

# Megazyme

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MEASUREMENT OF  
XYLANASE  
IN ANIMAL FEEDS

using

XYLANASE AX  
TABLETS

XYLAXFD 11/99



## INTRODUCTION:

Arabinoxylan is the major endosperm cell-wall polysaccharide of wheat and rye and 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*- $\beta$ -D-Xylanase (xylanase) is added to feeds to catalyse 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 enzyme during pelleting, binding of the enzyme to feed components and inhibition by soluble feed components.

The only biochemical methods with sufficient sensitivity and specificity to measure xylanase in feeds are viscometric assays and those employing Xylazyme AX test tablets (which contain dyed and crosslinked wheat arabinoxylan). The sensitivity of these two assay procedures is similar, however, viscometric assays are extremely tedious and allow only a limited number of assays to be performed concurrently. **In contrast**, assays employing Xylazyme AX tablets are rapid, reproducible and simple to perform.

It is generally accepted that xylanase enzymes which are best suited to feed applications have optimal activity at pH 6.0. Consequently, these enzymes are generally assayed at this pH in 0.1M sodium phosphate buffer. However, we have shown that sodium phosphate buffer extracts only a small proportion (< 20%) of the amount of enzyme added to the feed (in 'spiking' experiments). Consequently, we have evaluated a wide range of alternative extractants and extraction conditions and our final recommendations for the extraction and assay of xylanase in feeds is detailed below.

Of all the conditions and extractants evaluated, the best and most consistent results for feeds containing *Trichoderma* sp xylanases have been obtained using 0.1M acetic acid at room temperature. Optimal extraction of *Humicola* sp. xylanases is achieved with a buffer containing MES buffer salt (0.1M, pH 6.0; Sigma M-8250) and sodium dodecyl sulphate (SDS; 1% w/v).

## **KIT COMPONENTS:**

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

1. Xylazyme AX test tablets (200 tablets)
2. *A. niger* Control xylanase solution in 50% glycerol
3. *T. longibrachiatum* Control xylanase solution in 50% glycerol

## **EXTRACTION BUFFERS: (not enclosed)**

### **(A) Acetic acid (0.1M)**

Glacial acetic acid (5.75ml, 1.05g/mL) is added to 900ml of distilled water and the volume is adjusted to 1 litre.

### **(B) MES/SDS Buffer**

[MES (0.1M, pH 6.0) plus SDS (1%)]

MES free acid (Sigma M-8250; 19.5g is added to 900ml of distilled water and dissolved. The pH is adjusted to 6.0 with 1M sodium hydroxide, and SDS (lauryl sulphate, Na salt; Sigma L-4509; 10g) is added and dissolved. The volume is adjusted to 1 litre.

## **EQUIPMENT (RECOMMENDED):**

1. Glass test tubes (round bottomed; 16 x 100mm and 16 x 120mm)
2. Micro-pipettors eg: Gilson Pipetman 500 microlitre and 100 microlitre
3. Positive displacement pipettor eg: Eppendorf Multipette®  
- with 5.0mL Combitip® (to dispense 0.2ml aliquots of xylanase control in 50% glycerol)
4. Adjustable volume dispenser set at 5.0mL (to dispense Trizma Base solution)
5. Top-pan balance correct to 0.01g
6. Spectrophotometer set at 590nm
7. Vortex mixer (we recommend the Thermolyne Maxi-Mix II)
8. Thertman No. 1 (9cm) filter circles and filter funnels.

## EXTRACTION AND ASSAY OF XYLANASE IN FEED SAMPLES

### *Trichoderma* sp. Xylanases:

#### EXTRACTION:

1. A feed sample (approximately 100 grams) is milled to pass a 0.5mm screen and mixed thoroughly.
2. Samples of the above feed ( $0.5 \pm 0.01\text{g}$  in quadruplicate) are weighed into glass test-tubes (16 x 120mm).
3. Each sample is treated with 5ml of 0.1M acetic acid and stirred on a vortex mixer. To two of these tubes, water (0.2ml) is added with stirring, and to the other two tubes is added control *Trichoderma* sp. xylanase (0.2ml, 104mUnits) with vigorous and immediate stirring on a vortex mixer.
4. The slurries are left at room temperature with occasional stirring on a vortex mixer over the following 20 minutes.
5. Tubes are centrifuged (3,000rpm, 10 min) in a bench centrifuge and the supernatant is used directly in assays. Assays should be initiated within 30 minutes of obtaining these extracts to minimise loss of enzyme activity in these extracts.

#### ASSAY:

1. Aliquots of supernatant solutions (0.5ml) are accurately transferred (in duplicate) to glass test-tubes (16 x 100mm) and these tubes are **left at room temperature**.
2. To each tube a Xylazyme AX tablet is added (without stirring) and the tube is immediately placed in a water bath set at  $50^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$  and incubated for exactly 30 minutes.
3. After exactly 30 minutes, the tubes are treated with Trizma Base solution (5ml, pH ~9), stirred vigorously on a vortex mixer and stored at **room temperature** for 5 minutes.

**A. This treatment terminates the reaction.**

**B. The tubes must be stored at room temperature and not at  $50^{\circ}\text{C}$ , as the substrate is not stable under alkaline conditions at elevated temperatures (ie: absorbance values will increase due to substrate breakdown).**

4. Tubes are stirred on a vortex mixer and the slurry is filtered through a Whatman No. 1 (9cm) filter paper.
5. The absorbance of the filtrates are measured against a **Reaction Blank**.

The Reaction Blank is prepared by adding Trizma Base solution (5ml) to the feed extract (0.5ml), followed by the addition of a xylazyme AX tablet. The slurry is stirred and stored at room temperature for 5 minutes 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:

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

### WHERE:

Activity added = the amount of xylanase added to the feed slurry at the time of assay eg: 104 mUnits in the control xylanase solution (0.2ml).

SA = the reaction absorbance obtained for extracts of the feed to which no control xylanase was added.

TA = the total absorbance ie: the absorbance of extracts of the sample to which the control xylanase was added.

### EXAMPLE CALCULATION:

Sample	ABS/30 min. incubation
1. Starter control feed	0.000
2. Starter control + Trichoderma sp. xylanase	1.059
3. Sample 2 + 104mU xylanase (in the assay)	1.544

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

## WHERE:

- SA = absorbance of **extract of the sample**  
[assayed by the standard format (eg: 1.059)]
- TA = **total absorbance**; ie: the absorbance of extracts of the sample to which the additional xylanase (0.2ml of 520 mUnits/ml) was added (eg: Abs = 1.544)

## THUS:

Activity in feed (Units/0.5g);

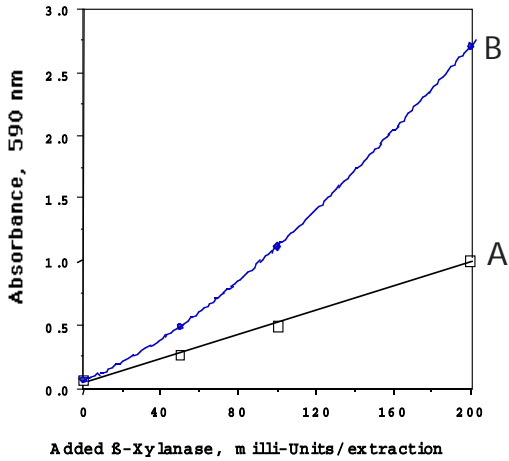
- =  $104/1000 \text{ Units} \times 1.059 / (1.544 - 1.059)$
- =  $0.104 \times 1.059 / 0.485 = \mathbf{0.227 \text{ Units / 0.5 grams}}$
- =  $0.227 \times 2000 = \mathbf{454 \text{ Units/Kg or 454,000 Units/ton}}$

## REFERENCE:

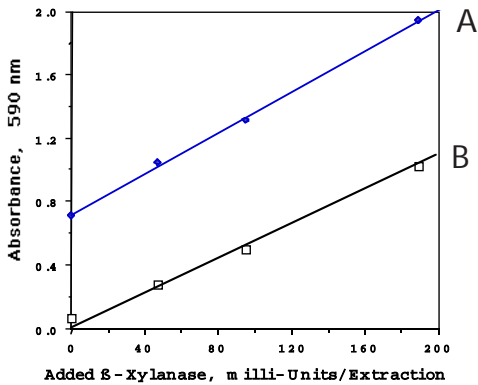
McCleary, B.V. "Problems in the measurement of  $\beta$ -xylanase,  $\beta$ -glucanase and  $\alpha$ -amylase in feed enzymes and animal feeds". In "**Proceedings of Second European Symposium on Feed Enzymes**" (W. van Hartingsveldt, M. Hessing, J.P. van der Lugt and W.A.C. Somers Eds.) Noordwijkerhout, 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 (<0.5mm) before use. The recovery of activity was approximately 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 which 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 recovery of enzyme added at the time of extraction, is linear and that the curves for the two feeds are approximately parallel.



**Figure 1:** Curves showing the recovery of *T. longibrachiatum* (pl 9.0) xylanase (as absorbance 590nm 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 0.1M acetic acid was quite surprising. Under these extraction conditions (0.5g feed per 5ml 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 which 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.

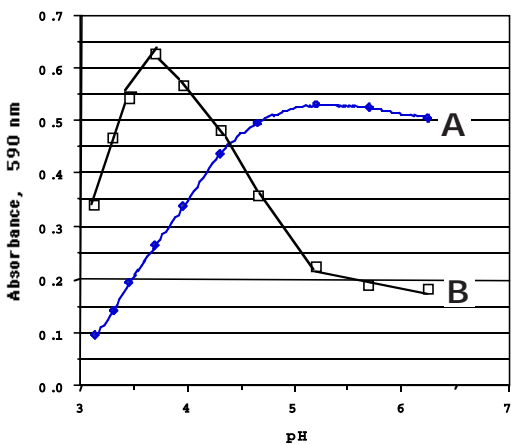


Figure 3. pH activity curves for the original *T. longibrachiatum* enzyme preparation (A) and for the xylanase fraction extracted from the feed sample (B).

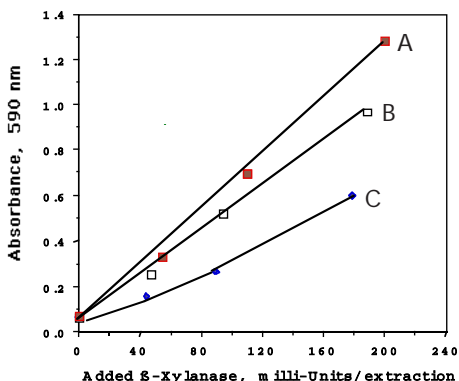


Figure 4. Binding of different highly purified xylanase enzymes to feed components. Extraction and assay conditions are as described in the text.

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



Megazyme International Ireland Ltd.,  
 Bray Business Park, Bray,  
 Co. Wicklow,  
 IRELAND

Telephone: (353.1) 286 1220  
 Facsimile: (353.1) 286 1264  
 Internet: [www.megazyme.com](http://www.megazyme.com)  
 E-Mail: [info@megazyme.com](mailto:info@megazyme.com)

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