



CELLULASE ANALYTICAL METHOD (ECU)

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- A. **Principle:** Endo-4- β -glucanase in the sample hydrolyzes the substrate Hydroxyethylcellulose, and the reducing sugars thus produced are assayed spectrophotometrically using dinitrosalicylic acid.
- B. **Equipment:**
1. pH Meter
 2. Constant Temperature Water Bath at $50.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$
 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: All solutions are prepared in deionized water, Milli-Q or equivalent.**
1. Citrate Buffer (0.05 M, pH 4.8): Prepare 0.05 M solutions of both citric acid ($\text{C}_6\text{H}_8\text{O}_7 \bullet \text{H}_2\text{O}$, 10.51 g/l) and sodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \bullet 2\text{H}_2\text{O}$, 14.71 g/l) in water. Adjust the pH of the 0.05M citrate solution to 4.8 with the 0.05 M citric acid solution (should require about 667 ml of citric acid solution per 1 liter of sodium citrate solution).
 2. Substrate: Dissolve 1.00 g hydroxyethylcellulose (Fluka AG) in citrate buffer and make the volume up to 100 ml. The powder is dissolved with magnetic stirring for at least one hour, after which it must stand 1 hour to clarify. Store at $+ 4^{\circ}\text{C}$ for a maximum of 2 weeks.
 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.

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- 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 0.200 – 0.250.
- b. Calculating Enzyme preparation:

$$\text{gram weight of sample} = \frac{10.01 \text{ ECU}}{\text{ECU (Target)}}$$

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 one of the test tube and mix with a vortex mixer. Continue enzyme addition at a sufficient interval to each tube except enzyme blanks.
- d. After exactly 10 minutes, add 3.0 ml of DNS reagent to each tube 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 tubes 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 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 respective enzyme blank.
- h. Determine the glucose concentration in the sample from the standard curve.

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3. Standard Curve (Daily):

- a. Prepare a 0.1 M glucose stock solution. Dissolve 1.802 g of glucose in citrate buffer and dilute with buffer to 100 ml in a volumetric flask. The solution is diluted in buffer as follows:

DILUTION	GLUCOSE $\mu\text{MOL/ML}$	ECU ²
1:25 (2ml/50ml)	4.0	6.67
1:15 (3ml/50ml)	6.0	10.01
1:10 (5ml/50ml)	10.00	16.67

- b. Do triplicate 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 200 μl 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 endo-1, 4- β -glucanase unit (ECU) is defined as the amount of enzyme producing one nmole of reducing sugars as glucose in one second (1 ECU = 1 nkat).
1. The corresponding endo-1, 4- β -glucanase activity (nkat/ml) is obtained by multiplying the glucose concentration ($\mu\text{mol/ml}$) by 1000, dividing by the reaction time (600s) and the enzyme dilution concentration in g/ml.

$$\text{ECU/g} = \frac{(\mu\text{mol/ml glucose produced}) \times 1000}{\text{enzyme conc. (g/ml)} \times 600 \text{ s.}}$$

G. Testing Accuracy Parameters:

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

H. References:

1. Bailey, M.J., Nevalainen, K.M.H, Enzyme Microb. Technology 3, 1981, 153-157.

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