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Endo-1,4-β-xylanase from *Bacillus subtilis* expressed in *Bacillus subtilis* (JECFA99-2)

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Endo-1,4-β-xylanase from *Bacillus subtilis* expressed in *Bacillus subtilis* (JECFA99-2)

New specifications prepared at the 99th JECFA (2024), published in FAO JECFA Monographs 34 (2025). An ADI "not specified" was established at the 99th JECFA (2024)

SYNONYMS	Endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo β 1,4 xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo 1,4-β-xylanase; β-D-xylanase
SOURCES	Produced by a genetically modified non-pathogenic and non toxigenic strain of *Bacillus subtilis* expressing the endo 1,4 β xylanase gene from *B. subtilis* under controlled submerged fed-batch pure culture fermentation. The endo 1,4 β xylanase is recovered from the fermentation broth by the separation of cellular biomass, concentration by ultrafiltration, purification by ion exchange chromatography and microfiltration. The final liquid product is formulated and standardized to the desired activity.
Active principles	Endo-1,4-β-xylanase
Molecular weight	With an apparent molecular weight of 20 kDa equivalent to the calculated molecular weight of the enzyme
Systematic names and numbers	4-β-D-xylan xylanohydrolase; IUBMB number: 3.2.1.8; CAS number: 9025-57-4.
Reaction catalysed	Endohydrolysis of 1,4-b-D-xylosidic linkages in xylans (including arabinoxylans) resulting in the generation of (1→4)-b-D-xylan oligosaccharides of different lengths.
Secondary enzyme activities	No significant secondary activities
DESCRIPTION	Brown liquid
FUNCTIONAL USES	Enzyme preparation Used as a processing aid in baking applications
GENERAL SPECIFICATIONS	Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Endo-1,4-β-xylanase activity	The sample shows endo-1,4-β-xylanase activity See description under TESTS

TESTS

METHOD OF ASSAY

Endo-1,4-β-xylanase activity

PRINCIPLE

The activity of endo-1,4-β-xylanase is determined using birchwood xylan as substrate. The analytical principle is based on hydrolysis of xylan to reducing sugars (xylose equivalents) at pH 6.0, 50 °C and 15 min. The released reducing sugars is measured spectrophotometrically at 570 nm. The endo 1,4 xylanase activity is quantified relative to a reference enzyme standard and expressed in Birchwood D(+) Xylanase Units/ml (BDXU/ml).

One BDXU is defined as the amount of enzyme that liberates 1 µmol of reducing sugars (xylose equivalents) from birchwood xylan per minute per ml at pH 6.0 and 50 °C.

MATERIALS AND EQUIPMENT

- Analytical balance (precision: 0.001 g)
- 96-well Microplate
- Spectrophotometer
- pH Meter
- Vortex mixer
- Thermostated water bath
- Positive displacement pipets with tips

REAGENTS AND SOLUTIONS

- Deionized water
- Citric acid monohydrated ($C_6H_{10}O_8$)
  Di-sodium hydrogen phosphate dehydrated ($Na_2HPO_4 \cdot 2H_2O$)
- Birchwood xylan
- Sodium hydroxide (NaOH)
- 37% Hydrochloric acid (HCl)
- D(+) xylose
- 2-hydroxy-3,5-dinitrobenzoic acid (DNS, $C_7H_4N_2O_7$)
- 29% Sodium hydroxide (NaOH)
  Potassium sodium tartrate tetrahydrate ($NaKC_4H_4O6 \cdot 4H_2O$)
- Reference endo-1,4-β-xylanase of known activity in BDXU/g

SOLUTIONS

50 mM Citrate Phosphate Buffer (pH 6.0)

- Weigh 10.5 g citric acid monohydrate and 8.9 g $Na_2HPO_4 \cdot 2H_2O$, respectively and dissolve in 600 ml deionized water in a 1 000-ml volumetric flask by mixing on shaker at 500 rpm.
- Adjust pH to 6.0 with 29% NaOH
- Make up the volume to 1 000 ml with deionized water.
- Store at 4 °C in a closed container. Stable for 1 week.

5% NaOH Solution (w/v)

- Weigh 3.0 g NaOH and dissolve in 60 ml deionized water in a beaker.
- Mix on shaker at 500 rpm to dissolution.

3% Birchwood Xylan Solution

- Weigh 6.0 g birchwood xylan and dissolve in 60 ml of 5% NaOH solution in a 250-ml beaker by mixing on shaker 300 rpm for 20 minutes.
- Add 100 mL of 50 mM citrate phosphate buffer and continue to mix.
- Adjust pH to 6.0 with 37% HCI.
- Quantitatively transfer to a 200-ml volumetric flask and make up to volume with deionized water.
- Prepare the solution on the day of assay.

40 mM Xylose

- Weigh 0.300 g D(+) xylose in a 50-ml volumetric flask and dissolve in a small amount of deionized water on shaker at 500 rpm.
- Make up the volume to 50 ml with deionized water and mix.
- Aliquot the solution in 1-ml tubes and freeze at -20 °C.

1 M NaOH Solution

Weigh 16.0 g NaOH and dissolve in 400 ml deionized water in a 600-ml beaker by mixing on shaker at 500 rpm to dissolution.

DNS Solution

Weigh 10.0 g DNS in a 1 000-ml beaker and add 300 ml deionized water.

Mix on shaker at 500 rpm and heat to 100 °C to dissolution.

Add 400 ml of 1M NaOH and continue to mix on shaker at 500 rpm.

Add 300 g potassium sodium tartrate tetrahydrate and continue to mix on shaker at 500 rpm to dissolution.

Transfer the solution to a 1 000-ml volumetric flask and make up the volume to 1 000 ml with deionized water.

Store in a glass bottle and cap. This solution can be stored in the dark at room temperature for up to one week.

Reference Endo-1,4-β-xylanase Solution

Weigh 1.0 g of the reference endo-1,4-β-xylanase standard in a 100-ml volumetric flask and let it dissolve completely in 50 mM citrate phosphate buffer by placing it at 4 °C overnight or mixing at room temperature for approximately 30 min.

Filter the solution with filter paper on ice.

Make a dilution with 50 mM citrate phosphate buffer to approximately 1.7 BDXU/ml so that its net absorbance falls within the linear range of the assay after the subtraction of the blank.

Place the standard solution on ice (stable for 8 hours).

Prepare on the day of assay.

Sample

Dilute each sample with 50 mM citrate phosphate buffer to obtain a final net absorbance between 0.45 and 0.55.

Place the diluted samples on ice; samples are stable for 8 hours.

Assay Procedure

Prepare the following dilutions of xylose standard according to the table below with 40 mM xylose standard solution:

Dilutions of xylose standard with 40 mM xylose standard solution

TUBE #	1 (BLANK)	2	3	4	5	6
Deionized water (µl)	100	80	60	40	20	0
40 mM xylose standard solution (µl)	0	20	40	60	80	100
BDXU/ml equivalent	0	8	12	24	32	40

Arrange test tubes in triplicate per sample. Add 100 µl of sample to each test tube. A maximum of 10 samples can be determined simultaneously per assay.

Set up duplicate blanks per sample.

Set up two reference endo-1,4-β-xylanase samples, in triplicate.

Add 700 µl of 3% birchwood xylan substrate solution to each test tube of xylose standards, reference endo 1,4 β-xylanase standards, samples and blanks.

Mix well for 5 seconds and incubate at 50 °C for 15 minutes.

Add 1 ml DNS solution to each tube to stop the reaction.

Add 100 µll of sample solution to each test tube of the blank.

Place all the tubes in a water bath at 95 °C for 15 minutes. Cool all the tubes for 5 minutes in an ice bath.

Transfer 300 µl each of xylose standard, samples, reference endo-1,4-β-xylanase standards and blanks, respectively into a 96-well microplate and measure the absorbance at 570 nm.

CALCULATION

To determine the net ΔAbsorbance, subtract the average blank from the absorbance reading of all standards and samples.

Prepare the standard curve using linear regression where net absorbance is on the y-axis and concentration of xylose (BDXU/ml) on the x-axis.

The net absorbance should be in the range between 0.45 and 0.55

The correlation coefficient must be ³ 0.99.

Determine the concentration of each sample from linear regression using the following equation:

$$\frac{\text{BDXU}}{\text{ml}} = \frac{(\Delta\text{Abs} - \text{b})}{\text{a}} \times \frac{\text{Dilution}}{15} \times \frac{A_{ref1}}{A_{ref2}}$$

Where:

ΔAbs: average absorbance of sample
(or reference endo 1,4 β xylanase standard) – average absorbance of blank

b: Y intercept of the calibration curve

a: Slope of the calibration curve

15: Time in minutes

Aref1: Known endo-1,4-β-xylanase activity of the reference standard

Aref2: Measured Endo-1,4-β-xylanase activity of the reference standard

Note: The ratio of Aref1/Aref2 must be between 0.9 and 1.1