Determination of β-Glucanase activity in feeds by viscosimetry

Note: The words "feed" and "feeds" can be replaced with "feedingstuffs" in this document.

1. Document history

Not relavant

2. Warnings

Refer to each product safety data sheet.
Wear suitable safety glasses and gloves.
Work under fume cupboard.

3. Scope

This method is applicable to determine endo-1,3(4)-β-glucanase activity in feeds by viscosimetry.

4. Normative references

Not applicable.

5. Definitions

Not applicable.

6. Principle and reactions

In this method are described the different operations to be performed to determine endo-1,3(4)-β-glucanase activity in feeds supplemented with enzymatic additives containing a β-glucanase activity, such as ROVABIO, using a viscosimetric method.

6.1. Principle

This method is specific to the determination of endo-1,3(4)-β-glucanase in feeds. The assay is based on the enzymatic hydrolysis of a barley β-glucan standard solution. The enzymatic activity is determined from the relative viscosity reduction.

6.2. Definition of the unit

Each unit of endo-1,3(4)-β-glucanase activity is equivalent to the quantity of enzyme that hydrolyzes the substrate, thus reducing the solution viscosity, in order to change the

relative fluidity by one unit without dimension per minute, according to the analysis conditions.

The standard β-glucanase activity determination is performed at pH 5.5 and 30 °C. However, an activity determination can also be performed at different pH and temperatures in order to characterize the protein. In this case, the buffer pH or even the buffer type may be modified, as well as the enzymatic reaction temperature.

6.3. Methodology

Significant interferences can occur during the endo-1,3(4)-β-glucanase activity determination in feeds. The interference level has, therefore, to be determined for each analysis. This interference determination is performed using a control sample (feed containing no enzyme), to which a known quantity of enzyme is being added. In order to perform the enzymatic analysis in the feed, the following information has to be known:

- The enzyme sample used in the feed production (product type, batch number),

[If the batch number is not provided, a retention enzyme, specific to the laboratory is used]

- The incorporation rate of the enzyme in the feed (L / t or g / t),
- A feed control sample (without enzyme),
- A formulated feed sample containing the enzyme,

The endo-1,3(4)-β-glucanase recovery percentage determination is performed in 5 steps:

1. Endo-1,3(4)-β-glucanase activity determination in the enzyme sample;
2. Endo-1,3(4)-β-glucanase activity determination in the control sample (without enzyme);
3. Performance of a determination using spiking:
    - perform an extraction of the control feed after having supplemented it with some enzyme at the same concentration than in the feed that has been produced,
    - determine the endo-1,3(4)-β-glucanase activity;
4. Endo-1,3(4)-β-glucanase activity determination in the treated feed;
5. Deduction of the recovery percentage in the feed additive.

Note: *If the control sample is not provided, refer to section 13.*

7. Reagents and materials

All reagents must be of analytical grade. Except when specifically indicated, the water used is chromatographic grade water. The water used in the water bath can be tap or distilled water.
The reagents used for the β-glucanase activity determination using the viscosimetric method are presented in annex 1. In every case but the β-glucan case, the identity as well as the purity of the reagents is more important than the supplier criteria.

7.1. Barley β-glucan at 1 % (w/V) (Substrate)

To prepare 100 mL of substrate, weigh exactly about 1.000 g to the nearest 0.001 g of β-glucan. Place 70 mL of water in a beaker, add a magnetic stirring bar and place on a heating magnetic stirrer. Stir vigorously and start heating. When the water is hot (but not boiling), add the β-glucan in avoiding the formation of lumps. When the liquid starts to boil, stop the heating, cover up the beaker with a watch glass and continue the stirring for 10 more minutes in order to completely dissolve the substrate. Then cool down to room temperature (20 - 25 °C) (at this step, the beaker can be placed onto a cold magnetic stirrer). Transfer the solution in a 100 mL volumetric flask. Add 10 mL of pH 5.5 1M sodium acetate buffer solution (7.4.). Rinse the beaker with water and transfer into the volumetric flask. Fill up the flask to volume using water.
Leave the solution to settle in a refrigerator for 18 hours prior use.
Keep the substrate in a refrigerator for a maximum of 2 weeks.

7.2. 1M sodium acetate

Dissolve approximately 82.0 g weighed to the nearest 0.2 g of anhydrous sodium acetate in 500 mL of water. Stir vigorously using a magnetic stirrer. Transfer in a 1L volumetric flask, rinse the beaker with water and transfer into the volumetric flask. Fill up the flask to volume using water.
Transfer into a bottle. Keep at room temperature for a maximum of 2 months.

7.3. 1M acetic acid

Weigh exactly approximately 60.1 g to the nearest 0.2 g of glacial acetic acid in a 100 mL beaker. Place a 500 mL beaker containing 400 mL of water onto a magnetic stirrer. Stir and pour slowly the previously weighed acetic acid. Transfer in a 1L volumetric flask. Rinse the beaker with water and transfer into the volumetric flask. Fill up the flask to volume using water.
Transfer into a bottle. Keep at room temperature for a maximum of 2 months.

7.4. pH 5.5 1M sodium acetate buffer solution

Using a calibrated pH meter, add some 1M acetic acid solution (7.3.) to the sodium acetate solution (7.2.) until pH 5.5 is reached.
Transfer into a bottle. Keep at room temperature for a maximum of 2 months.

7.5. pH 5.5 0.01M sodium acetate buffer solution

Pipette 10 mL of 1M sodium acetate buffer solution (7.4.) in a 1 L volumetric flask. Fill up to volume using water.

7.6. 1M sodium hydroxide solution

Weigh exactly about 40.0 g ± 0.1 g of sodium hydroxide in a 1 L beaker, add a magnetic stirring bar and place onto a magnetic stirrer. Under continuous stirring, gently add 750 mL of water. Transfer to a 1 L volumetric flask and fill up to volume using water.
Note: Ready to use solutions can also be used.

7.7. pH 6.0 MES / SDS (lauryl sulphate) buffer solution (0.1M MES, pH 6.0 ; 1 % (w/V) SDS (lauryl sulphate))

Weigh exactly about 97.5 g to the nearest 0.2 g of MES. Place 4500 mL water and a magnetic stirring bar in a 5 L beaker.
Add the MES and stir until complete dissolution.
With a calibrated pH meter, adjust the pH to pH 6.0 using 1M sodium hydroxide solution (7.6.).
Add 50.0 g weighed to the nearest 0.2 g of SDS (lauryl sulphate) and stir until complete dissolution.
In order to avoid the excessive formation of foam, transfer slowly into a 5 L volumetric flask.
Fill up to 5 L using water.

8. Apparatus

- Usual glassware and laboratory equipment;
- Grinder equipped with a 0.5 mm mesh opening grid;
- Disposable glass and plastic tubes with racks;
- P5000, P1000 and P200 GILSON pipettes;
- Stopwatch;
- Laboratory centrifuge (7000 rpm);
- Heating magnetic stirrer + magnetic stirring bars;
- Calibrated pH meter and analytical balance;
- Cold water bath (cold pack);
- Water bath at 30 °C (± 0.1 °C);
- Microviscosimeter (e.g. AMVn type ANTON PAAR microviscosimeter) + associated software;
- Ultrasonic bath;
- Multi-position magnetic stirrer;
- Filters and syringes.

References of the equipment used to date are listed in annex 2 as an example.

The whole of the equipment has to be used in a clean environment in order to avoid eventual contamination problems. The environment should be isolated from any concentrated enzyme sample and should be cleaned regularly. It is good practice to use pipettes specifically dedicated to this kind of analysis.

9. Sampling

Not applicable.

10. Procedure

10.1. Sample preparation

Feeds are ground with an ultra-centrifuge grinder equipped with a 0.5 mm mesh opening grid.

10.1.1. Feed control sample preparation

1- Grind, whenever possible, a minimum of 100 g of control feed.
2- Weigh exactly about 10.0 g to the nearest 0.5 g of ground feed and introduce them in a 250 mL conical flask. In order to avoid the excessive formation of foam, add slowly 100 mL of pH 6.0 MES / SDS buffer solution (7.7). Add a magnetic stirring bar and place into a cold water bath (water bath + cold pack) equipped with a multi-position magnetic stirrer. Stir for 30 minutes.
3- Transfer about 5 mL of this solution in a disposable plastic tube and centrifuge for 10 minutes at 7000 rpm and 15 °C.
4- Filter the supernatant then perform a dilution of the filtrated solution as follows: 1 mL in 2.5 mL of 0.01M pH 5.5 buffer acetate solution (7.5).
5- Determine the β-glucanase activity. The result will be recorded as **Control**.

Note: In the particular case of feeds made with T-Flex, weigh exactly about, whenever possible, 50 g ± 1 g of control feed into 500 mL of pH 6.0 MES / SDS buffer solution (7.7) then stir for 30 minutes at room temperature (steps 3, 4 and 5 remain unchanged).

Remark: The supernatant can be stored in cool conditions (15 °C maximum) before analysis.

10.1.2. Spiked sample preparation

1- Grind, whenever possible, a minimum of 100 g of control feed.
2- Weigh exactly about 10.0 g to the nearest 0.5 g of ground feed and introduce it into a 250 mL conical flask.
3- Spikes preparation:
The spikes to be performed are dependent upon the type of the feed additive used.

Spike with ROVABIO EXCEL LC (incorporation rate in the feed = 0.2 L / t).	Spike with ROVABIO EXCEL AP (incorporation rate in the feed = 50 g / t).
Introduce 0.5 mL of the additive in a 250 mL flask.	Introduce 0.25 g ± 10 mg of the additive in a 500 mL flask.
Fill up using water and homogenize. Take 1 mL and introduce it in the flask containing the 10 g of control feed.	

4- Add 100 mL of pH 6.0 MES / SDS buffer solution (7.7). Introduce a magnetic stirring bar and place in a cold water bath (water bath + cold pack) equipped with a multi-position magnetic stirrer. Stir for 30 minutes.
5- Transfer about 5 mL in a disposable plastic tube then centrifuge for 10 minutes at 7000 rpm and 15 °C.
6- Filter the supernatant then perform a dilution of the filtrated solution as follows: 1 mL in 2.5 mL of 0.01M pH 5.5 buffer acetate solution (7.5).
7- Determine the β-glucanase activity. The result will be recorded as **Spike**.

Note: For the particular case of feed containing T-Flex, weigh exactly about, whenever possible, 50 g ± 1 g of control feed, then perform a 5 mL spike. Add 500 mL of pH 6.0 MES / SDS buffer solution then stir for 30 minutes at room temperature (Steps 5, 6 and 7 remain unchanged).

Remark: The supernatant can be stored in cool conditions (15 •C maximum) before to be analyzed.

10.1.3. Enzymed feed sample preparation

1- Grind, whenever possible, a minimum of 100 g of enzymed feed.
2- Weigh exactly about 10.0 g to the nearest 0.5 g of ground feed and introduce it into a 250 mL conical flask. In order to avoid the excessive formation of foam, add slowly 100 mL of pH 6.0 MES / SDS buffer solution (7.7). Add a magnetic stirring bar and place in a cold water bath (water bath + cold pack) equipped with a multi-position stirrer. Stir for 30 minutes.
3- Transfer about 5 mL in a disposable plastic tube then centrifuge for 10 minutes at 7000 rpm and 15 °C.
4- Filter the supernatant then perform a dilution of the filtrated solution as follows: 1 mL in 2.5 mL of 0.01M pH 5.5 buffer acetate solution (7.5).
5- Determine the β-glucanase activity. The result will be recorded as **Treated**.

Note: For the particular case of feed containing T-Flex, weigh exactly about, whenever possible, 50 g ± 1 g of enzymed feed in 500 mL of pH 6.0 MES / SDS buffer solution (7.7) then stir for 30 minutes at room temperature (Steps 3, 4 and 5 remain unchanged).

Remark: The supernatant can be stored in cool conditions (15 •C maximum) before to be analyzed.

10.2. Sample assay procedure

10.2.1. β-glucanase activity determination in concentrated commercial formulations

A determination of β-glucanase activity is performed on the enzyme sample that has been supplemented to the feedstuff, using method T008. To do this, either:

- The batch number has been provided by the customer then the retention sample situated in the laboratory is used to perform the spike. In this case, the enzymatic

activity determination of the additive provided by the customer is not performed and the laboratory value is used instead.

- The batch number has not been indicated by the customer. A retention sample specifically selected by the laboratory for this purpose is used for the spike.

10.2.2. β-glucanase activity determination in the samples

Set the microviscosimeter with an appropriate capillary and a ball (see annex 3).

Before to start any enzymatic analysis, the capillary and the ball have to be calibrated against the buffer (buffer blank) and against the substrate (substrate blank). These calibrations should be performed each day an analysis is performed.

It is necessary to use clean equipment. The capillary has to be cleaned with water then dried with a flux of nitrogen between each analysis.

At the end of the day, clean the capillary and the ball using water, then ethanol then acetone and dry it with a flux of nitrogen.

10.2.2.1. Calibration against diluted buffer solution - Diluted buffer blank determination

Place 5 mL of pH 5.5 0.01M sodium acetate buffer solution (7.5) in a disposable sterile tube. Sonicate for 5 seconds. Place at 30 °C for 5 minutes.
Fill up the capillary (containing a calibrated ball) with 30 °C pH 5.5 0.01M sodium acetate buffer solution. Make sure that no air bubble remains in the capillary, then place it into the microviscosimeter.
The microviscosimeter will perform 60 measurements. The average drop time for the ***40 last measurements*** for the buffer is labelled Tw (value equal to about 12 s).

10.2.2.2. Calibration against the substrate - Substrate blank determination

Place 3 mL of water and 1 mL of 1 % barley β-glucan (7.1) in a disposable sterile tube. Sonicate for 5 seconds. Place at 30 °C for (at least) 5 minutes.
Add 1 mL of 30 °C pH 5.5 0.01M buffer acetate buffer solution (7.5) to the substrate. Mix using a vortex then fill up the capillary containing a calibrated ball with the diluted barley β-glucan solution (make sure that no air bubble is present in the capillary). Insert the capillary into the viscosimeter.
The viscosimeter will perform 30 measurements. The average drop time for the ***20 last measurements*** for the substrate is labelled Ts (value equal to about 24 s).

If the obtained value is too low or if the drop time decrease is lower than -10 msec / min, discard the substrate and prepare a new substrate solution.

10.2.2.3. Enzymatic activity determination for the sample

Place 3 mL of water and 1 mL of 1 % barley β-glucan (7.1) in a disposable sterile tube. Sonicate for 5 seconds then place the tube in a thermostated water bath set at 30 °C and leave to equilibrate for (at least) 5 minutes.

Add 1 mL of the enzymatic preparation to the 30 °C incubated substrate. Start the stopwatch. Mix using a vortex then fill up the capillary containing a calibrated ball with the mixture (make sure that no air bubble is present in the capillary). Insert the capillary into the microviscosimeter after exactly 1 minute.

The microviscosimeter will perform 30 measurements. At the end of the analysis, remove the capillary, clean it and then dry it with a flux of nitrogen.

11. Calculation

11.1. Determination of the enzymatic activity

Fr	=	relative fluidity
Tw	=	average drop time for the buffer (msec)
Ts	=	average drop time for the substrate (msec)
Tt	=	drop time during the assay at a given t time (msec)
t	=	assay time (minutes)
$T_{1/2}$	=	assay time (t) plus half (½) of Tt (minutes)
DF	=	dilution factor
V_T	=	total test volume (5 mL)
V_S	=	enzyme solution volume in the sample (1 mL)
Correction Factor	=	substrate factor (specific to each substrate batch)

① For the ***20 last measurements***, calculate Fr for each Tt

$$Fr = \frac{T_s - T_w}{T_t - T_w}$$

② Plot Fr = f ($T_{1/2}$)
③ Determine the linear regression slope
④ From it, determine the raw output activity: the obtained slope is proportional to the enzymatic activity present in the sample.

$$Raw\ Output\ Activity\ (U/g) = slope \times DF \times \frac{V_T}{V_S}$$

⑤ Determine the final activity

$$Activity\ (U/g) = Raw\ Output\ Activity \times Correction\ Factor$$

Note: When changing substrate, results could be different. Thus, apply a correction factor to get identical results as those obtained when using the former substrate.

11.2. Recovery percentage calculation

The enzymatic activity recovery percentage of the sample is deduced by applying the following calculation formula:

$$\% Rec. = \frac{Activity\ of\ the\ Treated\ Feed - Activity\ of\ the\ Control\ Feed}{Activity\ of\ the\ Spiked\ Control\ Feed - Activity\ of\ the\ Control\ Feed} \times 100$$

11.3. Normalised activity calculation

The activity is corrected in order to consider the interferences:

Normalised activity (U/kg) = % Rec. x Theorethical activity (U/kg)

11.4. Results interpretation

For the two series of analysis, calculate the difference between the two results:

$$Difference = \frac{Value_{max.} - Value_{min.}}{Value_{min.}} \times 100$$

If the difference is lower than or equal to 10 %, calculate the average of the two values to obtain the result.
If the difference is higher than 10 %, a third determination will have to be performed.

11.5. Non compliant results

Substrate blank: the viscosity reduction maximum (dropping time) that should be obtained during the calibration is - 10 msec / min. Lower values could indicate a contamination of the substrate or of the capillary with an enzyme. Clean thoroughly the capillary and repeat the calibration. If the problem remains, prepare a new substrate solution.

12. Precision

Refer to study report 2009VAL007.

13. Special cases

For a set to be complete, it must contain:

- a control sample
- the treated feed
- the enzyme (batch number) that was used during the feed production

However, the set may not contain any control feed. Two cases are then possible:

- The feed is essentially composed of wheat or maize. Then use a reference wheat or maize as control.
- The composition is unknown. In this situation, spike the treated with the same concentration as the theoretical concentration. Use an arbitrary control value such as 0 for example.

When the control is absent, the provided values are only indicative.

14. Annexes

Annex 1: Reagents list.
Annex 2: Equipment list.
Annex 3: AMVn type microviscosimeter settings.

15. Bibliography

N/A

Annex 1

List of reagents used and associated references for example

Reagent	Supplier	Product reference	Molecular weight (g / mol)
β-glucan	MEGAZYME LTD	P-BGBM	-
Anhydrous sodium acetate	PROLABO	27 653 292	82.03
Acetic acid	PROLABO	20 104 298	60.05
Acetone		CAS 97-64-1	

Annex 2

Equipment used and associated references for example

Consumables

P200 cones	GILSON	Ref.: D200ST
P1000 cones	GILSON	Ref.: D1000ST
13 mL tubes	SARSTEDT	Ref:: 55-518 PP
Hemolysis tubes	VWR	Ref.: 0512102
2.5 mL combitips	VWR	Ref.: 613-3521
Glassfiber multigrade syringe filters (Ø 0.45µm)	Millipore	Ref.: SLHVM25NK
Stickers	DIPÔLE	Ref.: ETQ5020TCGW
Resin film	DIPÔLE	Ref.: FR65AXR7

Equipment

Stopwatch
METTLER AE200 analytical balance, 0.1 mg precision
Mettler Toledo MP230 pH meter, 0.1 pH unit precision

GILSON pipettes	P200, 1 % precision	VWR	Ref: 01-285-055
	P1000	VWR	Ref: 01-285-077
	P5000	VWR	Ref: 01-285-102
4780 Eppendorf multipette		VWR	Ref: 01-302-011

Water bath, 0.1 °C precision
ANTON PAAR AMVn microviscosimeter + associated software
Ultra-centrifuge grinder
Centrifuge
Ultrasonic bath
Multi-position stirrer

Annex 3

Measurement equipment used and AMVn microviscosimeter settings

Apparatus type	AMVn type Anton Paar microviscosimeter	Supplier: ANTON PAAR
Capillary	1.6 mm internal diameter capillary	Supplier: ANTON PAAR
Ball	1.5 mm diameter steel ball	Supplier: ANTON PAAR
Measurement angle	75°	
Measurement temperature	30 °C	
Number of measurements for the water blank	60	The 20 first values are not taken into account in the calculations
Expected value for the water blank	About 12 seconds	
Number of measurements for the substrate blank	30	The 10 first values are not taken into account in the calculations
Expected value for the substrate blank	About 24 seconds	
Number of measurements for the sample assay	30	The 10 first values are not taken into account in the calculations