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BEECH XYLAN UNIT (BXU) ASSAY

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A. Principle: Xylanase in the sample hydrolyzes the substrate, beech xylan, and the amount of released reducing carbohydrate is determined spectrophotometrically using dinitrosalicylic acid. The method is suitable for measurement of enzymes samples containing *Aspergillus* or *Trichoderma* originated xylanase. The linearity of the assay should be checked when enzymes from other sources are analyzed.

B. Equipment:
1. pH Meter
2. Constant Temperature Water Bath at 50.0°C ± 0.5°C.
3. Boiling Water Bath approximately 100°C.
4. Analytical Balance
5. Spectrophotometer with wavelength setting at 540nm
6. Volumetric Flasks
7. Volumetric Pipettes
8. 25 ml screw cap tubes
9. Timer
10. Automatic Pipetters

C. Safety Precautions:
1. Utilize standard laboratory safety practices.
2. DNS- reagent is harmful by inhalation, contact with skin and eyes and if swallowed.

D. Reagents and Reagent Preparation: Volumes may be adjusted depending on requirements.
1. Citrate Buffer (0.05 M, pH 5.3) – dissolve 10.5 g of citric acid ($C_6H_8O_7 \cdot H_2O$) in about 800 ml of distilled water and adjust the pH with 1 M NaOH to 5.3. (The consumption should be about 110 ml.) Dilute to 1L with water in a volumetric flask.
2. Substrate – 1% beech xylan – Dissolve 1.0 g of Beech Xylan (Megazyme) by slowly adding into about 80 ml of citrate buffer already spinning. Heat to boiling point on a heating magnetic stirrer. Cool with continued stirring to room temperature. Dilute to volume in a 100 ml volumetric flask with citrate buffer. Store at 4°C for a maximum of 1 week or freeze in aliquots at -20°C. Mix well after thawing.
3. DNS reagent:(Stock Reagent)
   a. Cover a 2000 ml beaker with tinfoil to avoid light exposure.
   b. Place the beaker on the stir plate and add approximately 600 ml of distilled water and a stir bar.
   c. Use continuous magnetic stirring.
   d. Add 10.0 g of dinitrosalicylic acid, allow to dissolve.
   e. Gradually add 16.0 g of NaOH, allow to dissolve.
   f. Slowly add 300 g of Rochelle Salt over a 20-30 minute period.
   g. The solution may be cautiously warmed to a maximum temperature of 45°C to clear the solution.
   h. The solution may be filtered through a Whatman #1 Filter paper if needed.
   i. Allow the solution to cool.
   j. Analytically transfer the solution to a 1000 ml volumetric flask and dilute to volume with distilled water.

E. Procedure:
1. Enzyme Preparation:
   a. Dissolve an appropriate amount of enzyme preparation in Citrate Buffer. Use the same buffer if serial dilutions are required. The dilution of the enzyme should be used within 30 minutes. The final dilution concentration should correspond to an absorbance of approximately 0.10 – 0.40.
   b. Calculating Enzyme preparation:

$$\text{gram weight of sample} = \frac{16.7\ \text{BXU}}{\text{BXU (Target)}}$$

2. Enzyme Evaluation:
   a. Pipette 1.8 ml of substrate solution into 3 labeled 25 ml screw cap tubes, two for each enzyme test and one for the enzyme blank. (1A,1B, and 1C... is recommended.)
   b. Equilibrate at 50°C for 5 minutes.
   c. At zero time, start a timer and add 200 µl of suitably diluted enzyme solution to the first test tube and mix with a vortex mixer. Continue enzyme addition at sufficient interval to each tube except enzyme blanks.
   d. After exactly 5 minutes, add 3.0 ml of DNS reagent to both tubes and vortex.
   e. Add 3.0 ml of DNS reagent followed by 200 µl of sample solution to the enzyme blank tube.
   f. Remove the tubes from the water bath and place in a boiling water bath. After boiling for exactly 5 minutes remove the tubes and cool in cold water to room temperature.
   g. Read the absorbances in a 1- cm cuvette at 540 nm using air to set the spectrophotometer to zero. Correct the A_{540} value of each enzyme test by subtracting the reading of the respective enzyme blank.
   h. Determine the xylose concentration in the sample from the standard curve.
3. Standard Curve (Daily):
   a. Prepare a 10mM xylose stock solution. Dissolve 150 mg of xylose in citrate buffer and dilute with buffer to 100 ml in a volumetric flask. The solution is diluted in buffer as follows:

DILUTION	XYLOSE µMOL/ML	BXU
1:1 (0.150g/100ml)	10.0	33.3
1:2 (25ml/50 ml)	5.0	16.7
1:3 (16ml/50ml)	3.2	10.7
1:5 (10ml/50ml)	2.0	6.7

   b. Do duplicate assays of each standard dilution in the way as the enzyme blanks:
   c. Pipette into test tubes 1.8 ml of substrate.
   d. Add 3.0 ml of DNS and 200µl standard dilution.
   e. Prepare the reagent blank by adding 200 µl of citrate buffer instead of the standard dilution.
   f. Boil the tubes exactly 5 minutes, cool.
   g. Read the absorbencies of the filtrates in a 1- cm cuvette at 540 nm using air to set the spectrophotometer to zero. Correct the A_{540} value of each enzyme test by subtracting the reading of the average of the enzyme blanks.

F. Calculations:

1. Unit of activity: One xylanase unit (BXU) is defined as the amount of enzyme that produced reducing carbohydrates having a reducing power corresponding to one nmol xylose from beech xylan in one second under assay conditions (1 BXU = 1nkat).
2. The xylanase activity (BXU/g) is obtained by multiplying the xylose concentration (determined by the standard curve in µmol/ml) by 1000, dividing by the reaction time (300 s) and the enzyme dilution concentration in g/ml.

$$\text{BXU} = \frac{\text{Xylose Conc. (from curve) x 1000}}{\text{enzyme conc. (g/ml) x 300 s.}}$$

G. Testing Accuracy Parameters:

1. Range: Absorbance readings for each unknown sample must be bracketed by the absorbance readings of the standard curve.
2. Duplicate tests should not vary by more than 6%.

H. Reference:

1. Baily, M.J. and Poulanen, K. (1989). *Production of xylanases by strains of Aspergillus*. Appl. Microbiol. Biotechnol. 30:5-10