Determination of xylanase activity in premixes by viscosimetry

1. Document history

Not relevant

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 premix type samples.
Note: The words “premix” and “premixes” can be replaced with “premixtures” in this document.

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 xylanase activity in premixes supplemented with enzymatic additives containing a xylanase activity, such as ROVABIO, using a viscosimetric method.

6.1. Principle

This method is specific to the determination of endo-1,4-β-xylanase in premixes. Endo-1,4-β-xylanase hydrolyses the xylosidic linkages of the xylan. The test is set upon the enzymatic hydrolysis of xylosidic linkages of a solution containing soluble wheat arabinoxylan, a β-1,4-xylan polysaccharide substituted with arabinose. The enzymatic activity is proportional to the viscosity reduction of a wheat arabinoxylan solution in the presence of the enzyme to be determined.

6.2. Definition of the unit

Each unit of endo-1,4-β-xylanase activity is equivalent to the quantity of the 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 following conditions: pH 5.5 (or as indicated) and 30 °C (or as indicated).

The standard xylanase activity determination is performed at pH 5.5 and 30 °C. However, an activity determination can also be performed at different pHs 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.

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 xylanase activity determination using the viscosimetric method are presented in annex 1. In every case but the wheat arabinoxylan case, the identity as well as the purity of the reagents are more important than the supplier criteria.

7.1. Wheat arabinoxylan at 0.25 % (w/V)

To prepare 100 mL of substrate, weigh exactly about 0.250 g to the nearest 0.001 g of wheat arabinoxylan. 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 arabinoxylan in avoiding the formation of lumps. When the liquid start to boil, reduce the heating until the simmering point, cover up the beaker with a glass beaker cover and continue the heating for 10 more minutes in order to completely dissolve the substrate. Stop the heating and continue the stirring until the solution reaches room temperature (at this step, the beaker can be placed onto a cold magnetic stirrer). Transfer the solution in a 100 mL volumetric flask. Add 2.5 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.
A fresh substrate solution has to be prepared each analysis day.
The substrate is kept at room temperature.
At the end of the day, the excess substrate is discarded.

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.

8. Apparatus

- Usual glassware and laboratory equipment;
- Disposable glass and plastic tubes with racks;
- P5000, P1000, P200 GILSON pipettes and electronic HandyStep pipette or equivalent;
- Stopwatch;
- Heating magnetic stirrer + magnetic stirring bars;
- Calibrated pH meter and analytical balance;
- Water bath at 30 °C (± 0.1 °C);
- Vortex;
- Microviscosimeter (e.g. AMVn type from ANTON PAAR) + associated software;
- Ultrasonic bath.

The whole of the equipment has to be used in a clean environment in order to avoid any 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.

References of the equipment used to date are listed in annex 2 as an example. Any change of supplier reference should be validated through a comparative study. The measurement equipment used should have similar characteristics than the equipment described in this method.

9. Sampling

Not applicable.

10. Procedure

10.1. Sample preparation

Place approximately 4.50 g of premix weighed out to the nearest 0.05 g in 100 mL of pH 5.5 1M sodium acetate buffer solution.
Place a magnetic stirring bar, then extract the solution for 30 minutes at room temperature under continuous stirring.
Filter using a syringe filter.

Perform a dilution in pH 5.5 1M sodium acetate buffer solution in order to be within the 5 - 15 minutes test measuring interval:

$$\frac{\Delta \text{ (viscosity decrease)}}{\Delta \text{ time}} = -105 \pm 10 \; m\text{sec} / \text{min}$$

This corresponds to a 950 - 1150 msec decrease during the (approximate) 10 minutes measurement time.

10.2. Sample assay

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

Before to start an enzymatic analysis, the capillary and the ball have to be calibrated against water (**water blank**) and against the substrate (**substrate blank**). These calibrations against water and against the substrate are performed every 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, ethanol then acetone and dry them with a flux of nitrogen.

10.2.1. Calibration against water - Water blank determination

Place 5 mL of water 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 water. Make sure that no air bubble is present in the capillary, then place it into the microviscosimeter.
The microviscosimeter will perform 60 measurements. The drop time average for the 40 last measurements for water is labelled Tw (value equal to about 12 s).

10.2.2. Calibration against the substrate - Substrate blank determination

Place 4.8 mL of 0.25 % wheat arabinoxylan in a disposable sterile tube. Sonicate for 5 seconds. Place at 30 °C for (at least) 5 minutes.

Note: At the beginning of the day, it is possible to prepare several tubes containing the substrate solution (4.8 mL) and to leave them to incubate in the 30 °C water bath.

Add 0.2 mL of 30 °C water to the substrate (final concentration = 0.24 % w / v). Mix using a vortex then fill up the capillary containing a calibrated ball with the diluted wheat arabinoxylan 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 drop time average for the 20 last measurements for the substrate is labelled Ts (value equal to about 26 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 (7.1.).

10.2.3. Enzymatic activity determination for the sample

Place 4.8 mL of 0.25 % wheat arabinoxylan solution (7.1.) in a disposable sterile tube. Sonicate for 5 seconds. Place the tube in a thermostated water bath at 30 °C and leave to equilibrate for (at least) 5 minutes.

Add 0.2 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 viscosimeter after exactly 1 minute.

The viscosimeter will perform 30 measurements. At the end of the analysis, remove the capillary, clean it then dry it.

11. Calculation

11.1. Determination of the enzymatic activity

Fr	=	relative fluidity
Tw	=	average drop time for water (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 (0.2 mL)
CF	=	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.

$$\boxed{Raw\ output\ activity\ (U/g) = Fr\ slope \times DF \times \frac{V_T}{V_S}}$$

⑤ Determine the final activity

$$\boxed{Activity\ (U/g) = Raw\,Output\ Activity\ (U/g) \times CF}$$

11.2. Determination example

Calibrated ball	=	1.5 mm (steel 1.4125)
Enzyme dilution	=	4.4880 g in 100 mL then 0.88 mL in 50 mL (D = 1266)
Tw	=	11880 msec
Ts	=	26477 msec
Slope Fr	=	0.0092
CF	=	1.334
Activity	=	0.0092 x 1266 x (5/0.2) x 1.334
	=	**389 U / g**

11.3. Results interpretation

For the two series of analysis performed, calculate the difference between the two values:

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

If the difference is lower than or equal to 5 %, calculate the average of the two values to obtain the result.
If the difference is higher than 5 %, a third determination will have to be performed.

11.4. 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 the capillary by an enzyme. Clean thoroughly the capillary and repeat the calibration. If the problem remains, prepare a new substrate solution.

12. Precision

N/A

13. Special cases

Not applicable.

14. Annexes

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

15. Bibliography

N/A

Annex 1

List of reagents used and associated references for example

Name	Supplier	Product reference	Molecular weight (g / mol)
Arabinoxylan	MEGAZYME LTD	P-WAXYM	-
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

Item	Supplier	Reference
P200 cones	GILSON	Ref.: D200ST
P1000 cones	GILSON	Ref.: D1000ST
P5000 cones	GILSON	Ref.: D5000

Equipment

- Stopwatch (precision count-up / count-down) VWR Ref.: 609-0016
- METTLER AE200 analytical balance, 0.1 mg precision
- Vortex SCIENTIFIC INDUSTRIES, Vortex Genie 2 TYPE
- METTLER TOLEDO MP230 pH meter, 0.1 pH unit precision
- GILSON pipettes P200
P1000
P5000
- GRANT Y14 hot water bath, 0.1 °C precision
- HAAKE or RHOVISC or ANTON PAAR (AMVn) microviscosimeter + associated software

Annex 3

Measurement equipment used and 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 26 seconds	
Number of measurements for the sample assay	30	The 10 first values are not taken into account in the calculations