3. Determination of activity of Hydrolytic enzyme

Aim

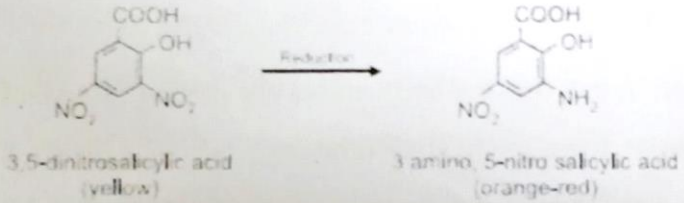
To determine the activity of hydrolytic enzyme cellulase by kinetic characterization (K_m and V_{max}).

Introduction

Sugars with reducing property (arising out of the presence of a potential aldehyde or keto groups) are called reducing sugars. Some of the reducing sugars are glucose, galactose, lactose and maltose. The DNS method is one of the classical and widely used methods for the quantitative determination of reducing sugars.

Principle

Reducing sugars have the property to reduce many of the reagents. One such reagent is 3,5-dinitrosalicylic acid (DNS). 3,5-DNS in alkaline solution is reduced to 3 amino 5 nitro salicylic acid.



Reagents and instruments required

Equipment: Flasks, Spectrophotometer, Sample tubes, Micropipette

Chemicals:

1. Tri sodium citrate
2. Citric acid
3. Carboxymethyl cellulose
4. D-Glucose
5. 3,5-Dinitrosalicylic acid (DNS)
6. Sodium potassium tartrate tetrahydrate
7. Sodium hydroxide
8. Hydrochloric acid

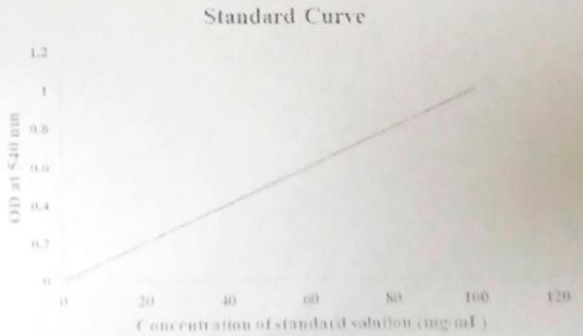
Reagents:

1. **Preparation of DNS reagent:** 1g of DNS is dissolved in 50ml of distilled water. Add 30g of sodium potassium tartrate tetrahydrate. Then add 20ml of 2N NaOH, which turns the solution to transparent orange yellow colour. The final volume is made to 100 ml with the distilled water.
2. **0.1M Citrate buffer (pH 4.8):**
    1. Prepare 80 mL of distilled water in a suitable container.
    2. Add 1.554 g of Tri sodium citrate to the solution.
    3. Add 0.906 g of Citric Acid to the solution.
    4. Adjust solution to final desired pH using HCl or NaOH.
    5. Add distilled water until volume is 100 mL.
3. **Standard Glucose Solution:** Stock: 100 mg in 100 mL distilled water.
4. **Substrate solution (CMC):**100 mg in 100 mL of 0.1 M citrate buffer.
5. **Enzyme solution (Cellulase):** 1 mg in 10 mL of 0.1 M citrate buffer.

Procedure

1. Standard curve plotting

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1mL of the working standard solution into a series of test tubes.
2. Make up the volume in standard tubes to 1 mL with distilled water.
3. Pipette out 1 mL distilled water in a separate tube to set a blank.
4. Add 1mL of 3,5-dinitrosalicylic acid (DNS) reagent to each tube.
5. Then place the tubes in a boiling water bath for 5 minutes.
6. Take the tubes out and cool the tubes to room temperature.
7. Then the absorbance was measured at 540 nm using UV-Visible spectrophotometer.
8. Plot the standard curve between concentration of the standard glucose solution (mg/mL) and optical density (OD) at 540 nm.



2. Activity of hydrolytic enzyme on different substrates

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1mL of the substrate solution (CMC) into a series of test tubes.
2. Make up the volume to 1 mL with distilled water in each test tube.
3. Add 1 mL enzyme (cellulase) to all the test tubes (excluding Control) and again the tubes were incubated at 50^0 C for 30 minutes in the water bath.
4. A control is also prepared in the same manner except the addition of enzyme (add 1 mL of distilled water instead of enzyme).
5. After the incubation the tubes were removed from the water bath and the reaction was stopped by adding 4 mL of DNS reagent to each tube.
6. Then place the tubes in a boiling water bath for 5 minutes.
7. Take the tubes out and cool the tubes to room temperature.
8. Then the absorbance was measured at 540 nm using UV-Visible spectrophotometer.
9. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose.
10. Enzyme activity (IU/ml of enzyme) is defined as the amount of enzyme required for the formation of 1 µmol of sugar from CMC per minute.
11. A graph was drawn by plotting the substrate concentration on X-axis and optical density on Y-axis.
12. Draw Lineweaver Burk plot. From the graph calculate the values of K_m and V_{max}.

S.No.	Substrate Concentration (mg/mL)	Enzyme Concentration (mg/mL)	OD at 540 nm (DNS method)	Sugar (glucose) released (mg/mL)	Sugar (glucose) released (µmol)	Enzyme activity (IU/mL of enzyme)

References:

1. Iqbal, H., Ahmed, I., Zia, M. and Irfan, M. (2011) Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by *Trichoderma viride* under SSF and its