

Megazyme

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endo-1,4- β -D- XYLANASE

ASSAY PROCEDURES

T-XAX 11/14

1. Xylazyme AX Tablets
2. Viscometric Assay
3. Reducing-Sugar Assay



GENERAL INTRODUCTION:

Xylanase (*endo*-1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) finds widespread application in chicken feed supplements, in bread improver mixtures and in the enzymic bleaching of wood pulp (Nissen *et al.*, 1992¹). Historically, xylanase activity has been measured with the DNS reducing sugar assay (Bailey, 1988²) with purified xylan from oat spelts, larchwood or birchwood as substrate. However, 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 insoluble, dyed, crosslinked arabinoxylan or 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.^{3,4}
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, this assay should, perhaps, be established as a reference method.⁵
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, the reducing sugar procedure we recommend is based on the Nelson/Somogyi⁶ procedure, and this is included. The Nelson/Somogyi procedure is preferred because, unlike many other procedures (such as the DNS method²), it gives close to stoichiometric colour response for the same molar concentration of oligosaccharides of varying degrees of polymerisation.

Of the methods described in this booklet, we recommend the Xylazyme AX procedure because of its convenience, reliability and accuracy. If a soluble, dyed, xylan substrate is preferred, Azo-Xylan (Birchwood) is the substrate of choice (see separate booklet). With Azo-Xylan (Birchwood) (Cat. no. **S-AXBP**), the reproducibility of the assay is better than with Xylazyme AX, but sensitivity is much lower.

Xylazyme AX Tablet Test 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- β -D-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)]

Add 60.0 g of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water. Adjust the pH of this solution to pH 4.7 by the addition of 4 M (16 g/100 mL) NaOH solution. Then adjust the volume to one litre. Store at room temperature.

(B) Acetate Extraction/Dilution Buffer [Sodium acetate (25 mM, pH 4.7) containing sodium azide (0.02%)]

Add 25 mL of **concentrated acetate buffer** (A) to 950 mL of distilled water. Adjust the pH of the solution to 4.7 by dropwise addition of 2 M HCl acid. Add 0.2 g of sodium azide and dissolve and then adjust the volume to one litre. Store at room temperature.

(C) Concentrated Phosphate Buffer [Sodium Phosphate (0.5 M, pH 6.0)]

Add 156 g of sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) to 900 mL of distilled 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 50 mL of **concentrated phosphate buffer** (C) to 940 mL of distilled water. Adjust the pH to 6.0 with 1 M HCl or 1 M NaOH. Add 0.2 g of sodium azide and dissolve and adjust the volume to one litre. Store at 4°C.

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.

ENZYME STANDARDS:

Purified *Aspergillus niger* and *Trichoderma longibrachiatum* xylanases are supplied in the "Xylanase Assay Kit" (Cat. no. **K-XYLS**). Dilute 1 mL of this solution to 100 mL 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 BSA (Sigma Cat. no. C-2153) at 0.5 mg/mL and 0.02 g of sodium azide. These solutions are stable to repeated freezing and thawing cycles.

NOTES:

1. Xylazyme AX tablets contain AZCL-wheat arabinoxylan, i.e. they are based on wheat arabinoxylan, the native substrate.
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. Alternatively, activity can be standardised 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 is measured against this blank.
4. The standard curve relating xylanase activity to absorbance at 590 nm is not always linear (refer to Figs. 1-3; pages 6 and 7).
5. Stirring of the test tube on addition of the Xylazyme AX tablet to the enzyme solution gives a slight (about 5%) increase in absorbance values, 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, Tris buffer salt solution (pH ~ 9) is recommended. However, since some xylanase enzymes still have some activity at pH 9-10 (e.g. *Humicola insolens* xylanase in Novo Biofeed Plus), it is then advisable to use 2% (w/v) tri-sodium phosphate (pH 11) as the stopping reagent. In these cases, the stopped reaction should be filtered 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. Add 0.5 mL of the solution obtained in step 1 to 4.5 mL of extraction/dilution buffer B or D (i.e. dilute 10-fold). Repeat this process of dilution until a concentration suitable for assay is achieved. For example, certain industrial enzyme preparations require a dilution of the **original preparation** of 10,000-fold.
3. For chicken feeds, extraction of enzyme is complicated by binding of enzyme to feed components (such as cellulose) and/or the presence of xylanase inhibitor proteins. Consequently, extraction conditions may vary depending on the nature of the feed mixture and the particular xylanase employed. Please refer to separate booklet (Xylanase in Animal Feeds; **K-XYLS**) for information on the measurement of xylanase in these materials.

STOPPING REAGENTS:

A. Tris Buffer Salt Solution (2% w/v; pH 9.0)

Dissolve 20 g of tris buffer salt (Megazyme Cat. no. **B-TRIS500**) in 1 L of distilled water. Store this solution at room temperature.

B. Tri-Sodium Phosphate [(Na₃PO₄·12H₂O); 2% w/v, pH 11]

Dissolve 20 g of tri-sodium phosphate in 900 mL of distilled water. Adjust the pH to 11.0 with 4 M HCl and the volume to 1 L. Store this solution at room temperature.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (16 x 120 mm, ~16 mL capacity, round bottomed).
2. Micro-pipettors, 1,000 µL (e.g. Gilson Pipetman®).
3. Positive displacement pipettor, e.g. Eppendorf Multipette® with 5.0 mL Combitip® (to dispense 0.50 mL aliquots of enzyme concentrate).
4. Adjustable volume dispenser set at 10.0 mL (for Tris buffer salt or tri-sodium phosphate).
5. Bench centrifuge (required speed 3,000 rpm)(approx. 1,500 g).
6. Analytical and top-pan balances.

7. Vortex mixer (e.g. IKA MS2® Minishaker).
8. Thermostated 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 0.50 mL aliquots of suitably diluted enzyme preparation [in sodium acetate buffer (25 mM, pH 4.7) or sodium phosphate buffer (25 mM, pH 6.0)] to the bottom of 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. **Do not stir** the suspension.
3. Terminate the reaction **exactly 10 min** after the addition of the tablet by adding 10.0 mL of Tris buffer salt solution (2% w/v, pH ~ 9.0); or 10.0 mL of tri-sodium phosphate solution (2% w/v, pH 11.0; refer to Note 6, page 3). Stir the tube vigorously on a vortex mixer.
4. Leave the tubes sit at **room temperature** for about 5 min and then stir the contents 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 2% Tris buffer salt solution (or 2% tri-sodium phosphate solution) 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 *A. niger* and *T. longibrachiatum* on Xylazyme AX tablets and on Wheat Arabinoxylan are shown in Figs. 1-3. Details of the Somogyi reducing sugar assay are given on page 13 of this booklet.

CALCULATION OF ACTIVITY:

Xylanase activity in the sample being assayed is determined by reference to the appropriate standard curve or regression equation to convert absorbance (590 nm) into milli-Units of activity per assay.

The regression equation varies slightly from batch to batch of Xylazyme AX tablets. To minimise this inconvenience, large batches of tablets are prepared.

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:

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

$$100 = \text{initial extraction volume (i.e. 100 mL per g of solid).}$$

$$2 = \text{conversion from volume assayed (0.5 mL) to 1 mL of extract.}$$

Dilution = further dilution of the initial extraction solution.

In all cases, **One Unit** of enzyme activity is the amount of enzyme required to release one micromole of 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).

NOTE: The regression equation shown in Fig. 1 should be used only over the absorbance range 0.2-2.0.

$$\text{milli-Units/assay (i.e. 0.5 mL)} = 55.0 \times \text{Abs.} + 0.8; R = 0.99$$

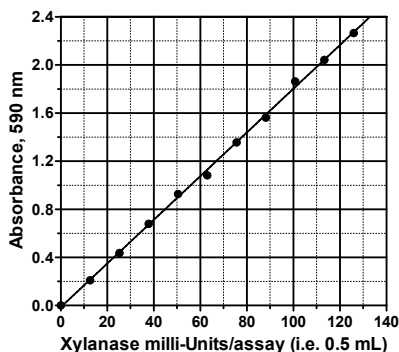


Figure 1. Pure *A. niger* xylanase standard curve on Xylazyme AX (Lot 120101) at pH 4.7. Reaction stopped with Tris buffer salt.

milli-Units/assay (i.e. 0.5 mL) = 39.2 x Abs. +1.4; R=0.99

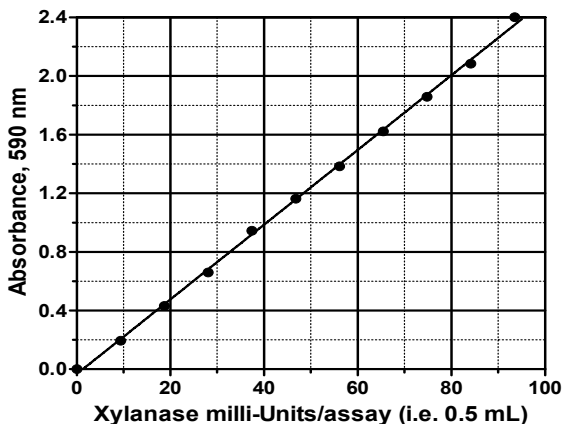


Figure 2. *T. longibrachiatum* xylanase (E-XYTR3; pl 9.0 form) standard curve on Xylazyme AX (Lot 120101) at pH 6.0. Reaction stopped with tri-sodium phosphate (pH 11.0).

milli-Units/assay (i.e. 0.5 mL) = 1.1 + 24.1 x Abs. - 2.6 x Abs.²;
R=0.99

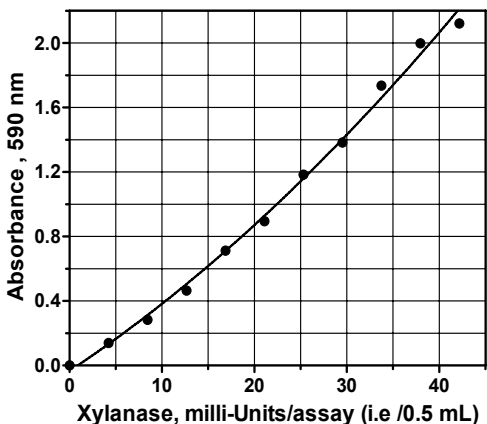


Figure 3. *T. longibrachiatum* xylanase (pl 5.5 form) standard curve on Xylazyme AX (Lot 120101) at pH 4.7. Reaction stopped with Tris buffer salt.

Viscometric Assay of β -Xylanase using Wheat Arabinoxylan

INTRODUCTION:

This method describes a viscometric assay for the measurement of *endo*-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, 20-30 cSt) in appropriate buffer (sodium acetate or sodium 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: ~ 20-30 cSt (1% w/v; Ostwald C-Type viscometer, 30°C)

Sugar composition: arabinose 38%; xylose 62%

Purity: > 95%

Starch content: < 0.1%

β -Glucan content: < 0.1% **Protein:** ~ 4.0%

Moisture: < 5.0%

Description: White, odourless powder

PREPARATION OF ARABINOXYLAN SUBSTRATE:

Accurately weigh 1 g of wheat arabinoxylan into a 120 mL dry pyrex beaker. Wet the sample with 6 mL of 95% v/v ethanol and then add 80 mL of cold water. Add a magnetic stirrer bar to the beaker and place the beaker on a magnetic stirrer-hotplate and heat at 120°C with vigorous stirring. Loosely cover the beaker with aluminium foil and heat with stirring for approx. 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 sodium acetate buffer (1 M, pH 4.7) or 20 mL of sodium phosphate buffer (0.5 M, pH 6.0) and adjust the pH to the desired value. Adjust the volume to 100 mL, and store in a well sealed Duran® glass bottle. Prevent microbial contamination by adding a few drops of toluene to the substrate solution. 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 ± 0.005 .
3. Bench centrifuge (required speed 3,000 rpm).
4. Filter funnels using Whatman No. 1 paper.
5. Magnetic stirrer-hotplate.
6. Water bath equilibrated at $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$.
7. Ostwald viscometer: Standard U-tube viscometer type C.
8. Stop clocks.

REAGENTS:

1. Wheat arabinoxylan [1% w/v in 100 mM sodium acetate buffer (pH 4.7) or sodium phosphate buffer (pH 6.0)].
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 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. Mill samples in a centrifugal mill (apparatus 1).
2. Weigh exactly 1.00 g of flour or powdered enzyme preparation into a 250 mL Erlenmeyer flask and add 100 mL of appropriate extraction buffer, stir and allow the enzyme to extract over 15 min at room temperature.

3. Filter an aliquot of the slurry through a Whatman No. 1 (9 cm) filter circle, or centrifuge at 3,000 rpm for 10 min. Store the filtrate in an ice bath or in a refrigerator. For longer periods of time, store 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. Prepare liquid enzyme samples by adding 1.0 mL of the preparation to 9 mL of appropriate buffer (10-fold dilution). Further dilute this solution as for extracts of powder 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 (η) 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:

- η_{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 g 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 per g or per 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 g 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

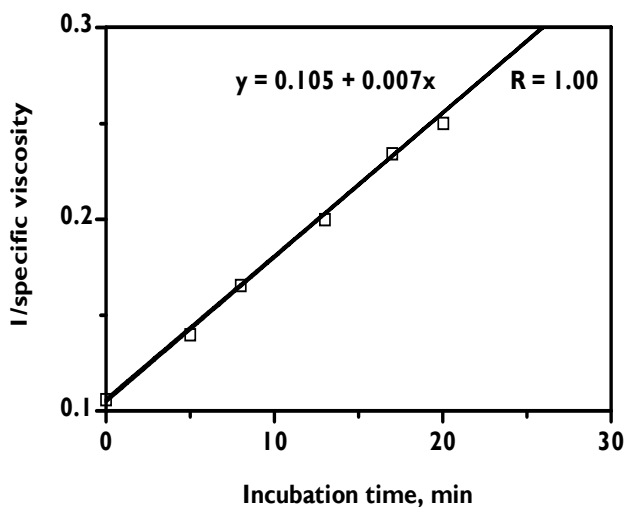


Figure 4. Plot of reciprocal viscosity ($1/\eta$) against incubation time.

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 (Δ) of 0.06 to 0.60.

A Modified Somogyi Reducing Sugar Assay for the Measurement of β -Xylanase using Wheat Arabinoxylan as Substrate

REAGENTS:

- A. Dissolve 25 g of anhydrous sodium carbonate plus 25 g of sodium potassium tartrate and 200 g of anhydrous sodium sulphate in 800 mL of demineralised water and adjust the volume to one litre. Filter the solution if necessary.
- B. Dissolve 30 g of copper sulphate pentahydrate in 200 mL of demineralised water containing 4 drops concentrated sulphuric acid.
- C. Dissolve 50 g of ammonium molybdate in 900 mL of demineralised water and carefully add 42 mL of concentrated sulphuric acid.
Separately dissolve 6 g of sodium arsenate heptahydrate in 50 mL of water, and then add this to the above solution. Adjust the volume of the solution 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 5-fold with demineralised water just before use (this is stable at 4°C for about 4 weeks).

PREPARATION OF ARABINOXYLAN SUBSTRATE:

Prepare this as described on page 8 of this booklet.

ASSAY PROCEDURE:

1. Add 0.5 mL aliquots of wheat arabinoxylan solution (1% w/v) [in 100 mM sodium acetate buffer (pH 4.7) or 100 mM sodium phosphate buffer (pH 6.0)] to glass test tubes (16 x 120 mm) and pre-equilibrate these at 40°C for 5 min.
2. Add 0.2 mL aliquots of suitably diluted and pre-equilibrated (at 40°C for 5 min) enzyme preparation to the substrate solution, stir the solution on a vortex mixer and incubate at 40°C.

3. Terminate 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 $\mu\text{g}/0.2$ 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. Incubate all tubes, including reaction tubes, standards and reagent blanks, in a boiling water bath for 20 min.
2. Remove the tubes from the water bath, allow them to cool over 5 min and add 3 mL of Reagent E. Stir the tubes vigorously and allow them to stand at room temperature for 15 min. Again stir the tubes.
3. Measure the absorbance of all tubes at 520 nm against the Reagent Blank.

CALCULATIONS:

β -Xylanase Activity (Units/mL)

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

where:

ΔE = 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 μg of xylose

$$= \frac{50 (\mu\text{g of xylose})}{\text{absorbance for } 50 \mu\text{g of xylose}}$$

$\frac{I}{150}$ = conversion from μgrams of xylose to μmoles

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