XYLANASE
(Xylazyme AX)

ASSAY PROTOCOL

K-XYLS

09/22

(200 Assays per Kit)

INTRODUCTION:

Arabinoxylan is the major endosperm cell-wall polysaccharide of wheat and rye. It is found in significant proportions in most cereal solutions and slurries of high viscosity, and in animal nutrition, it reduces the rate of nutrient absorption from the gut. *endo*-β-D-Xylanase (xylanase) is added to feeds to catalyse the depolymerisation of this polysaccharide. It can be demonstrated that *endo*-cleavage by xylanase of just one bond per thousand in the arabinoxylan backbone can significantly remove viscosity properties.

Of the carbohydrase enzymes used as feed supplements, one of the most difficult to measure has been xylanase. These problems are attributed to several factors, including the low levels of enzyme added to the feed, inactivation of the enzyme during pelleting, binding of the enzyme to feed components, and the presence of specific xylanase inhibitors.

The only biochemical methods which are sufficiently sensitive, specific, and robust to measure xylanase in feeds are viscometric assays and those employing dyed xylan or arabinoxylan polysaccharides. Viscometric assays are tedious, whereas assays employing dyed xylan substrates are rapid, reproducible, and simple to perform. We recommend the use of either Xylazyme AX tablets or Azo-Wheat Arabinoxylan (Azo-WAX). Xylazyme AX-based assays are about 5-fold more sensitive than assays employing Azo-WAX. However, this latter substrate does have sufficient sensitivity in most applications, and results are slightly more reproducible than with Xylazyme AX.

It is generally accepted that xylanase enzymes that are best suited to feed applications have optimal activity at pH 6.0. Consequently, these enzymes are generally assayed at this pH in 100 mM sodium phosphate buffer. In recovery experiments, however, we found that sodium phosphate buffer extracts only a small proportion (< 20%) of the amount of enzyme added to the feed. Thus, a wide range of alternative extractants and extraction conditions have been evaluated. For feeds containing *Trichoderma* sp. xylanases, the best and most consistent results have been obtained using 100 mM acetic acid or 100 mM sodium acetate buffer (pH 4.7) at room temperature. Optimal extraction of *Humicola* sp. xylanases was achieved with a buffer containing 100 mM MES buffer (pH 6.0) and 1% w/v sodium dodecyl sulphate (SDS).

KITS:

Kits containing the required reagents to measure xylanase in animal feeds are available from Neogen. These kits contain:

Bottle 1: *A. niger* control xylanase in 50% (v/v) glycerol (activity at 40°C and pH 4.7 stated on vial).

Bottle 2: *T. longibrachiatum* control xylanase in 50% (v/v) glycerol (activity determined at 40°C and pH 6.0 stated on vial).

Bottle 3: Xylazyme AX test tablets (200 tablets).

EXTRACTION BUFFERS (not enclosed):

(A) Acetic acid (0.1 M)
Add 5.8 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water and adjust the volume to 1 L. This is **Buffer A**.

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

EQUIPMENT (Recommended):

1. Glass test tubes (round bottomed; 16 x 100 mm and 16 x 120 mm).
2. Micro-pipettors, e.g. Gilson Pipetman 200 µL and 500 µL.
3. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.2 mL aliquots of xylanase control in 50% (v/v) glycerol].
4. Adjustable volume dispenser set at 5.0 mL (to dispense **Buffer B**).
5. Top-pan balance, correct to 0.01 g.
6. Spectrophotometer set at 590 nm.
7. Vortex mixer.
8. Whatman No. 1 (9 cm) filter circles and filter funnels.

EXTRACTION AND ASSAY OF XYLANASE IN FEED SAMPLES:

***Trichoderma* sp. Xylanases:**

EXTRACTION:

1. Mill feed samples (approx. 50 g) to pass a 0.5 mm screen and mix thoroughly.
2. Weigh 0.5 g (± 0.01 g) of each sample in quadruplicate into glass test tubes (16 x 120 mm).
3. Add 5 mL of **Buffer A** (see page 2) to each sample and stir on a vortex mixer. Add 0.2 mL of distilled water to two of these tubes with stirring. To the other two tubes add 0.2 mL of control xylanase solution (approx. 60-80 mU/0.2 mL; see vial label) with vigorous and immediate stirring on a vortex mixer.
4. Incubate the slurries at room temperature and stir occasionally over the following 20 min.
5. Centrifuge the tubes at 1,500 *g* for 10 min in a bench centrifuge and use the supernatant directly in the assays. Assays should be initiated within 30 min of obtaining these extracts to minimise loss of enzyme activity in the extracts.

ASSAY:

1. Accurately transfer 0.5 mL aliquots of supernatant solutions (in duplicate) to glass test tubes (16 x 100 mm) **at room temperature.**
2. Add a Xylazyme AX tablet (without stirring) to each tube and immediately place the tubes in a water bath set at 50 ± 0.1°C and incubate for exactly 30 min.
3. After exactly 30 min, add 5 mL of **Buffer B** (see page 2), stir vigorously on a vortex mixer, and store at **room temperature** for 5 min.

NOTE:

1. This treatment terminates the reaction.

2. The tubes must be stored at room temperature and not at 50°C, as the substrate is not stable under alkaline conditions at elevated temperatures (i.e. absorbance values will increase due to substrate breakdown).

4. Stir the tubes on a vortex mixer and filter the slurry through a Whatman No. 1 (9 cm) filter paper.

5. Measure the absorbance of the filtrates at 590 nm against a **Reaction Blank.**

Prepare the Reaction Blank by adding Buffer B (see page 2) (5 mL) to the feed extract (0.5 mL), followed by a Xylazyme AX tablet. Stir the slurry and store at room temperature for 5 min before filtration through Whatman No. 1 filter paper.

A single Reaction Blank is required for each feed sample.

CALCULATION OF ACTIVITY:

The level of xylanase in the flour sample is calculated as follows:

$$\textbf{Activity in feed sample (0.5 g)} = \textbf{Added activity} \times \frac{\textbf{SA}}{\textbf{TA - SA}}$$

where:

Added activity	=	the amount of xylanase added to the feed sample slurry at the time of assay. (Calculate from the stated activity value on the vial which is given in mU/mL. For example, if the activity value is 386 mU/mL and 0.2 mL is used in the assay the value is 77 mU).
SA	=	the reaction absorbance obtained for extracts of the feed sample to which no control xylanase was added.
TA	=	the total absorbance, i.e. the absorbance of extracts of the feed sample to which the control xylanase was added.

EXAMPLE CALCULATION:

Sample	**Abs/30 min. incubation**
1. Reaction blank	0.000
2. SA	0.859
3. TA (SA + 77 mU xylanase)	1.299

Thus:

Activity in feed (U/0.5g)

= 77/1000 Units x 0.859/(1.299 - 0.859)

= 0.077 x 0.859/0.440 = **0.150 U/0.5 grams**

= 0.150 x 2000 = **300 U/Kg or 300,000 Units/ton**

NOTE: Through the equation, the activity calculated is at 40°C and the pH at which the particular enzyme was standardised, e.g. *A. niger* xylanase at pH 4.7 and *T. longibrachiatum* xylanase at pH 6.0.

REFERENCE:

McCleary, B. V. Problems in the measurement of β-xylanase, β-glucanase and α-amylase in feed enzymes and animal feeds. "*Proceedings of Second European Symposium on Feed Enzymes*" (W. van Hartingsveldt, M. Hessing, J. P. van der Lugt and W. A. C. Somers, Eds.), Noordwijkerhout, Netherlands, 25-27 October, 1995.

APPENDIX:

Information on the percentage recovery of *Trichoderma* sp. xylanases added to feeds was obtained by performing incubations and assays under the standard conditions for feeds, with four levels of added enzyme, in the presence and absence of feed in the extraction mixture. The pelleted feed which was used was milled (< 0.5 mm) before use. The recovery of activity was approx. 40-50% of the added activity **(Figure 1)**. The line obtained for "enzyme without added feed" is curved, whereas the line for enzyme recovered from "enzyme/feed mixtures" is linear. In **Figure 2**, results are shown for a feed sample that had been sprayed with enzyme after pelleting and for a sample of the same feed which had not been sprayed with enzyme. It is apparent that the recovery of enzyme added at the time of extraction is linear and the curves for the two feeds are approximately parallel.

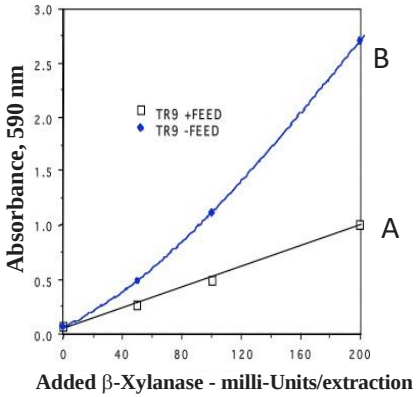

Figure 1: Curves showing the recovery of *T. longibrachiatum* (pI 9.0) xylanase (as absorbance 590 nm on hydrolysis of Xylazyme AX) from feed/enzyme mixtures (A), in comparison to measured activity in preparations free of added feed (B).

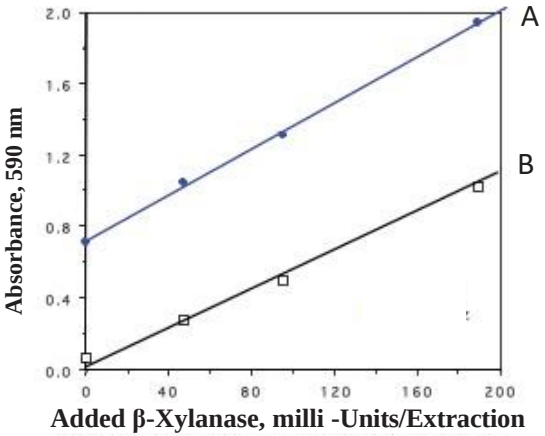

Figure 2: Effect of enzyme added at the time of assay on the measured absorbance values. The two feed samples analysed were identical except that one was sprayed with enzyme post-pelleting (A) and the other was not (B).

The observation that the best extractant for *T. longibrachiatum* xylanase was 100 mM acetic acid was quite surprising. Under these extraction conditions (0.5 g feed per 5 mL of 0.1 M acetic acid), a final extraction pH of 4.0 $\pm$ 0.1 is obtained, whereas the optimal pH for activity of this enzyme is 6.0 (with only 70% of maximal activity at pH 4.9; refer to **Figure 3**). This result suggests that the extractant either selectively solubilises a particular form of the enzyme (i.e. the pI 5.5 form which has a pH optima of 4.0) or that it extracts some other component that associates with the xylanase resulting in a change in pH activity characteristics. The first possibility has been discounted based on the observations that:

1. the pI 5.5 form of the enzyme represents only a small proportion of the total xylanase in the mixture which was evaluated, and
2. when the pI 5.5 and 9.0 forms of *T. longibrachiatum* xylanases were separated, purified, and evaluated in binding studies, it was found that the pI 5.5 form binds to the feed more strongly (lower recoveries; see **Figure 4**) than does the pI 9.0 form.

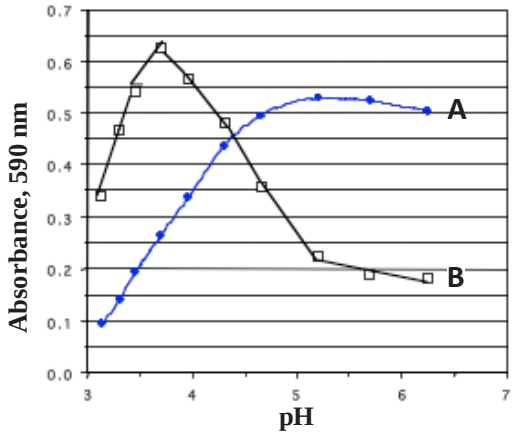

Figure 3. pH activity curves for the original *T. longibrachiatum* enzyme preparation (A) and for the xylanase fraction extracted from the feed sample (B).

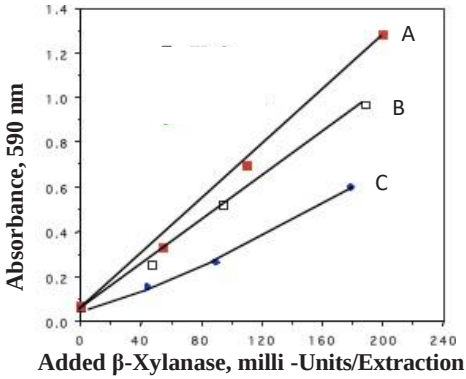

Figure 4. Binding of different highly purified xylanase enzymes to feed components. Extraction and assay conditions are as described in the text.

A. *A. niger* xylanase;
B. *T. longibrachiatum* xylanase pI 9.0 form;
C. *T. longibrachiatum* xylanase pI 5.5 form.

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