4. PROCEDURES

4.1 MATERIALS REQUIRED

4.1.1 Buffer: - 0.1 M sodium acetate buffer (pH 4.0).

4.1.2 Substrate Solution: - *p*-Nitrophenyl-β-Glucopyranoside (10 mM).
Weigh 301 mg of p-Nitrophenyl-β-Glucopyranoside into a 150 ml pyrex beaker and add 95 ml of 0.1 M sodium acetate buffer (pH 4.0). Stir the solution until the substrate dissolves (about 10 min). Adjust the volume to 100 ml with 0.1 M sodium acetate buffer (pH 4.0). Add 0.02 g of sodium azide to prevent microbial contamination. Store the solution in a sealed Duran bottle. Take about 10 ml into a polypropylene tube for day-to-day use. Do not routinely use from the substrate stock. Store both containers at 4°C.

4.1.3 Enzyme Preparation:
With a Gilson micropipette accurately dispense 1.0 ml of well suspended enzyme solution into a 100 mL volumetric flask and adjust to volume with 0.1 M sodium acetate buffer (pH 4.0). Sequentially dilute the preparation by adding 0.5 mL of enzyme solution to 4.5 mL of buffer (i.e. 10-fold), mix the solution well, and repeat this step to get a dilution suitable for assay.
Perform these dilutions with a Gilson pipette set to 0.5 mL (for the enzyme preparation) and an Eppendorf Multipette with the 12.5 mL tip (setting 5 for 2.5 mL plus setting 4 for 2 mL) to deliver the 4.5 mL of buffer.

4.2 ASSAY PROCEDURE:

4.2.1 Pre-incubate substrate solution (0.2 mL) in glass test-tubes (16 x 100 mm) at 40°C for 5 min.

4.2.2 Pre-incubate suitably diluted enzyme solution (about 5 mL) at 40°C for 5 min.

4.2.3 Add 0.2 ml of enzyme solution to the bottom of tubes containing substrate solution (0.2 mL) on carefully timed intervals (about 15 sec), mix the tube contents vigorously for 5 sec., and incubate the tubes (in quadruplicate) for exactly 10 min.

4.2.4 At the end of the incubation time, terminate the reaction by adding 3.0 ml of 2% tri-sodium phosphate solution (pH 12.0) with vigorous stirring.

4.2.5 Prepare a Reaction Blank solution (in duplicate) by adding 3.0 mL of 2% tri-sodium phosphate solution (pH 12.0) to 0.2 mL of substrate solution, mix vigorously, and then add 0.2 mL of enzyme solution with vigorous stirring.

4.2.6 Measure the absorbance values (400 nm) as follows:

- zero the spectrophotometer with distilled water.
- Measure the absorbance of the Reaction Blank against the water and record this value (for future reference only).
- Then zero the spectrophotometer with the Reaction Blank.
- Measure the absorbance of all other solutions (reaction and standards) against the Reaction Blank.

4.2.7 Calculate activity as follows:

$$\text{Units / mL} = \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{100}{1.0} \times \text{Dilution}$$

$$= \Delta E_{400} \times 9.39 \times \text{Dilution}$$

where:

ΔE_{400} = Absorbance (reaction) – Absorbance (blank).
Incubation Time = 10 min.
Total volume in cell = 3.4 ml
Aliquot assayed = 0.2 ml
E_{mM} of *p*-nitrophenol in 2% tri-sodium phosphate = 18.1
Extraction volume = 1.0 ml of enzyme in total volume of 100 ml (Original Extract).
Dilution = further dilution of the Original Extract.

NOTE:- For a β-glucosidase solution of 40 Units/ml, the further dilution of the "Original Enzyme Extract" is 10-fold.