α-AMYLASE (thermostable) from *BACILLUS LICHENIFORMIS* containing a MODIFIED α-AMYLASE GENE from *B. LICHENIFORMIS*

New specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add. 11 (2003). An ADI "not specified" was established.

SOURCES

Produced by submerged fed-batch pure culture fermentation of a genetically modified nonpathogenic and nontoxigenic strain of *Bacillus licheniformis*. The sequence of the alpha-amylase gene, originally derived from *B. licheniformis,* has been modified to confer thermostability on the enzyme. The *B. licheniformis* production strain was developed using a host strain derived from a nonpathogenic and nontoxigenic *B. licheniformis* ancestral strain.

Active principles

Alpha-amylase (synonym: glycogenase)

Systematic names and numbers

1,4-alpha-D-glucan glucanohydrolase; E.C. 3.2.1.1; CAS No. 9000-90-2

Reactions catalysed

Endohydrolysis of 1,4-alpha-glucosidic linkages in amylose and amylopectin forming dextrins and oligosaccharides

DESCRIPTION

Brown liquid. Contains stabilizing and antimicrobial compounds.

FUNCTIONAL USES

Enzyme preparation.
Starch hydrolysis in the production of, e.g., sweeteners, ethanol, and beer.

GENERAL SPECIFICATIONS

Must conform to the General Specifications for Enzyme Preparations used in Food Processing (see Volume Introduction)

CHARACTERISTICS

IDENTIFICATION

Alpha-amylase

The sample shows alpha-amylase activity
See description under TESTS

TESTS

Alpha-amylase activity

Principle

Alpha-amylase catalyzes the hydrolysis of ethylidene-G_7PNP (4,6-ethylidene(G_7)-*p*-nitrophenyl(G_1)-α,D-maltoheptaoside) to, for example, G_2-PNP and G_3-PNP, where G = glucose and PNP = *p*-nitrophenol. G_2-PNP and G_3-PNP are then hydrolyzed to glucose and *p*-nitrophenol by added alpha-glucosidase. Para-nitrophenol is measured spectrophotometrically at 405 nm. Alpha-amylase activity is determined relative to an alpha-amylase standard with known activity and is expressed in Kilo Novo alpha-amylase Units (Termamyl) (KNU(T)). One KNU(T) is the amount of alpha-amylase which, under standard conditions (pH 7.1; 37^o), dextrinizes 5.26 g starch dry substance per hour. One KNU(T) corresponds to the amount of alpha-amylase that hydrolyses 672 micromoles of ethylidene-G_7PNP per minute under standard conditions (pH 7.1; 37^o).

The quantification limit of the method is approximately 0.3 KNU(T)/g.
(Note: The method can be adapted for manual execution. Any suitable spectrophotometer may be used in place of a centrifugal analyzer.)

Apparatus
Centrifugal analyzer (Cobas Fara, Roche, or equivalent)
Diluter (Hamilton Microlab or equivalent)

Reagents
(Note: Use only deionised water)
Alpha-amylase standard (available from Novozymes A/S)
Starch dry substance
Alpha-glucosidase reagent and the substrate, *4,6-ethylidene(G_7)-p-nitrophenyl(G_1)-α,D-maltoheptaoside* may be obtained as a kit for the centrifugal analyzer.
BRIJ 35 solution, 15%: Add 1000 ml BRIJ 35 solution (polyoxyethylene 23 lauryl ether) to a 2000 ml volumetric flask. Add water to volume.
Stabilizing stock solution: Place 882 g $CaCl_2 \cdot 2H_2O$ in a 2000ml volumetric flask. Add 33 ml of 15% BRIJ 35 solution and water to volume.
Stabilizing solution (1%): Pipette 20 ml of the stabilizing stock solution into a 2000 ml volumetric flask. Add water to volume.

Standard and sample solutions
Standard alpha-amylase solutions: Dilute the alpha-amylase standard to 0.450 KNU(T)/ml as follows:
Accurately weigh the calculated quantity of the standard. Place the standard in a 200 ml volumetric flask and add water to approximately two thirds of the volume. Add 2 ml of the stabilizing stock solution. Add water to volume. This is a standard stock solution. Prepare the standard working solutions by diluting the standard stock solution with the stabilizing solution (1%) as shown in the table below. Use the diluter and vials compatible with the centrifugal analyzer.

Sample No.	Enzyme Stock Solution (µl)	Stabilizing solution (1%) (µl)	KNU(T)/ml
1	20	580	0.0150
2	30	570	0.0225
3	40	560	0.0300
4	50	550	0.0375
5	60	540	0.0450

The standard solutions can be stored in a refrigerator for one day.
"Void" standard: In a vial, mix 85.5 microliters of the alpha-amylase standard stock solution with 514 microliters of the stabilizing solution (1%). The solution can be stored for one day in a refrigerator.

Alpha-amylase control sample: Accurately weigh an alpha-amylase preparation of known activity, add to a 250 ml volumetric flask and make up to volume with the stabilizing solution (1%). If necessary, dilute the sample with the stabilizing solution (1%) again to obtain the final alpha-amylase activity within the range of the standard curve. Place the solution in a vial. The solution can be stored in a refrigerator for one day.

Alpha-amylase test samples: Accurately weigh out each sample into individual 250 ml volumetric flasks and add the stabilizing solution (1%) to volume. If necessary, dilute the sample with the stabilizing solution (1%) again to obtain the final activity of approximately 0.03 KNU(T)/ml.

Procedure

1. Pour the substrate solution into a 4 ml reagent container placed in the reagent rack of the centrifugal analyzer.
2. Pour alpha-glucosidase solution into a 15 ml reagent container placed in the reagent rack.
3. Place vials containing standard solutions and the control sample in the calibration rack.
4. Place the vial containing the void standard in the first position in the calibration rack. (Note: The void standard is used to condition the pipette before use. It is not used in the determination of alpha-amylase activity).
5. Place vials containing the test samples in the sample rack.
6. Set up the analysis program and start the analysis

Analysis

The analysis is performed automatically by the centrifugal analyzer. The empty rotor of the analyzer rotates until the temperature reaches 37^{o}. Twenty microliters of the test sample or control sample, 10 microliters of water, and 250 microliters of the alpha-glucosidase reagent are pipetted into cavities in the rotor. The rotor rotates for 10 seconds. During that time, the reagents are centrifuged into horizontally-oriented cuvettes. Then, 25 microliters of the substrate and 20 microliters of water are added to each cuvette. The rotor rotates again and the substrate is mixed with the content of each cuvette. The absorption is measured for the first time after 120 seconds and then every five seconds. A total of 37 measurements are made for each cuvette.

Calculations

The analyzer reads the alpha-amylase activity of the test samples from the standard curve and calculates the results in KNU(T)/ml. Calculate the alpha-amylase activity of each test sample in KNU(T)/g using the following formula:

$$Activity(KNU(T))/g = \frac{S \times V \times F}{W}$$

Where

S = analysis result in KNU(T)/ml
V = volume of the volumetric flask used to dilute the test sample (ml)
F = dilution factor used in the second dilution of the test sample (ml/ml)
W = weight of the test sample (g)