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Lab. 4 Quantitative Screening of Spore-Forming Bacteria Producing Protease

Introduction

The Folin-Ciocalteu assay for proteolytic enzyme activity measures the release of tyrosine and tryptophan residues from protein substrates during proteolysis. These aromatic amino acids react with the Folin-Ciocalteu reagent, producing a blue-colored complex whose intensity is proportional to the concentration of released residues. The absorbance is typically measured at 660–750 nm using a spectrophotometer. This assay is commonly used for general protease activity determination due to its sensitivity and ability to quantify proteolysis by monitoring amino acid release.

Objective

The objective of the practical session is to select, from the previously isolated bacteria, those with significant proteolytic activity through colorimetric measurement of enzymatic activity.

Required material and reagents

- Freshly prepared bacterial cultures previously isolated on NB
- Nutrient broth (NB)
- 250 ml Erlenmeyer flasks
- Shaker-incubator (37°C)

Standard Curve

- 0.01% tyrosine solution
- 4% TCA (Trichloroacetic Acid) solution
- 50% diluted Folin-Ciocalteu reagent
- Distilled water
- 2% Na_2CO_3 solution in 0.1N NaOH
- Graduated pipettes (1, 2, and 5 ml)

Enzymatic Activity Assay

- Beakers
- Automatic micropipettes
- 0.2M phosphate/citrate buffer, pH 5
- Substrate: 2.5% casein solution in 0.02M sodium citrate
- 4% TCA solution
- 2% Na_2CO_3 solution in 0.1N NaOH
- 50% diluted Folin-Ciocalteu reagent
- UV-Visible spectrophotometer
- Vortex mixer
- Test tubes
- Centrifuge
- Water bath
- Funnel
- Filter paper
- pH meter

I. Production fermentation

Inoculate the nutrient broth (10%) with the bacterial isolates previously selected for secondary screening. Incubate at 37°C with moderate agitation for 24 hours.

II. Standard Curve

The standard range is prepared from a stock solution of tyrosine, diluted to achieve concentrations expressed in mg/ml.

Tubes	1	2	3	4	5	6
Stock Solution of Tyr (ml)	0	0.1	0.2	0.3	0.4	0.5
TCA (ml)	0.5	0.4	0.3	0.2	0.1	0
Na_2CO_3 (ml)	2.5	2.5	2.5	2.5	2.5	2.5
Agitation and incubation for 10 min at room T°						
Folin diluted to 50%	0.5	0.5	0.5	0.5	0.5	0.5

- After vigorous shaking, let the mixture stand for 30 minutes at room temperature.
- The absorbance measured at 750 nm is used to plot the standard curve.

III. Enzymatic Activity Assay of Bacterial Cultures

- Take 2 ml from each culture, centrifuge, and use the supernatant as the enzyme extract.

Step 1: Preparation of the Reaction Mixture

Reagent	Assay (ml)
Enzyme extract	1
Phosphate buffer	1,5
Substrate (caseine)	2,5
Shake and incubate in a water bath at 37ºC for 30min	
TCA	2,5
Let the mixture stand for 10min at room temperature	
Filtrate	

Note: The reaction is stopped by adding TCA.

Step 2: Measurement of Enzymatic Activity

Reagent	Tube 1 Blank (ml)	Tube 2 assay (ml)
Filtrate	-	0,5
TCA	0,5	-
Na_2CO_3 solution	2,5	2,5
Folin reagent	0,5	0,5
Shake and let the mixture stand for 30min, measure the absorbance at 750nm		

- Plot the standard curve: OD = f([Tyr]).
- Establish a numerical relationship between optical density and tyrosine concentration.
- Deduce the corresponding concentration by referencing the tyrosine standard curve.
- Calculate the protease activity, knowing that proteolytic activity is expressed in IU, where 1 IU = µg of tyrosine released per 1 ml of enzyme extract per hour.