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ASSAY OF PULLULANASE using RED-PULLULAN

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PRINCIPLE:

This assay procedure is specific for *endo*-acting pullulanase and limit-dextrinase (from grain) activity. On incubation of Red-Pullulan with pullulanase or limit-dextrinase the substrate is depolymerised by an *endo*-mechanism to produce low-molecular-weight dyed fragments which remain in solution on addition of ethanol to the reaction mixture. High-molecular weight material is removed by centrifugation, and the colour of the supernatant is measured at 510 nm. Pullulanase in the assay solution is determined by reference to a standard curve. Although this substrate can be used to measure limit-dextrinase, we recommend the use of Limit-Dextrizyme tablets (cat. no. T-LDZ) which form the basis of an assay with a greater sensitivity.

SUBSTRATE:

The substrate is partially depolymerised pullulan which is dyed with Procion Red MX-5B to an extent of approx. one dye molecule per 30 sugar residues.

Add 0.5 g of powdered substrate to 25 mL of 0.5 M potassium chloride solution and stir vigorously at room temperature until it completely dissolves.

This substrate is not stable at room temperature for extended periods, and thus should be stored at 4°C between use. For long term storage, it is recommended that the substrate be stored in a well sealed glass bottle and be overlain with a few drops of toluene to prevent microbial growth.

BUFFER SOLUTION:
(Sodium Acetate buffer, 200 mM, pH 5.0)

Add 12.0 g of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water. Adjust the pH to 5.0 by the addition of 5 M (20 g/100 mL) sodium hydroxide solution. Approximately 100 mL is required. Adjust the volume to 1 litre.

ENZYME PREPARATION:

Add 1.0 g of powdered enzyme preparation or 1.0 mL of liquid enzyme preparation to 90 mL of 200 mM sodium acetate buffer (pH 5.0) in a 100 mL volumetric flask and adjust the volume to 100 mL. Add a magnetic stirrer bar and gently stir the solution/slurry at room temperature over 15 min. Filter an aliquot of the solution, if necessary, and dilute further in 200 mM sodium acetate buffer (pH 5.0), as required to obtain the correct concentration for assay.

ASSAY PROCEDURE:

Pre-equilibrate enzyme solution at 40°C for 5 min.
Add 1.0 mL of pre-equilibrated enzyme solution to 0.5 mL of pre-equilibrated Red Pullulan solution. Stir the mixture and incubate at 40°C for exactly 10 min.

Terminate the reaction and precipitate high-molecular weight substrate by the addition of 2.5 mL of ethanol (95 % v/v) [or industrial methylated spirits (IMS, 95 % v/v)] with vigorous stirring for 10 sec on a vortex mixer. Allow the reaction tubes to equilibrate at room temperature for 10 min. Stir the tubes again and then centrifuge at 1,000g for 10 min.

Pour the supernatant solution directly from the centrifuge tube into a spectrophotometer cuvette and measure the absorbance of blank and reaction solutions at 510 nm against distilled water. Activity is determined by reference to a standard curve.
The blank is prepared by adding ethanol to the Red-Pullulan substrate before addition of the enzyme. Usually, a single blank is required with each set of determinations.

STANDARD CURVE:

Standard curves for pure *Bacillus acidopullulyticus* pullulanase, *Bacillus licheniformis* pullulanase and *Klebsiella planticola* pullulanases on Red Pullulan (Lot 61201) are shown in Figs. 1-3, respectively. Enzyme activity is standardised using borohydride reduced pullulan (1.0 % w/v) as substrate in 100 mM sodium acetate buffer (pH 5.0) at 40°C using the Nelson/Somogyi reducing sugar method.

One Unit of activity is defined as the amount of enzyme required to release one μmole of D-glucose reducing-sugar equivalents per minute from borohydride reduced pullulan, under the defined assay conditions (pH 5.0, 40°C).

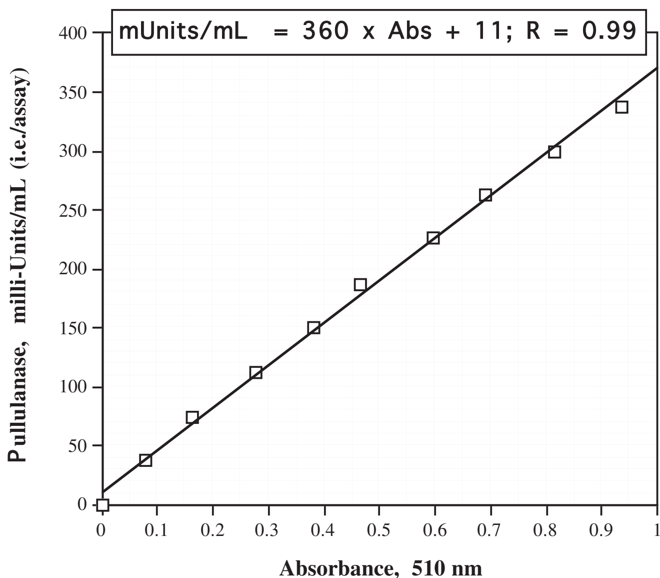

Figure. 1. *Bacillus acidiopullyticus* pullulanase on Red-Pullulan (Lot 61201).

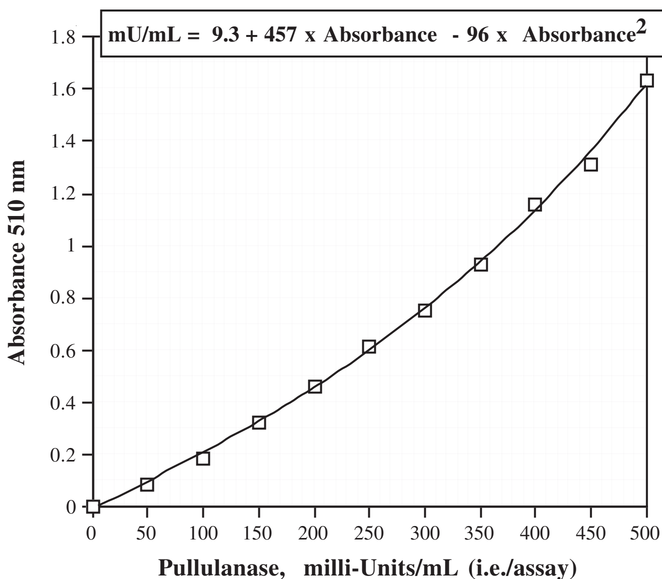

Figure. 2. *Bacillus licheniformis* pullulanase on Red-Pullulan (Lot 61201).

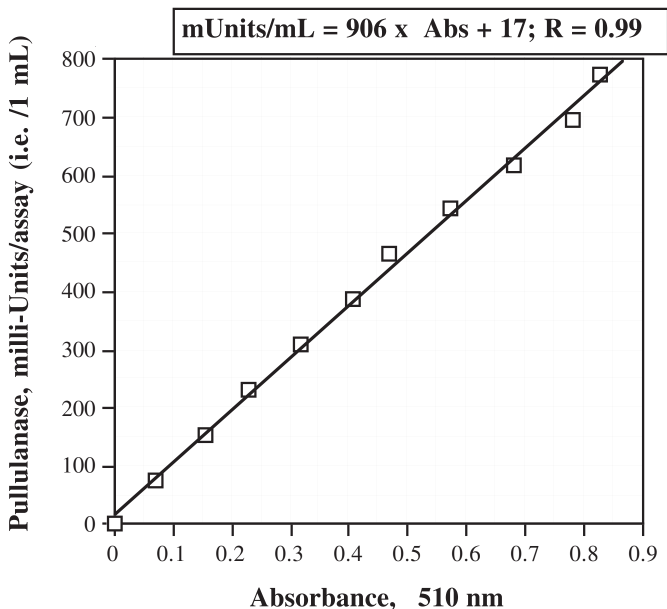

Figure. 3. *Klebsiella planticola* pullanase on Red-Pullulan (Lot 61201).

CALCULATIONS:

Units/mL of original solution

$$= \text{milli-Units per assay (ie. per 1.0 mL)} \times 100 \times \frac{1}{1000} \times \text{Dilution.}$$

where:

milli-Units per assay is determined by reference to the Standard Curve.

100 = initial extraction or dilution volume (e.g. 1 mL per 100 mL).

$\frac{1}{1000}$ = conversion from milli-Units to Units.

Dilution = further dilution of the original enzyme extract.

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