

MIXED β -GLUCANASE, CELLULASE AND XYLANASE FROM RASAMSONIA EMERSONII (TENTATIVE)

New tentative specifications prepared at the 80th JECFA (2015) and published in FAO JECFA Monographs 17 (2015). An ADI “not specified” was established at the 80th JECFA (2015).

Information required:

- *method to determine the identity for β -glucanase, including data from a minimum of five batches, using the method described;*
- *method to determine the identity for cellulase, including data from a minimum of five batches, using the method described;*
- *a non-proprietary method to determine the identity and activity for xylanase that can be used by control laboratories and data from a minimum of five batches, using the method described.*

SOURCES

The mixed β -glucanase, cellulase and xylanase enzyme preparation is produced by submerged fermentation of a non-pathogenic and non-toxicogenic strain of *Rasamsonia emersonii*. The enzyme is recovered from the fermentation broth. The recovery process includes the separation of cell mass by vacuum drum filtration or centrifugation. The enzyme is concentrated by ultrafiltration and/or evaporation. The mixed enzyme concentrate is then formulated with glycerol, and potassium sorbate, added to standardise and stabilise the preparation, respectively.

Active principles	β -Glucanase, cellulase, and xylanase
Systematic names and numbers	<p>β-Glucanase: 3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase EC No.: 3.2.1.6 CAS No.: 62213-14-3</p> <p>Cellulase: 4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase EC No.: 3.2.1.4 CAS No.: 9012-54-8</p> <p>Xylanase: 1,4-β-D-xylan xylanohydrolase EC No.: 3.2.1.8 CAS No.: 9025-57-4</p>
Reactions catalysed	<p>β-glucanase: Endohydrolysis of (1\rightarrow3) or (1\rightarrow4) linkages in β-D-glucans</p> <p>Cellulase: Endohydrolysis of (1\rightarrow4)-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans. Will also hydrolyse (1\rightarrow4) linkages in β-D-glucans that also contain (1\rightarrow3) linkages.</p> <p>Xylanase: Endohydrolysis of 1,4-β-D-xylosidic linkages in xylans</p>
Secondary enzyme activities	Hemicellulase, pentosanase, arabinase, amylase, pectinase, protease
DESCRIPTION	Light brown to dark brown liquid.

FUNCTIONAL USES Enzyme preparation

The β -glucanase, cellulase, and xylanase enzyme preparation is used as a processing aid in brewing, grain processing and in the production of potable alcohol. The action of these enzymes allows for decreased viscosity, which in turn improves filterability, yield and consistency of product quality. The enzyme preparation is also used in grain processing to degrade cell wall components, in order to improve mechanical treatments.

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

CHARACTERISTICS**IDENTIFICATION**

β -Glucanase activity The sample shows β -glucanase activity.
Method information required

Cellulase activity The sample shows cellulase activity.
Method information required

Xylanase activity The sample shows xylanase activity.
Method information required

TESTS

β -glucanase and cellulase activity

Principle

Enzyme activity is measured by a method based on the reduction of viscosity of a beta-glucan substrate at pH 5.60 and 45°, in the presence of β -glucanase and cellulase. This is measured using a calibrated Ubbelohde viscosimeter. Activity is expressed in Beta-Glucanase Fungique (BGF) units. One BGF unit is the amount of enzyme per milliliter (ml) of reaction mixture (15 ml substrate and 2 ml enzyme solution) that causes a change in viscosity of the substrate with a speed giving a slope of 0.147 per min under the conditions of the test, when the reaction time is plotted against the inverse of the falling time.

Apparatus

Ubbelohde No. 1C Viscosimeter, with a circulation flow constant of approximately 0.03, or equivalent
Analytical balance, accuracy to within 0.001 g
Waterbath

Description of Ubbelohde Viscosimeter:

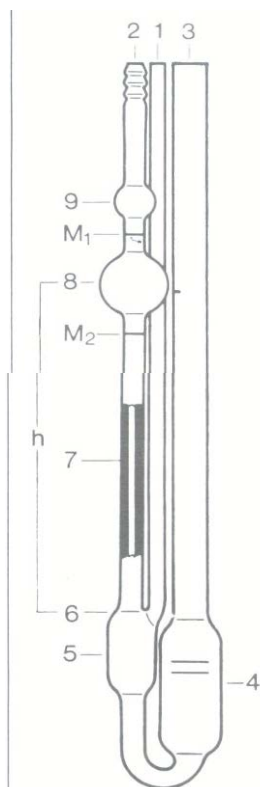


Figure legend:

1. Ventilating tube
2. Capillary tube
3. Filling tube
4. Reservoir
5. Reference level vessel
6. Dome-shaped top part
7. Capillary
8. Measuring sphere
9. Pre-run sphere
- M1. Upper timing mark
- M2. Lower timing mark

Reagents and solutionsPotassium dihydrogen phosphate (KH_2PO_4)

Phosphoric acid, 85%, P-BGBM beta-glucan from barley; viscosity 20–30 cst. Megazyme

Sodium Hydroxide, 1 mol/l

Ultrahigh quality water, resistance >18.2 mega Ohm/cm, TOC < 500 µg/l.

Phosphoric acid 1.0 mol/l

Slowly and while stirring continuously add 67 ml phosphoric acid 85% to approximately 500 ml water in a 1 l volumetric flask. Make up to volume and mix. This solution may be kept for 2 months at room temperature.

Phosphate Buffer, 1.0 mol/l, pH 5.00

Dissolve 13.6 g potassium dihydrogen phosphate in approximately 80 ml water in a 100 ml volumetric flask. If necessary heat while dissolving and allow cooling to ambient temperature. Adjust the pH to 5.00 by adding phosphoric acid solution (1 mol/l) or sodium hydroxide solution (1 mol/l). Make up to volume with water and mix. This solution may be kept for 2 months.

Beta-glucan substrate solution

Use a reserved batch of beta-glucan, with a known substrate batch factor, Sf. (This is the factor used to bridge new substrates to the existing substrate used for calibration).

Dissolve 1.0 g beta-glucan in approximately 30 ml water in a 100 ml glass Erlen Meyer flask. Stir for approximately 1 h. Next place the solution in the bath with boiling water for 5 min and allow cooling to ambient temperature. Add 10.0 ml phosphate buffer 1 mol/l, pH 5.00 and quantitatively transfer to a 100 ml volumetric flask with water. Make up to volume with water and mix.

Check the pH of the solution. It must be 5.60 +/- 0.05. Prepare a new solution when pH is out of range. Only use a freshly prepared solution.

Control

β -glucanase and cellulase of known activities are used as assay controls.

Samples

Allow the sample to attain room temperature. Weigh, accurately to within 0.0001 g, and in duplicate an amount of sample corresponding to 10 000 BGF in a 100 ml volumetric flask. Dissolve in water by stirring on a magnetic stirrer. Make up to volume with water and mix. Dilute 0.25 ml of these solutions with 4.75 ml water in a centrifuge tube and mix. The activity of the sample should fall between 3.4 and 6.8 BGF/ml.

Procedure

Allow the Ubbelohde viscosimeter to equilibrate at 45° for at least 20 min. Place a reagent tube containing 15.0 ml of beta-glucan substrate in the 45° water bath and allow it to equilibrate for at least 20 min.

Start a stopwatch (Stopwatch 1) set to T= 0 min, and leave this running throughout the assay to record incubation time. Add 2.0 ml of sample solution to the equilibrated substrate at T = 0 min. Mix on a tube shaker.

Fill the reservoir of the viscosimeter through Filling Tube to a level between the two marks on the reservoir (refer to the Description of Ubbelohde Viscosimeter, if necessary).

Measure the viscosity of this solution at every three min for a 15 min period, by following step 5 - 9. This will result in 5 measurements in total. At T = ~ 2.5 min close Ventilating Tube (with finger) and execute suction on Capillary Tube to fill the Capillary Tube up to the Pre-run sphere (refer to the Description of Ubbelohde Viscosimeter, if necessary).

Stop suction, remove finger from Ventilating Tube and start Stopwatch 2 when the liquid reaches the Upper Timing mark (M1).

Read the time on Stopwatch 1 (do not stop stopwatch 1!). This will be Rt1 for the calculations.

Allow the liquid to run down to the Lower Timing mark, M2. Stop Stopwatch 2 when the Lower Timing mark, M2 is reached. This will be Vt1 in the calculation.

Repeat this procedure every three min. Record Rt2 to Rt5 and Vt2 to Vt5. Repeat Steps 1-9 using a mixture of 15.0 ml substrate and 2.0 ml water as the substrate blank. Record the viscosity as Vtb.

Repeat Steps 1-9 using a mixture of 17.0 ml water as the water blank. Record the viscosity as Vtw.

Repeat Steps 1-9 if the enzyme activity is outside the linear range of the assay, with appropriate dilutions.

Calculations

Calculate the Reaction Time (T1-T5), in sec, accurately to 0.01 sec using the equations:

$$\begin{aligned} T1 &= \{Rt1 + (\frac{1}{2} Vt1)\} \\ T2 &= \{Rt2 + (\frac{1}{2} Vt2)\} \\ T3 &= \{Rt3 + (\frac{1}{2} Vt3)\} \\ T4 &= \{Rt4 + (\frac{1}{2} Vt4)\} \\ T5 &= \{Rt5 + (\frac{1}{2} Vt5)\} \end{aligned}$$

Calculate the average time for drop in viscosity, for the five measuring points of the substrate blank in sec, using the equation:

$$Vt_{bl} = (\text{Sum of } Vt \text{ 1 to 5}) / 5$$

Calculate the average time for drop in viscosity, for the five measuring points of the water blank in sec, using the equation:

$$Vt_w = (\text{Sum of } Vt \text{ 1 to 5}) / 5$$

For each of the five measuring points of sample, calculate X using the equation:

$$X = Vt_{bl} / (Vt - Vt_w).$$

For each of the measuring points, plot the T values (obtained in Step 1, x axis) against the corresponding X (obtained in Step 4, y-axis). Calculate the slope according to the linear equation

$$y = Px + b$$

From this line calculate the slope, P.

Calculate the enzyme concentration in the incubation mixture as follows:

$$C = (W / 100) \times (0.25 / 5.00)$$

where

C is Sample Concentration in g/ml

W is Sample weight, g

0.25/5.00 is Dilution Factor

100 is Volume of Dilution, ml

Calculate the enzyme activity of the sample as follows:

$$\beta\text{-Glucanase Activity, BGF/g} = (P \times 60 / 0.147) \times (17/2) \times (1/C) \times Sf$$

where

P is Slope from Step 5

60 is Conversion of sec to min

0.147 is Conversion Factor from definition of Enzyme Activity

17/2 is Correction Factor for incubation mixture

C is Sample Concentration from Step 6

Xylanase activity

The sample shows xylanase activity.
Method information required