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Sandstedt, Kneen, and Blish (S.K.B) MODIFIED ANALYTICAL METHOD

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A. Principle: This procedure is used to accurately determine the α-amylase activity of enzyme preparations derived from *Aspergillus niger, Aspergillus oryzae, Rhizopus oryzae* and similar fungal derived enzymes. The assay is based on the time required to obtain a standard degree of hydrolysis. The degree of hydrolysis is measured by comparing the iodine color of the hydrolysate with the reference color standard.

B. Equipment:

1. Analytical Balance
2. Volumetric flask
3. Disposable test tubes
4. Volumetric pipettes
5. 50 ml Erlenmeyer flask or 50 ml screw cap tubes
6. Water bath set at 30° ±0.1°C
7. Automatic Pipetters
8. Digital timer or Stopwatch
9. Reference Color Standard: Use a special Alpha-Amylase Color Disk (Orbeco Analytical Systems, 185 Marine Street, Farmingdale, NY 11735, Catalog N. 620s-5). Alternatively, prepare a color standard by dissolving 25.0 g of cobaltous chloride ($CoCl_2 \cdot 6H_2O$) and 3.84 g of potassium dichromate in 100 ml of 0.01N hydrochloric acid. This standard is stable indefinitely when stored in a stoppered bottle or comparator tube.
10. Comparator: Use either the standard Hellige comparator (Orbeco, Catalog No. 607) or the pocket comparator with prism attachment (Orbeco, Catalog No 605 AHT). The comparator should be illuminated with a 100-W frosted lamp placed 6 inches from the rear opal glass of the comparator and mounted so that direct rays from the lamp do not shine into the operator's eyes.

C. Safety Precautions:

1. Utilize standard laboratory safety practices.
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 and Reagent Preparation: (Volumes prepared may be adjusted as needed for testing.)

1. Stock Iodine Solution: Dissolve 2.2 g of iodine and 4.4 g of potassium iodide in distilled water in a 50 ml beaker with stir bar. Quantitatively transfer the solution to a 100 ml volumetric flask and dilute to volume with distilled water. 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. A smaller total volume (50) may be prepared.
2. Working Iodine Solution: Dissolve 10 g of potassium iodide in approximately 150 ml of 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 to volume with distilled water. Cover the solution with tinfoil to protect from light. This is the working solution used in the test.

3. Starch Substrate Buffer Solution: Dissolve 9.3 g NaCl, 69 g KH_2PO_4, and 4.8 g Na_2HPO_4 in distilled water, transfer this solution to a one liter volumetric flask and dilute to volume with distilled water. The pH of the concentrate buffer solution should be 5.3. This solution may be used for 6 months.
4. Buffered Starch Substrate:
   a. Place a 600 ml beaker with a stir bar on a hot plate, and fill to approximately 250 ml with distilled water.
   b. Heat to boiling on high power with adequate stirring.
   c. Weigh 10.00 g (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.
   **The dry weight of J.T. Baker's Starch may be determined by following the general Procedure for Loss on Drying as described in the current revision of AM-001 or by determining the moisture as described in the current revision of AM-037. The dry weight necessary may be calculated by the equation below.

$$\text{Weight (equivalent to10.00 g dry weight)} = 10.00\text{ g} \times \frac{100}{\%\text{ dry matter of starch(g)}}$$

   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 50 ml of Starch Substrate Buffer Solution (3) and dilute with water to volume. (The pH of this solution should be 5.3.)
5. Enzyme Diluent (0.2% $CaCl_2$ dihydrate solution): Dissolve 4.0 g of $CaCl_2 \cdot 2H_2O$ in approximately 400 ml of distilled water. Transfer this solution to a two-liter volumetric flask and dilute to volume with distilled water.

E. Procedure :

1. Enzyme Standard Preparation:
   a. Due to a change in the starch production, a one-to-one correlation in activity is no longer valid for the SKB. Therefore, a factor must be utilized to obtain the correct result. A standard sample of specified activity according to the pre-starch production change must be run to determine the factor. Enzyme Development Corporation's current standard's specified activity is listed on the container. (example below: 10,978 SKB/g) When the standard is run, the specified activity is compared to the assay-determined activity and the ratio yields the factor for the substrate preparation:

$$\text{i.e. } \frac{\text{(Specified Initial Act.) } 10{,}978}{\text{(Experimental Act.) } 7{,}800} = 1.407\text{ (Factor)}^*$$

   *This factor must be determined on the day of analysis, and can be used for all subsequent testing that day.

   b. Calculating Enzyme preparation:
   To determine the necessary dilution for the standard,

$$\text{g/ml standard} = \frac{240\text{ mg starch digested}}{\text{(20.0 min) (theoretical activity of standard)}} \times \text{SKB Factor} \div 10\text{ ml aliquot}$$

   (Use the 10,978 for the theoretical activity)
   This gives you the concentration necessary for the final dilution.

Therefore,

$$\frac{240 \text{ mg starch digested}}{(20.0 \text{ min.})\ (10{,}978)} \times 1.40 = 0.000153 \text{ g/ml in the final dilution} \div 10 \text{ ml aliquot}$$

The respective serial dilutions would be 0.153g/100ml X 10ml/100ml=0.00015g/ml

c. Weigh a quantity of the SKB standard enzyme preparation and dilute appropriately with the Enzyme Diluent, as described on the bottle, or by calculation using a previous factor. Use the final dilution for the 10 ml aliquot in the Enzyme evaluation.
d. The activity of the standard and the corresponding SKB Factor must be determined prior to preparation of all other enzyme samples, and should be used in determining the dilutions for all other samples.

2. Enzyme Sample Preparation:
   a. Calculating enzyme preparation:

$$\text{g/ml sample} = \frac{240 \text{ mg starch digested}}{20.0 \text{ (min)} \times \text{SKB(Target)}} \times \text{SKB Factor *} \div 10 \text{ ml aliquot}$$

*based on the determination of the actual experimental activity of the standard

   b. Dissolve an appropriate amount of enzyme in Enzyme Diluent buffer. Use the same buffer if serial dilutions are necessary.

3. Enzyme Evaluation:
   a. Pipet 20 ml of the Buffered Starch Substrate in a 50 ml Erlenmeyer flask or screw cap tube. Place the flask or tube in a water bath at 30°C and allow it to equilibrate for at least 15 minutes.
   b. Prepare a series of 15 x 100 mm test tubes with 5 ml of the Working Iodine Solution. Cover the tubes with tin foil or place in a dark cabinet to protect from light.
   c. Pipet a 10 ml aliquot of the enzyme preparation from above into a 50 ml tube containing the Buffered Substrate Solution, while starting a stopwatch or digital timer. Mix the tube or flask gently by inverting or swirling.
   d. After 10 minutes of elapsed time, begin to withdraw a 1 ml aliquot of the enzyme substrate mixture from the tube, and add it to one of the tubes of Working Iodine Solution at a timed intervals (on the minute preferably). The reaction mixture and iodine should be mixed immediately and transferred to a square Hellige Comparator tube for comparison with the Reference Color Standard.
   e. If the solution is yellow or lighter than the Reference Color Standard on the initial reading, the enzyme is more active than thought, and needs to be diluted further. The dilutions previously prepared may be used and diluted further with Enzyme Diluent and pipetted to a new substrate tube.
   f. Additional one ml aliquots of the reaction mixture are removed and mixed with the Working Iodine Solution and compared with the Reference Color Standard disk at approximately one-minute intervals. The square Comparator tube used for this color comparison work is merely emptied between successive readings.
   g. When nearing the end point, a 1 ml aliquot should be withdrawn at a shorter time interval (preferably a quarter or half minute). The color of the solution should change from a dark purple or brown to a medium red- orange, as it becomes close to the endpoint.
   h. As the solution gets closer in color to the endpoint, one tube may be darker than the Comparator disk, and a second one will be lighter than the Reference Color disk. Under these conditions, record the end point at the nearest quarter minute. This corresponds to the dextrinizing time for the sample.
   i. If the solution is still purple or brown after 30 minutes, a dilution prior to the final dilution should be tested or a new enzyme preparation should be made targeting a lower activity.

F. Calculations:

1. Unit of Activity: One SKB unit is the amount of enzyme that will dextrinize starch under assay conditions.
2. SKB equals the milligrams of starch digested multiplied by the SKB Factor and divided by the weight of the sample added and time required for dextrinization.

$$\text{SKB/g} = \frac{\text{mg. of starch digested}}{\text{(g enzyme)(dextrinizing time)}} \times \text{SKB Factor} = \frac{240}{(W)(T)} \times \text{SKB Factor*}$$

W = Weight in grams of enzyme in 10 ml aliquot
(g/ml enzyme from Enzyme Preparation from above X 10)
T = Dextrinizing time (in quarter minutes)

*All experimental results must be multiplied by the factor to obtain the correct result. Since this factor is used in the overall SKB equation, and is used in determining the weight of enzyme used, the factor eventually cancels out of the equation.

3. Example: If the dextrinizing time is19.25 minutes, the g/ml enzyme is 0.0024, and the SKB Factor is 1.387 the calculation is:

$$\frac{240}{19.25 \text{ min.} \times .0024} \times 1.387 = 7{,}205 \text{ SKB/g}$$

G. Testing Accuracy Parameters:

1. If recommendations concerning enzyme strength are followed and if there has been no apparent inactivation of the enzyme, the end point for the reaction will fall within a 10 to 30 minute time interval.

H. References:

1. Sandstedt, Kneen, and Blish, Cereal Chemistry 16,172 (1939)

Attachments: Not Applicable

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