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FCC ALU (FCC IV ACID LACTASE - β-galactosidase)

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A. Principle: This procedure is used to determine lactase activity of enzyme preparations derived from *Aspergillus oryzae* var. The assay is based on a 15 minutes hydrolysis of an o-nitrophenyl-β-D-galactopyranoside substrate at 37° C and pH 4.5.
Note: The enzyme assay procedure is not intended to be used solely with enzymes derived from any source organisms. Applicability of the assay to these organisms or enzyme sources should be considered informational and not a requirement.

B. Equipment:
1 Volumetric Pipettes
2 Volumetric Flasks
3 25 ml screw capped test tubes
4 Constant temperature water bath at 37° C ± 0.1° C
5 Spectrophotometer set to read at 420 nm
6 pH Meter
7 Timer
8 Analytical Balance
9 Automatic Pipetters

C. Safety Precautions:
1 Utilize standard laboratory safety practices.
2 Acetic Acid: Pipetting should be preformed in the Fume hood.
3 o-Nitrophenol: Causes irritation, harmful if inhaled.

D. Reagent and Reagent preparation:
1 Acetic Acid 2N: Add 11.5 ml of glacial acetic acid into a 100 ml volumetric flask and dilute to volume with distilled water. Mix well, and store in a refrigerator.
2 4.0 N Sodium Hydroxide: Dissolve 16.0 g of sodium hydroxide in approximately 50 ml of distilled water and dilute to volume in a 100 ml volumetric flask or dilute an appropriate volume of stock 10 N stock NaOH. (Other 4N NaOH solutions may be used.)
3 Acetate Buffer: Combine 50 ml of 2.0 N Acetic Acid and 11.3 ml of 4.0 N Sodium Hydroxide in a 900 ml of distilled water, verify that the pH is 4.50 ± 0.05 using a pH meter, adjust if necessary with 2.0 N Acetic Acid or 4.0 N Sodium Hydroxide and dilute to volume in a 1000 ml volumetric flask with distilled water.
4 Sodium Carbonate Solution 1%: (Only necessary when analyzing standard curve) Dissolve 10.0 g of Sodium Carbonate in approximately 250 ml of distilled water and dilute to volume in a 1000 ml volumetric flask with distilled water.
5 Sodium Carbonate Solution 10%: Dissolve 10.0 g of Sodium Carbonate in approximately 50 ml of distilled water, quantitatively transfer the solution to a 100 ml volumetric flask and dilute to volume with distilled water.
6 Substrate: (Protect from light during and after preparation)
a. Dissolve 370.0 mg of o-nitrophenyl-β-D-galactopyranoside in approximately 50 ml of Acetate Buffer.
b. Quantitatively transfer the solution to a 100 ml volumetric flask and dilute to volume with Acetate Buffer. Protect from light
c. Perform the assay procedure within 2 hours of Substrate preparation.

E. Procedure
1 Enzyme Preparation:
a. Dissolve an appropriate amount of enzyme preparation in distilled water. Use distilled water if serial dilutions are necessary. Prepare a solution from the test sample preparation such that 1 ml of the final dilution will contain between 0.15 and 0.65 lactase units.
b. Calculating Enzyme preparation concentration:

$$\text{Sample concentration (g/ml)} = \frac{\text{0.2 Acid Lactase Units}}{\text{FCC-ALU (Target)}}$$

c. Weigh an appropriate amount of enzyme in grams, and dilute to the desired concentration using serial dilutions. **Fungal Lactase powders should be weighed at approximately 0.55g**, and liquids should be weighed >1.0g for greater accuracy.

d. Perform the assay procedure within 2 hours of dissolution of the Enzyme Preparation.
If the sample has a theoretical activity of 100,000 FCC-ALU/g:

$$\text{Sample concentration} = \frac{\text{0.2units}}{\text{100,000U/g}} = 0.000002$$

Therefore, the following serial dilutions would be made,
Dilution 1: 0.55g enzyme / 250ml total volume
Dilution 2: 4ml of dilution #1 / 250ml total volume
Dilution 3: 3ml of dilution #2 / 50 ml total volume
The concentration is calculated 0.55g/250ml X 4ml/250ml X 3ml/50ml = 0.00000212g/ml

If the sample has a theoretical activity of 2,000 FCC-ALU/g:

$$\text{Sample concentration} = \frac{\text{0.2units}}{\text{2,000U/g}} = 0.0001$$

Therefore, the following serial dilutions would be made,
Dilution 1: 0.55g enzyme / 250ml total volume
Dilution 2: 5ml of dilution #1 /100ml total volume
The concentration is calculated 0.55g/250ml X 5ml/100ml = 0.00011g/ml

2 Enzyme Evaluation:
a. Pipette 2 ml of the ONPG Substrate solution into 2 labeled screw cap test tubes for each sample and reagent blank.
b. Place the tubes in the 37° C water bath for a minimum of 10 minutes.
c. At zero time, add 0.5 ml of the Enzyme Preparation (use 0.5 ml distilled water for the reagent blank) to each tube. Vortex the tubes and immediately return them to the bath. Allow sufficient time between injections.
d. Allow the tubes to incubate in the 37° C water bath for exactly 15 minutes.
e. At 15 minutes add 2.5 ml of 10% Sodium Carbonate to stop the reaction. Vortex the tubes vigorously.
f. Add 20 ml of distilled water to each tube and mix thoroughly.
g. Using a suitable Spectrophotometer, record and print the Optical Density of each sample at 420nm using distilled water to zero the instrument.

3 Standard Curve: (System suitability) Yearly
a. 2.0 mM o-Nitrophenol Stock: Transfer 139.0 mg of o-Nitrophenol to a 50 ml beaker, add 10 ml of USP alcohol (95% ethanol) and swirl to dissolve. Quantitatively transfer the solution to a 500 ml volumetric flask and dilute to volume with 1% sodium carbonate.
b. 0.10 mM Standard Solution: Pipette 5.0 ml of the 2.0 mM o-Nitrophenol Stock solution into a 100 ml volumetric flask and dilute to volume with 1 % sodium carbonate solution.
c. 0.14 mM Standard Solution: Pipette 7.0 ml of the 2.0 mM o-Nitrophenol Stock solution into a 100 ml volumetric flask and dilute to volume with 1 % sodium carbonate solution.
d. 0.18 mM Standard Solution: Pipette 9.0 ml of the 2.0 mM o-Nitrophenol Stock solution into a 100 ml volumetric flask and dilute to volume with 1 % sodium carbonate solution.
e. Determine the absorbance of the three o-Nitrophenol Standards at 420nm in 1-cm cell, using a suitable spectrophotometer. Use water to zero the instrument. Calculate the millimolar extinction, ε, for each o-Nitrophenol Standard (0.10, 0.14, and 0.18 mM).

$$\varepsilon = A_n/C$$

A_n = the absorbance of each o-nitrophenol standard at 420 nm
C = the corresponding concentration of o-nitrophenol standard

f. ε for each standard should be approximately 4.60 per mM. Perform a linear regression analysis of the absorbance readings of the three o-Nitrophenol Standards versus the o-nitrophenol concentrations (0.10, 0.14, and 0.18 mM.). The r^2 should be greater than or equal to than 0.99.

g. Determine the mean ε of the three o-Nitrophenol Standards for use in the calculations below.

F. Calculations:

1 Unit of Activity: One acid lactase unit (ALU) is defined as that quantity of enzyme that will liberate o-nitrophenol at a rate of 1 μmol/min under the conditions of the assay.

2 Calculate the activity (lactase activity per gram) of the enzyme preparation taken for analysis as follows:

$$ALU/g = [(A_s - B)(25)]/[(\varepsilon)(15)(W)]$$

A_s = absorbance reading for the Enzyme Test Preparation
B = average of absorbance readings for the reagent blank
25 = final volume, in ml, of the diluted incubation mixture
ε = mean absorptivity of the o-Nitrophenol Standards per μM
15 = incubation time in minutes
W = weight in g of original enzyme preparation contained in the 0.5 ml aliquot of Test Preparation (final concentration of the enzyme dilution multiplied by 0.5, the volume of enzyme injected.)

G. Testing Accuracy Parameters:

1 Enzyme preparations are diluted to a concentration between 0.15 and 0.65 ALU/ml.

2 A Reference Material (RM) preparation should be evaluated with each substrate preparation. The RM should not deviate from its predetermined value by more than ± 5.0%.

H. Reference:

1 Food Chemicals Codex, Current Edition.