

**Enzymatic Assay of PULLULANASE
(EC 3.2.1.41)**

PRINCIPLE:

Pullulan + H₂O $\xrightarrow{\text{Pullulanase}}$ Maltotriose

CONDITIONS: T = 25°C, pH 5.0, A_{546nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

- A. 20 mM Sodium Acetate Buffer, pH 5.0 at 25°C
(Prepare 100 ml in deionized water using Sodium Acetate, Trihydrate, Sigma Prod. No. S-8625. Adjust to pH 5.0 at 25°C with 1 M HCl.)
- B. 2.0% (w/v) Pullulan Solution
(Prepare 10 ml in Reagent A using Pullulan, Sigma Prod. No. P-4516.)
- C. 2.0 mM Glucose Standard Solution
(Prepare 10 ml in deionized water using D-(+)Glucose, Anhydrous, Sigma Prod. No. G-8270.)
- D. 16 mM Copper Sulfate, 1.3 mM Sodium Sulfate, 226 mM Sodium Carbonate, 190 mM Sodium Bicarbonate and 43 mM Sodium Potassium Tartrate (Copper Soln)
(Prepare 1 liter in deionized water using Cupric Sulfate Pentahydrate, Sigma Prod. No. C-7631; Sodium Bicarbonate, Sigma Prod. No. S-8875; Sodium Sulfate, Anhydrous, Sigma Prod. No. S-9627; Sodium Carbonate, Anhydrous, Sigma Prod. No. S-2127; and Sodium Potassium Tartrate Tetrahydrate, Sigma Prod. No. S-2377.)¹
- E. 40 mM Molybdic Acid, 19 mM Arsenic Acid and 756 mM Sulfuric Acid (Ars-Mol)
(Prepare 1 liter in deionized water using Molybdic Acid, Ammonium Salt Tetrahydrate, Sigma Prod. No. M-0878; Arsenic Acid, Sodium Salt, Sigma Prod. No. A-6756; and Sulfuric Acid, Sigma Prod. No. S-1526.)²

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REAGENTS: (continued)

F. Pullulanase Enzyme Solution
(Prepare a solution containing 0.40 - 0.80 unit/ml
Pullulanase in Reagent A.)

PROCEDURE:

Pipette (in milliliters) the following reagents into 4-dram vials:

	Test	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std Blank
Reagent A (Buffer)	0.35	0.35	0.40	0.30	0.20	0.10	----	0.50
Reagent B (Pullulan Soln)	0.05	0.05	----	----	----	----	----	----

Mix by swirling and equilibrate to 25°C. Then add:

Reagent F (Enzyme Soln)	0.10	----	----	----	----	----	----	----
Reagent C (Glucose Std)	----	----	0.10	0.20	0.30	0.40	0.50	----

Mix by swirling and incubate for exactly 10 minutes. Then add:

Reagent D (Copper Soln)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Reagent F (Enzyme Soln)	----	0.10	----	----	----	----	----	----

Mix by swirling and incubate in a boiling water bath for 60 minutes. Remove the vials and let cool to 20°C. Then add:

Reagent E (Arsenomolybdate soln)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
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Shake and mix the vials until the foaming stops and any precipitate present is dissolved. Then add:

Deionized Water	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00
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Mix by inversion and record the $A_{546\text{ nm}}$ for the Test, Blank, Standards and Standard Blank using a suitable spectrophotometer.

CALCULATIONS:

$$\Delta A_{546\text{ nm}} \text{ Std} = A_{546\text{ nm}} \text{ Std} - A_{546\text{ nm}} \text{ Std Blank}$$

Prepare a standard curve by plotting the $\Delta A_{546\text{ nm}}$ of the Glucose Standards versus micromoles of glucose.

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CALCULATION: (continued)

Sample Determination:

$$\Delta A_{546\text{nm}} \text{ Test} = A_{546\text{nm}} \text{ Test} - A_{546\text{nm}} \text{ Blank}$$

Determine the μ moles of maltotriose (measured as glucose reducing equivalent) using the Standard curve.

$$(\mu\text{moles of maltotriose released})(\text{df})$$

$$\text{Units/mg protein} = \frac{\hspace{15em}}{(10)(0.1)}$$

df = Dilution factor

10 = Time (in minutes) of assay as per the Unit Definition

0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will liberate 1.0 μ mole of maltotriose (measured as glucose) from pullulan per minute at pH 5.0 at 25°C.

FINAL ASSAY CONCENTRATION:

In a 0.50 ml reaction mix, the final concentrations are 20 mM sodium acetate, 0.2% (w/v) pullulan and 0.04 - 0.08 unit pullulanase.

REFERENCES:

- Somogyi M., (1952) *J. Biol. Chem.* **195**, 19-23
Somogyi M., (1945) *J. Biol. Chem.* **160**, 61-68
Nelson N., (1944) *J. Biol. Chem.* **153**, 375-380

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NOTES:

1. Sodium Sulfate, Sodium Carbonate, and Sodium Potassium Tartrate are dissolved in approximately 500 ml of deionized water. Cupric Sulfate is dissolved in approximately 100 ml of deionized water and slowly added to the above solution to avoid precipitation. Sodium Bicarbonate is dissolved first in deionized water and then added to the above solution. Dilute the solution to 1 liter. If a precipitate forms, it should be removed by filtration prior to use. Store in an amber bottle and avoid exposure to direct sunlight. Store at room temperature.
2. Molybdcic Acid is dissolved in approximately 300 ml of deionized water. Add Sulfuric Acid slowly. Caution, this is an exothermic reaction! Arsenic Acid is dissolved in approximately 300 ml of deionized water and is added to the above solution. The solution is diluted to a total volume of 1 liter and incubated at 37°C for 48 - 72 hours. If a precipitate forms, it should be removed by filtration prior to use. Store in an amber bottle and avoid exposure to direct sunlight. The solution expires six months after preparation. Store at room temperature in an exhaust hood.
3. The method of assaying for the presence of reducing sugars, described here, is that of Somogyi/Nelson.
4. This assay is based on the cited references.
5. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.