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REFERENCE AMYLASE UNIT ANALYTICAL METHOD (RAU)

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A. Principle: Bacterial amylase hydrolyzes starch into dextrins and maltose. Iodine is added to the hydrolysate at increasing times of incubation and the transmission of the color formed is measured spectrophotometrically. The time needed until the transmission is equal to that of the color reference, is a measure for the enzyme activity.

B. Equipment:

1. Volumetric Pipettes
2. Volumetric Flasks
3. Automatic pipetters
4. 50 ml screw capped test tubes
5. Constant temperature water bath at 30° C ± 0.2 C
6. Spectrophotometer set to read at 620 nm
7. pH Meter
8. Analytical Balance

C. Safety Precautions:

1. Utilize standard laboratory safety procedures.
2. Iodine: Poison. Fatal if swallowed or inhaled. Use ventilation hood, and appropriate PPE. Wash skin immediately upon contact and notify manager.
3. Potassium Iodide: Irritant. May cause fetal effects. Avoid prolonged exposure if pregnant.

D. Reagents:(Volumes may be adjusted depending on requirements)

1. Phoshate Buffer pH 6.6 (Stock Solution): Dissolve 10.7 g of potassium dihydrogen phosphate anhydrous, analytical reagent and 8.2 g of dipotassium hydrogen phosphate anhydrous analytical reagent in distilled water, adjust pH to 6.60 ± 0.005 with 1N HCL or NaOH and quantitatively transfer the solution to a 1L volumetric flask and dilute to volume with distilled water. Prepare a new buffer when the pH is not within the range.
2. Calcium Chloride Solution (Stock Solution): Dissolve 43.3 g of calcium chloride 6 aq. in distilled water quantitatively transfer the solution to a 1L volumetric flask and dilute to volume with distilled water.
3. Magnesium Chloride Solution (Stock Solution): Dissolve 14.0 g of magnesium chloride 6 aq. in distilled water quantitatively transfer the solution to a 1L volumetric flask and dilute to volume with distilled water.

4. Sodium Bicarbonate Solution(Stock Solution) Dissolve 21.0 g of sodium bicarbonate in distilled water quantitatively transfer the solution to a 1L volumetric flask and dilute to volume with distilled water.
5. RAU Buffer: To approximately 500 ml of distilled water in a 1L volumetric flask add 10 ml (in order listed) of each Stock Solution: Calcium Chloride Stock Solution, Magnesium Chloride Stock Solution and Sodium Bicarbonate Stock Solution and dilute to volume with distilled water
6. Stock Iodine Solution: Weigh 5.50g of iodine and 11.00 g of potassium iodide, and transfer quantitatively to a 250 ml beaker with stir bar using distilled water. Using distilled water, transfer the solution to a 250 ml volumetric flask and, dilute to volume and mix thoroughly. Transfer this solution to a dark brown bottle for storage. This solution, if stored in a dark cabinet, will normally be stable for about one month.
7. Working Iodine Solution: Dissolve 10 g of potassium iodide in distilled water, add 1.0 ml of the stock iodine solution with a volumetric pipette, transfer the solution to a 250 ml volumetric flask and dilute with distilled water to volume. Cover the solution with tinfoil to protect from light. This is the working solution used in the test.
8. Starch Substrate:
   a. Place a 600 ml beaker with a stir bar on a hot plate, and fill to approximately 250ml with distilled water.
   b. Heat to boiling on high power with adequate stirring.
   c. Weigh 10.00gm (dry weight basis) of J.T. Baker's Starch which replaced J. T. Baker's Lintner Starch (Special for Diastatic Powder Determination) in a 50 ml beaker.
   d. Add approximately 30 ml of distilled water and stir with a glass stir rod to produce a slurry.
   e. Slowly add the starch slurry to the boiling water from step b.
   f. Return the solution to a rolling boil with continuous stirring and hold the starch suspension at the boiling point for exactly two minutes.
   g. Quantitatively transfer the starch solution to a 500 ml volumetric flask and cool to room temperature. * Surface dehydration, which results in flakes of insoluble starch, can be eliminated by rapidly transferring the hot starch solution to the volumetric flask before cooling in a water bath. The solution should be stirred continuously during cooling.
   h. Add 10 ml of Phosphate Buffer Solution and dilute with water to volume
9. Color reference: Introduce approximately 60 ml of distilled water into a beaker. Add exactly 1 ml of hydrochloric acid 1.00N, 25.0 g of cobaltous chloride 6 aq, analytical reagent and 3.84 g of potassium bichromate, analytical reagent and dissolve the solids. Quantitatively transfer the solution to a 100 ml volumetric flask and dilute to volume with with distilled water. Store the solution in a brown glass bottle in a refrigerator. The solution will keep for a few months.
   Note: When in doubt, check the strength of the cobaltous chloride 6 aq according to Ph. Eur. Vol. I, page 60 (1969). Specification ≥ 99.0 %.

E. Procedure

1. Enzyme Preparation:
   a. Dissolve an appropriate amount of enzyme preparation in RAU Buffer. Use the same buffer if serial dilutions are required dilute to a concentration between 2 and 3 RAU per ml.
   b. Calculating Enzyme Preparation:

$$\text{Weight of sample} = \frac{400}{15.5 \text{ min.} \times \text{RAU (target)}} \div 10$$

c. Note: For evaluation of all blends made with starch and any sample containing predominantly starch at a concentration greater than 75%, the sample must be filtered to remove the starch from the injections solution.

i. Draw up at least 10 ml of the final dilution into a 20 ml syringe.

ii. Attach a 1.0µm pore size syringe filter to the tip of the syringe.

iii. Gently push the liquid through the filter until "any" resistance is felt. At this point change to a new filter, and continue until at least 10 ml of liquid has been filtered.

iv. Ensure that the filtrate is visually clear.

v. The filtrate is your enzyme solution for injection.

2. Enzyme Evaluation:
   a. For each sample solution to be measured, place in the water bath at 30°C one 50 ml conical flask containing 20.0 ml of Starch Substrate
   b. Close the flask and allow to equilibrate for 10-20 minutes.
   c. Pipette 5.0 ml of Working Iodide solution into approximately 15 disposable culture tubes.
   d. Starting at time zero inject exactly 10 ml of sample solution into the conical flask containing the starch substrate, close the flask, mix by swirling and immediately return it to the water bath.
   e. After exactly 11 minutes incubation, transfer exactly 1 ml of incubation solution to a tube containing 5 ml of iodine solution and mix. Immediately measure the absorbance of the color formed at 620 nm in a 1 cm cell, repeat this procedure at 1 minute intervals until a reading is found higher than and lower than the reading for the color reference of 0.5410.

F. Calculation:

1. Unit of Activity: One Reference Amylase Unit (RAU) is the quantity of enzyme converting 1.0 mg of starch (100 % of dry matter) per minute in standardized conditions into a product having a transmission at 620 nm after reaction with an iodine solution of known strength, equal to the one of a color reference.
2. Calculate the relation between the transmission (Y-axis) and the incubation time (X-axis) Determine the **(T)** time necessary for the sample solution to reach the same transmission as the one found for the color reference (0.5410) accurately to within 0.01 minute.
3. Calculate the alpha amylase activity in RAU in the flask as follows:

$$\text{RAU} = \frac{400}{\mathbf{T} \times \text{enzyme conc. (g/ml)}} \div 10$$

G. Testing Parameters:

1. The incubation time needed for the sample solution to reach a transmission equal to the one of the reference between 11 and 25 minutes may be used to find the approximate activity of unknown preparations. Final test, however, should be determined in the range of 11 and 20 minutes.
2. The method described here is applicable for enzymes produced by Bacillus subtilis variety, but cannot be used for samples containing beta amylase.
3. A Reference Material is assayed with each run to ensure method accuracy.