reading as the elapsed time from mixing the enzyme/substrate solutions to the mean of the falling time.
5. The viscosity ( $\eta$ ) of the reaction digest is proportional to the falling number according to the following equation:

$$\eta \text{ digest} = \frac{t \text{ digest} - t \text{ solvent}}{t \text{ solvent}}$$

where:

$\eta$  digest = specific viscosity of the digest

$t$  digest = falling time in seconds of the digest

$t$  solvent = falling time in seconds of the buffer (100 mM)

**Note:** The falling time of the buffer is identical to that of water under the conditions used.

## CALCULATIONS:

Calculate the reciprocal viscosity ( $1/n_{sp}$ ) (Table 1) and plot  $1/n_{sp}$  against incubation time (in min) (Fig. 4). From the linear graph, determine the slope (**A**) in terms of increase in reciprocal viscosity **per hour**.

If the slope is "**A**" IRV Units for 1 mL of enzyme solution from an extract of 1 gram of dry sample with 100 mL of buffer (and then diluted); or alternatively, 1 mL of liquid enzyme diluted to 100 mL with buffer (and then further diluted); **then** the increase in reciprocal viscosity per hour/g or mL of original preparation is:

$$\text{IRV Units} = A \times 100 \times \text{Dilution}$$

where:

**A** = slope from graph in terms of increase in reciprocal viscosity per hour.

**100** = 1 gram of original enzyme preparation is extracted with 100 mL of buffer (100 mM), or 1 mL of liquid enzyme preparation is diluted to 100 mL with extraction/dilution buffer.

**Dilution** = further dilution of the extract or diluted liquid enzyme concentrate required to get an appropriate enzyme concentration for assay.

**Table 1: Results and calculated data for the determination of reciprocal viscosity values.**

Incubation time (min)	Time to flow (t) (seconds)	Specific viscosity $n_{sp}=(t-t_0)/t_0$	$1/n_{sp}$
0	302.75	11.75	0.085
5	247.12	9.41	0.106
10	211.34	7.90	0.127
14	190.76	7.04	0.142
17	174.47	6.35	0.157
21	162.45	5.84	0.171

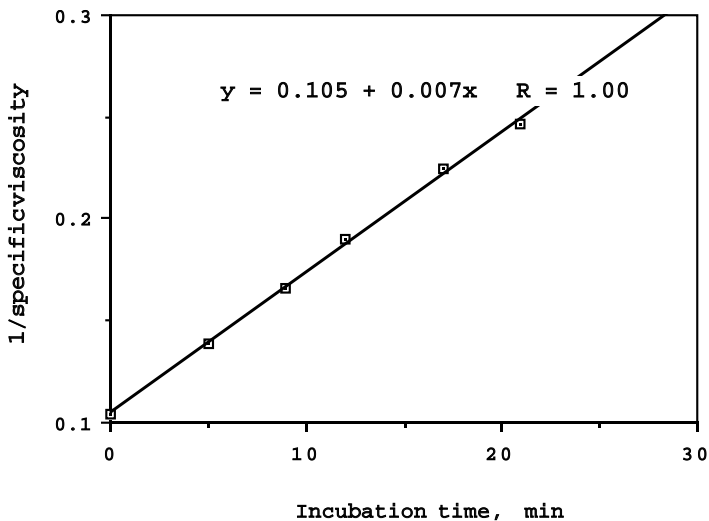


Figure 4. Plot of reciprocal viscosity (1/n) against incubation time.

Studies on *Trichoderma* sp, *Aspergillus niger* and *Humicola* sp. xylanases have shown a linear correlation between enzyme units (Somogyi reducing sugar method) and IRV Units over IRV slope values (A) of 0.06 to 0.60.

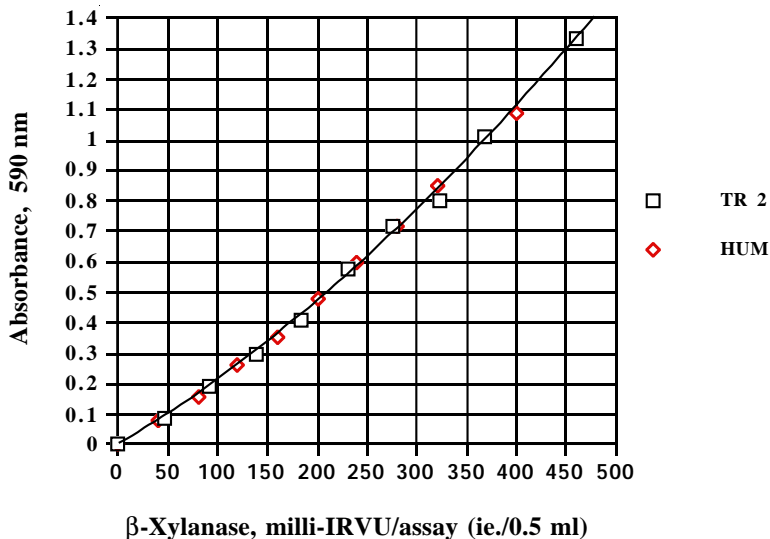


Figure 5. Standard curves for pure *H. insolens*, and *T. longibrachiatum* xylanases on Xylazyme AX (Lot 40602) at pH 6.0.

# A MODIFIED SOMOGYI REDUCING-SUGAR ASSAY for the measurement of $\beta$ -XYLANASE USING WHEAT ARABINOXYLAN AS SUBSTRATE

## REAGENTS:

- A. 25 g anhydrous sodium carbonate, plus 25 g sodium potassium tartrate, and 200 g of anhydrous sodium sulphate are dissolved in 800 mL of demineralised water and the volume is adjusted to one litre. The solution is filtered if necessary.
- B. 30 g of copper sulphate pentahydrate is dissolved in 200 mL of demineralised water containing 4 drops concentrated sulphuric acid.
- C. 50 g of ammonium molybdate is dissolved in 900 mL of demineralised water and 42 mL of concentrated sulphuric acid is added carefully.  
6 g of sodium arsenate heptahydrate is dissolved separately in 50 mL of water, and this is added to the above solution. The volume of the solution is adjusted to 1 litre. If necessary, warm the solution to 55°C to give complete dissolution of the components.
- D. Add 1 mL of reagent B to 25 mL of reagent A.
- E. Dilute solution C five-fold with demineralised water just before use (this is stable at 4°C for about 4 weeks).

## PREPARATION OF ARABINOXYLAN SUBSTRATE:

This is prepared as described on page 9 of this booklet.

## ASSAY PROCEDURE:

1. Pre-equilibrate aliquots (0.5 ml) of wheat arabinoxylan (1% w/v) [in 100 mM sodium acetate buffer (pH 4.7) or 100 mM sodium phosphate buffer (pH 6.0)] at 40°C for 5 min.
2. Add aliquots (0.2 mL) of suitably diluted and pre-equilibrated enzyme preparation to the substrate solution, stir the solution on a vortex mixer and incubated at 40°C.

3. Terminated the reaction after 5, 10, and 15 min by the addition of 0.5 mL of Reagent D, with vigorous stirring of the reaction mixture on a vortex stirrer.
4. Prepare a **Reaction Blank** by adding 0.5 mL of Reagent D to the substrate solution before addition of the enzyme preparation.
5. Prepare a **Xylose Standard Curve** by mixing 0.2 mL of xylose solutions (0-50  $\mu$ grams/0.2 mL) with 0.5 mL of substrate solution and 0.5 mL of Reagent D.
6. Prepare a **Reagent Blank** by mixing 0.2 mL of buffer solution with 0.5 mL of substrate solution and 0.5 mL of Reagent D.

### COLOUR DEVELOPMENT:

1. All tubes, including reaction tubes, standards and reagent blanks, are incubated in a boiling water bath for 20 min.
2. The tubes are removed from the water bath, allowed to cool over 5 min and treated with 3 mL of Reagent E. The tubes are stirred thoroughly and allowed to stand at room temperature for 15 min, at which time they are stirred again.
3. The absorbance of all tubes are measured at 520 nm against the Reagent Blank.

### CALCULATIONS:

#### $\beta$ -Xylanase Activity (Units/mL)

$$= \Delta E \times \frac{1}{\text{Incubation time}} \times F \times 5 \times \frac{1}{150}$$

where:

$\Delta E$  = absorbance of the reaction solution read against the reaction blank

Incubation time = time of incubation of the enzyme with the substrate

F = a factor to convert absorbance to  $\mu$ grams of xylose  
 =  $\frac{50 \text{ (micrograms of xylose)}}{\text{absorbance for 50 micrograms of xylose}}$

$\frac{1}{150}$  = conversion from  $\mu$ grams of xylose to  $\mu$ moles

# AZO-WHEAT ARABINOXYLAN FOR ASSAY OF *endo*-XYLANASE

## PRINCIPLE:

This assay procedure is specific for *endo*-1,4- $\beta$ -D-xylanase activity. On incubation of Azo-Wheat Arabinoxylan with *endo*-xylanase, the substrate is depolymerised by an *endo*-mechanism to produce low-molecular weight dyed fragments which remain in solution on addition of industrial methylated spirits (IMS) to the reaction mixture. High-molecular-weight material is removed by centrifugation, and the colour of the supernatant is measured. *endo*-Xylanase in the assay solution is determined by reference to a **Standard Curve**.

## SUBSTRATE:

The substrate is prepared by dyeing highly purified, and partially depolymerised wheat arabinoxylan with Remazolbrilliant Blue dye. This substrate is supplied in a soluble ready-to-use form. It may tend to precipitate slightly from solution on extended storage at 4°C. Thus, before dispensing, the substrate should be warmed to room temperature and thoroughly mixed by vigorous shaking. It should be dispensed with a positive displacement dispenser (eg. Eppendorf Multipette®).

## DISSOLUTION:

Add powdered substrate (1 gram) to a 200 ml beaker, and wet with 3 ml of IMS. Add 95ml of water as the slurry is stirred and heated on a hot-plate stirrer. Increase the solution temperature to about 95°C, and then turn the heat off. Continue to stir the solution until the powder completely dissolves (about 15 min). Cool the solution to room temperature and adjust the volume to 100ml. Add sodium azide (0.02g) as a preservative. Store the solution at 4°C between use. Under these conditions and excluding contamination, the substrate is stable for several years.

Before use, mix the substrate solution by shaking to resuspend any material which may have settled on standing.

The solution is viscous, so it should preferably be dispensed with a positive displacement dispenser (e.g. Eppendorf Multipette<sup>R</sup> with a 5.0 mL Combipip).

## PRECIPITANT SOLUTION:

Industrial methylated spirits (95% v/v).

## ENZYME EXTRACTION AND DILUTION:

With liquid enzyme preparations, add a sample (1.0 ml) (using a positive displacement dispenser if the solution is viscous), to **extraction / dilution buffer** (49 ml, pH 4.7) and mix thoroughly. This is termed the **Original Extract**. Dilute an aliquot of **Original Extract** (1.0 ml) 10-fold by addition to 9.0 ml of **extraction / dilution buffer**. This process of dilution is repeated until an enzyme dilution suitable for assay is obtained. For example, for the industrial enzyme preparation **Finizym** (from *Aspergillus niger*; Novo Nordisk, Denmark), a dilute of the original extract approximately 100-fold.

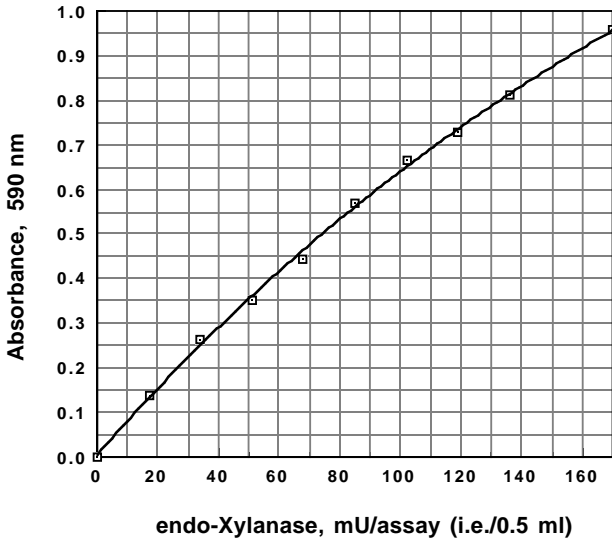
With powder samples, add the preparation (1.0 g) to **extraction / dilution buffer** (50 ml, pH 4.6) and gently mix the slurry over 15 min, or until the sample is completely dispersed or dissolved. Centrifuge this solution (the **Original Extract**) at 1,000 *g* for 10 min, or filter through Whatman No. 1 (9 cm) filter circles. This extract is then further diluted with **buffer**, as for the liquid enzyme samples.

## ASSAY PROCEDURE:

1. Pre-equilibrate aliquots (0.50 mL) of substrate solution, in glass test-tubes (16 x 100 mm) at 40°C for 5 min.
2. Initiate the reaction by the addition of pre-equilibrated enzyme solution (0.5 mL) in sodium acetate buffer (100 mM, pH 4.7)[or 0.1M phosphate buffer (pH 6.0)]. Stir the mixture on a vortex mixer and then incubated at 40°C for exactly 10 min.
3. Terminate the reaction after 10 min, and precipitate high-molecular weight, non-depolymerised substrate by adding 2.5 mL of industrial methylated spirits (95% v/v) with vigorous stirring on a vortex mixer for 10 sec .
4. Allow the reaction tubes to equilibrate to room temperature over 10 min, mix again, and then centrifuged at 3,000 rpm (1,000 *g*) for 10 min on a bench centrifuge.
5. Read the absorbance of the supernatant solution at 590nm, and determine the enzyme activity by reference to a standard curve. The reaction blank is prepared by adding industrial methylated spirits (2.5 mL) to the substrate solution (0.5 mL) before addition of the enzyme (0.5 mL). Usually, only a single blank is required with each set of determinations. Typical blank absorbance values at 590 nm are less than 0.05.

## STANDARD CURVE:

A typical standard curve for *Aspergillus niger* xylanase on Azo-wheat arabinoxylan (Lot 50201) is shown in Figure 6. To use this Standard Curve, the assay conditions described above must be strictly adhered to.



**Figure 6.** Standard curve for *Aspergillus niger* endo-xylanase on Azo-wheat arabinoxylan (Lot 50201). Assay conditions are described in the text.

One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of xylose reducing-sugar equivalents (Somogyi reducing-sugar method) from wheat arabinoxylan (1% w/v) in one minute at 40°C and pH 4.5.

## CALCULATION OF ACTIVITY:

*endo-Xylanase* activity is determined by reference to the standard curve to convert absorbance to milliUnits of activity per assay (i.e. per 0.5ml) on arabinoxylan, and then calculated as follows:

### Units/ml or gram of Original Preparation:

$$= \text{milliUnits (per assay i.e. per 0.5 ml)} \times 2 \times 50 \times \frac{1}{1000} \times \text{Dilution}$$

where:

2 = conversion from 0.5 ml to 1.0 ml.

50 = the volume of buffer used to extract the original preparation (i.e. 1.0g/50ml or 1.0ml of enzyme added to 49ml of buffer).

$\frac{1}{1000}$  = conversion from milliUnits to Units.

Dilution = further dilution of the **original extract**.



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