Contains:

- protease
- L-glutaminase
- Leucine aminopeptidase
- sucrase
- amylase
- cellulase
- lipase
- catalase
- carboxypeptidase

Protease Assay


Original method: pH 7.0, temp. 37°C.

0.10 to 0.25 ml culture supernatant is diluted with resp. 0.90 to 0.75 ml Tris-buffer (100 mM, pH 7.2) and equilibrated at 30°C in a tube. 0.5ml of a pre-warmed 1.25% (w/v) azoalbumine (Sigma) solution is added and the contents are mixed. After 30-60 minutes incubation at 30°C the reaction was stopped by the addition of 1.0 ml 10% (w/v) trichloroacetic acid. The precipitate is removed by centrifugation. To 1.0 ml of the supernatant is added 1.0 ml 0.5 M NaOH immediately before reading the absorbance at 440 nm. The protease activity in the supernatant is considered to be due to alkaline serine protease and expressed in absorbance units per ml kecap x hour. Blanks are prepared for every sample, with the same amounts of sample with buffer, after which is added the TCA and only then followed by the azoalbumine.

Cal. : (OD440/ml.hr) = 5 x OD440 / (time x sample volume)
Glutaminase/ Leucine amino peptidase assay


buffer: 2.5 mM of L-y-glutamyl-p-nitroanilide (Sigma), 100 mM Tris-HCl buffer (pH 7.2)  
(or L-y-leucine-p-nitroanilide for LAP)

The assay mixture contained the buffer and enzyme preparation (50-100µl) in a final volume of 1 ml. Dilutions are made in 100 mM Tris-HCl, with the substrate (pH 7.2). After incubation at 30°C for 30 min. or more, the enzyme reaction is stopped by the addition of 2 ml of 1.5 N acetic acid to the mixture, and then the absorbance at 410 nm is measured (a yellow colour will develop). The molar extinction coefficient of p-nitroaniline is 8.3 x 10³ M. cm⁻¹.

For every sample a blanc has to be prepared in the same way as the measurement sample but in this case the reaction is directly stopped at t=0 by the addition of the acetic acid. Activity is expressed as umol / ml.hr:

\[ \text{Activity} = \frac{3 \times E_{410}}{(8.3 \times \text{time} \times \text{sample size (in ml))}} \]
Determination of sucrase and amylase

Buffer: 100mM NaAc (pH 5.2)
Substrate: 0.5% starch (amylase) or 0.5% sucrose (sucrase)

Assay:
- 50 - 200 ul sample
- 750 - 600 ul buffer
- 200 ul substrate
After the addition of the substrate and mixing, the mixture is incubated at 30 C for 0.5 - 2 hrs. The reaction is stopped by heating at 100 C for 5 minutes. Released glucose is measured by the GOD-PAP test.
For every sample a blank is prepared in the same way as for the activity measurement but with 200 ul water replacing the substrate.

enzyme activity is expressed as umol/ml.hr:
= mM glucose / (time x sample size (in ml))

Determination of glucose

Glucose Oxidase catalyzes the reaction:

\[
glucose + O2 \rightarrow gluconic acid + H2O2
\]

H2O2 reacts by the action of peroxidase with 4-aminophenazon and phenol to a coloured product of which the extinction can be measured at 510 nm.

Procedure

The determination is done by the Boehringer testkit.

Dissolve the contents of bottle 1 in 200 ml demi, add the contents of bottle 2. Keep the solution in the dark at 4oC for max. 4 weeks. Make a standard solution of 2 mM glucose.
Add to a cuvette
- 0.2 ml blank, standard or sample
- 2.0 ml reaction mixture
Incubate 45-60 minutes at room temperature in the dark.
Lipase assay

Original method: no salt, temp 35oC.

Buffer: 0.09 M NaAc, 0.01M acetic acid pH 5.5
Test solution: 20 ml glyceroltrioleate, emulsified in a solution of 8.0 gr sodium taurocholate, 120 ml acetate buffer and 20 ml 2.2% CaCl, 20 g NaCl. The volume is made up to 200 ml.

10 ml test solution is mixed with 5 ml extract and incubated at 30oC for 60 min, with gentle shaking. The reaction is terminated by adding ethanol (40 ml). The mixture is then titrated with alcoholic 0.02 N KOH against phenolphthalein. Conditions are arranged so that the titre used in the calculation lay between 7 and 20 ml. The unit of enzyme activity is the amount of enzyme which liberates 1.0 mg of oleic acid per minute.
Cellulase Assay


Orginal method: temp. 50°C, no salt, pH 5.0.

Required: 1% Sodium carboxymethylcellulose, 10% NaCl in 0.01 N acetate buffer pH 5.5 (HAc and NaAc) and 1% 3,5 dinitrosalicylic acid

CM-cellulose solution and sample are preheated at 30°C and mixed by putting together 0.5 ml of each solution. They were incubated for 10’ at 30°C. 1% 3,5 dinitrosalicyclid acid reagent (1.0 ml) is then added, mixed and the tube immersed in boiling water for 5 min. The tube is cooled with running tap water and 10 ml water is added, the whole mixed and the absorbance is measured at 540 nm. The control is a sample which contained in stead of enzyme, 0.5 ml water. Reducing sugars in the extract were determined separately and subtracted from the results obtained in the assay.

1 unit is that amount of enzyme that produces 1.0 mg of reducing sugar calculated as glucose under the above conditions.
Determination of catalase activity

Principle

Catalase catalyses the reaction:

\[ 2 \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2 \]

Procedure

Flood a plate culture of the organism in question with the \( \text{H}_2\text{O}_2 \) solution. Development of gas bubbles is watched with a magnifying glass.
Or: Mix 1 ml of \( \text{H}_2\text{O}_2 \) solution with 1 ml of culture withdrawn from a broth culture. The evolution of gas bubbles, caused by the liberation of free oxygen, indicates the presence of catalase in the culture.
Determination of Carboxypeptidase activity

Based on: Bergmeister
Orginal method: pH 7.6, room temperature, no salt

Principle

N-Cbz-aa-aa + water  N-Cbz-aa  + aminoacid
The disappearence of the second aminoacid binding can be measured at 232 nm, the separate aminoacid can be measured with ninhydrine.

Solutions

- 0.15 M Tris-HCl, pH5.5
- 40 mM CoCl₂, 600 mM NaCl
- 30 mM Cbz-aa-aa (pH 7 - 8 with NaOH)
- Acetate-cyanide reagens:
  0.360 g NaAc, 0.01 g NaCN and 67 ml HAc with water to 1000 ml. This most be older than a week, store at 4°C.
- ninhydrine reagens: 0.750 g in 25 ml ethyleenglycolether
- 50% ethanol

Procedure

Put in a quarz-glass-cuvette:
- 0.50 ml Tris-buffer
- 0.50 ml CoCl, NaCl solution
- 1.00 ml sample
mix and incubate 15 minutes, then add 1.00 ml Cbz-aa-aa, mix and incubate again 30 min exactly. Measure extinction at 232 nm with time intervals of 1 -2 minutes.

Colour methode (extended method of the previous one)
After 30 minutes take 0.10 ml incubation mix and add:
- 0.90 ml water\n- 0.50 ml acetate-cyanide solution
put for 2 minutes at 100°C
Add 0.50 ml ninjhydride reagens, put 15 minutes at 100°C, cool down and add 3.00 ml ethanol. Measure at 570 nm.
Calculations

UV-methode: volume activity = 35200 * F * ΔE/min
a 100% hydrolysis gives a decrease of 0.85 in extinction.

Farb methode: use a phenylanaline standard (10.33 mg in 50 ml buffer)