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LACTASE NL ANALYTICAL METHOD (YLU)

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A. Principle: This procedure is used to determine the neutral lactase activity of enzyme preparations derived from *Kluyveromyces marxianus* var. *lactis* and *Saccharomyces* sp. The assay is based on a 10 minute hydrolysis of an o-nitrophenyl-β-D-galactopyranoside (ONPG) substrate at 30.0° C ± 0.1° and at pH 6.50.

B. Equipment:

1. pH Meter
2. Constant Temperature Water Bath at 30.0° ± 0.1°C
3. Analytical Balance
4. Spectrophotometer with wavelength setting at 420nm
5. Volumetric Flasks
6. Volumetric Pipettes
7. 25 ml screw cap tubes
8. Timer
9. Automatic Pipetters

C. Safety Precautions:

1. Utilize standard laboratory safety practices.
2. o-Nitrophenol: Causes irritation, harmful if inhaled.

D. Reagents and Reagent preparations:

1. Magnesium Solution: Dilute 2.465 g of magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) in about 80 ml of distilled water. Transfer the solution into a 100 ml volumetric flask, dilute to volume with distilled water, and mix.
2. EDTA Solution: Dissolve 0.186 g of disodium EDTA dihydrate ($C_{10}H_{14}N_2Na_2O_8.2H_2O$) in about 80 ml of distilled water. Transfer the solution into a 100 ml volumetric flask, dilute to volume with distilled water and mix.
3. P-E-M Buffer: Dissolve 17.6 g of potassium dihydrogen phosphate (KH_2PO_4) and 12.2 g of dipotassium hydrogen phosphate (K_2HPO_4) in about 1800 ml of water. Add 20.0 ml of Magnesium Solution and 20.0 ml EDTA Solution. Transfer the solution into a 2000 ml volumetric flask, dilute to volume with water, and mix. The pH should be 6.50 ± 0.05.

4. ONPG Substrate: (Protect from light) Dissolve 250.0 mg ONPG (use lot currently in use) in about 80 mL of P-E-M Buffer. Transfer the solution to a 100 ml volumetric flask, dilute to volume with P-E-M Buffer.
   Prepare, at most, 2 hr. before testing.
5. Sodium Carbonate Solution: Dissolve 50.0 g of sodium carbonate anhydrous (Na_2CO_3) and 37.2 g of disodium EDTA dihydrate ($C_{10}H_{14}N_2Na_2O_8.2H_2O$) in about 900 ml of water. Transfer the solution into a 1000 ml volumetric flask, dilute to volume with water, and mix.

E. Procedure:

1. Enzyme Preparation
   a. Dissolve an appropriate amount of enzyme preparation (at least 1g) in P-E-M Buffer. Use the same buffer if serial dilutions are needed so that 1 ml of this final dilution contains between 0.035 and 0.12 YLU
   b. Calculating enzyme preparation concentration:

$$\text{g. weight of enzyme} = \frac{0.07}{\text{Target YLU/g}}$$

   c. Weigh an appropriate amount of enzyme in grams, and dilute to the desired concentration using serial dilutions. Generally powders should be weighed >0.1g and liquids should be weighed >1.0g for greater accuracy.

   Example: The sample has a theoretical activity of 10,000 /g.

$$\text{Sample concentration} = \frac{0.07}{10{,}000\text{U/g}} = 0.000007$$

   Therefore, the following serial dilutions would be made,
   dilution 1: 0.7g enzyme/ 100ml total volume (0.007g/ml)
   dilution 2: 10 ml of dilution 1/ 100 ml total volume (0.1)
   dilution 3: 10 ml of dilution 2/ 100 ml total volume (0.1)
   dilution 4: 10 ml of dilution 3/ 100 ml total volume (0.1)
   The concentration is calculated 0.007g/ml (dil1) X 0.1 X 0.1 X 0.1=0.000007
   d. Perform the assay procedure within 2 h of dissolution of the enzyme preparation.

2. Enzyme Evaluation
   a. Pipette 5 ml of the ONPG Substrate solution into 3 labeled screw cap test tubes. (1A, 1B and 1C... for each sample number is recommended)
   b. Place the tubes in the 30° C water bath for a minimum of 5 minutes but not more than 15 minutes.
   c. At zero time, add 1.0 ml of the enzyme solution to each of the first two tubes (1A and 1B...etc.), vortex the tubes and immediately return them to the bath. Allow sufficient time between injections (One minute between injections is recommended.)
   d. Allow the tubes to incubate in the 30° C water bath for exactly 10 minutes.
   e. At 10 minutes, add 2 ml Sodium Carbonate Solution to the tubes (1A and 1B...etc)

f. Vortex the tubes vigorously and hold them at room temperature.
g. To the third tube (1C...etc) add the Sodium Carbonate Solution immediately followed by 1 ml of the original enzyme solution to act as the sample blank.
h. Determine the absorbances of all tubes at 420 nm in a 1-cm path length cell, with a suitable spectrophotometer, using water as the blank. Print the results.

3. Standard Curve: (System Suitability performed yearly in triplicate)
   a. Standard o-Nitrophenol Solution: 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 into a 1000 ml volumetric flask, dilute to volume with distilled water and mix.
   b. Pipette 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 14.0 ml portions of Standard o-nitrophenol solution into a series of 100 ml volumetric flasks.
   c. Add 25 ml of Sodium Carbonate Solution to each flask, Dilute each to volume with P-E-M Buffer, and mix.
   d. The dilutions contain respectively 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, and 0.14 μmol/ml of o-nitrophenol.
   e. Determine the absorbance in triplicate of each dilution at 420 nm in a 1-cm path length cell, with a suitable spectrophotometer, using water as the blank. For each dilution, plot absorbance against μmol of o-nitrophenol (this must result in a straight line through the origin). Divide the absorbance of dilution by μmol of o-nitrophenol to obtain the extinction coefficient (ε) at that dilution (the slope of the line is the extinction coefficient). Average the seven values thus calculated (this should result in a value of 4.60 ± 0.05).

F. Calculations:

1. Unit of Activity: One Yeast Lactase Unit (YLU) is defined as that quantity of enzyme that will liberate 1.0 μmol/min of o-nitrophenol under the conditions of the assay.
2. Calculate the activity of the enzyme preparation taken for the analysis as follows:

$$YLU/g = [(A \times 8)/(\varepsilon \times 10 \times W)],$$

A	=	absorbance reading for the sample
8	=	volume (ml), of the incubation mixture after termination
ε	=	extinction coefficient
10	=	incubation time (minutes)
W	=	weight in g of final enzyme preparation (concentration).

G. Testing Accuracy Parameters

1. Enzyme preparations are diluted to a concentration between 0.035 and 0.12 YLU units/ml.
2. A Reference Material is assayed each day to assure method accuracy