PULLULANASE FROM *BACILLUS DERAMIFICANS* EXPRESSED IN *BACILLUS LICHENIFORMIS*

New specifications prepared at the 74th JECFA (2011) and published in FAO JECFA Monographs 11 (2011). An ADI "not specified" was established at the 74th JECFA (2011).

SYNONYMS Pullulan α-1,6-glucanohydrolase; amylopectin 6-glucanohydrolase, bacterial debranching enzyme; α–dextrin endo-1,6-glucosidase; debranching enzyme; R-enzyme

SOURCES Pullulanase is produced by submerged fed-batch fermentation of a genetically modified strain of *Bacillus licheniformis* which contains a gene coding for pullulanase from *Bacillus deramificans.* The enzyme is recovered from the fermentation broth. The recovery process includes the separation of cellular biomass and concentration by ultrafiltration. The final product is formulated using food-grade stabilizing and preserving agents and is standardized to the desired activity.

Active principles: Pullulanase

Systematic names and numbers: Pullulan 6-α-glucanohydrolase; EC 3.2.1.41; CAS No. 9075-68-7

Reactions catalysed: Hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen

Secondary enzyme activities: No significant levels of secondary enzyme activities

DESCRIPTION Amber liquid

FUNCTIONAL USES Enzyme preparation.
Used in the hydrolysis of carbohydrates in the manufacture of starch hydrolysates (maltodextrins, maltose and glucose), high fructose corn syrup, beer, and potable alcohol.

GENERAL SPECIFICATIONS Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Pullulanase activity: The sample shows pullulanase activity.
See description under TESTS.

TESTS

Enzyme activity

Principle

Pullulanase catalyses the hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen. The activity of pullulanase is determined by measuring a degree of hydrolysis of the insoluble Red Pullulan substrate, which is solubilized due to hydrolysis by pullulanase. The insoluble residue is precipitated with ethanol and removed by centrifugation. The absorbance of the supernatant is measured at 510 nm. The colour intensity is proportional to the enzyme activity. Pullulanase with known activity is used as a standard.

The enzyme activity is expressed in Acid Stable Pullulanase Units (ASPU). One ASPU is defined as the amount of pullulanase that liberates reducing sugars equivalent to 0.45 µmol of glucose per minute from pullulan at pH 5.0 and a temperature of 40°.

Apparatus

Spectrophotometer (510 nm)
Water bath with thermostatic control (40±1^{o})
pH meter
Microcentrifuge
Positive displacement pipette and tips (250 µl)
Repeater pipette with 12.5 ml or 50 ml tips

Reagents and solutions

(Note: use deionized water)

Sodium acetate buffer (0.2 M, pH 5.0): Weigh 16.406 g of anhydrous sodium acetate and dissolve in 900 ml of water. Adjust the pH to 5.00±0.05 with glacial acetic acid. Bring the volume to 1000 ml with water and mix. The solution can be stored at 4^{o} for one month.

Red Pullulan substrate solution (2%): Weigh 1.00 g of Red Pullulan (Megazyme) into a 50-ml conical tube and dissolve in 50 ml of the sodium acetate buffer by stirring with a magnetic stirring bar for approximately 20-30 min. The solution can be stored at 4^{o} for two weeks.

Standard pullulanase solution: Use pullulanase standard solution with certified activity expressed in ASPU/ml (available from Danisco US Inc., Genencor Division, Rochester, NY, USA). Dilute the solution with the sodium acetate buffer to obtain three working standard solutions with concentrations that fall within the linear range of the assay after subtracting blank (typically 0.95 – 1.80 ASPU/ml). The assay is linear within the absorbance range of 0.4 -1.0. The standard solutions should be kept on ice and used within 2 h.

Sample solution: Dilute the pullulanase samples with the sodium acetate buffer to obtain a net absorbance (after subtracting blank) within the linear range of the assay. The solutions should be kept on ice and used within 2 h.

Procedure

1. Prepare duplicate microcentrifuge tubes in a rack for each standard solution, sample and blank. Using a positive displacement pipette, dispense to appropriate tubes 250 µl of standard solutions, control sample (pullulanase solution with known activity) and test sample. Add 250 µl of the sodium acetate buffer to the blank tube.
2. Add to each tube 250 µl of 2% Red Pullulan substrate solution with a repeater pipette and vortex for 3 sec.
3. Incubate all tubes in a water bath at 40±1^{o} for exactly 20 min.
4. Remove from the water bath and immediately add 1.0 ml of absolute ethanol using a repeater pipette. Vortex for 3 sec.
5. Leave all tubes at room temperature for 5-10 min.
6. Centrifuge the tubes in a benchtop centrifuge for 10 min.
7. Transfer the supernatant from each tube to a 1.5 ml cuvette. Zero the spectrophotometer with absolute ethanol and measure the absorbance for each cuvette at 510 nm. Subtract the absorbance of the blank from the absorbance readings for all standards and samples.
8. Prepare the standard curve using linear regression. The correlation coefficient must be ≥0.99.
9. Determine the pullulanase concentration of each sample solution from the standard curve.
10. Calculate the pullulanase activity for each sample of the pullulanase preparation in ASPU/g as follows:

$$Activity\ (ASPU/g) = C \times D$$

where

C is the pullulanase concentration of each sample determined from the standard curve (ASPU/ml); and

D is the dilution factor calculated by dividing the sample volume after dilution (ml) by the initial sample weight (g).