Determination of xylanase activity in Rovabio 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 any sample (liquid or powder).

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 using a viscosimetric method.

6.1. Principle

The activity of endo-1,4-β-xylanase is determined using a viscosimetric method. Endo-1,4-β-xylanase catalyses the hydrolysis of xylosidic linkages in the wheat arabinoxylan substrate to yield xylose, and reduces consequently the viscosity of the sample solution. The viscosity decrease of the sample solution, expressed in terms of a drop time, is a measurement of the endo-1,4-β-xylanase activity and is determined using a falling ball viscosimeter at pH = 5.5 and 30°C.

6.2. Definition of the unit

Each unit of endo-1,4-β-xylanase 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 per minute 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 this viscosimetric method are presented in annex 1. In every case but the arabinoxylan case, the identity and 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 watch glass 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 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 sterile tubes with stoppers;
- P5000, P1000 and P200 GILSON type pipettes and electronic *HandyStep* pipette;
- Stopwatch;
- Heating magnetic stirrer + magnetic stirring bars;
- Calibrated pH - meter and analytical balance;
- Water bath capable of 30.0 °C (± 0.1 °C);
- Vortex;
- Microviscosimeter (e.g. HAAKE or RHOVISC or AMVn type ANTON PAAR microviscosimeter) + 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 better practice to use pipettes specifically dedicated to this type 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

Every enzymatic preparation will be diluted in water. Try to minimize the dilution errors (no small enzyme quantities in big water volumes). In every case, it is better practice to weigh out the enzyme to be analyzed, even for a liquid enzyme. The sample density, measured at 20 °C, will be used to calculate the activity in U / mL of the product.

The enzyme is extracted at room temperature under magnetic stirring, for 10 min. for AP formulations, for 30 min. for AP T. FLEX formulations and manually for LC formulations.

Classical assay portions are:

- approximately 0.600 g weighed to the nearest 0.001 g for AP formulations, in 250 mL water;
- approximately 0.900 g weighed to the nearest 0.001 g for LC formulations, in 100 mL water;
- approximately 0.900 g weighed to the nearest 0.001 g for LC2 formulations, in 200 mL water.

If necessary, dilute the extract to reach an enzyme concentration within the linearity range of the method.

HAAKE or RHOVISC type viscosimeter:

The enzyme has to be diluted in order to be within the 5 - 15 minutes test-measuring interval:

$$\frac{\Delta\,(\text{viscosity decrease})}{\Delta\,\text{time}} = -50 \pm 5\ \text{msec} / \text{min}$$

This corresponds to a 450 - 550 msec decrease during the (approximate) 10 minutes measurement time. It may also be necessary to check that the relative fluidity slope obtained during the analysis is between 0.01 and 0.02.

Note: The enzymatic activity determination is controlled using a software. Some areas have to be completed for each new analysis. In the "density" zone, make sure that 1 is entered rather than the actual product density. All results provided by the software are then expressed in U / g of product.

AMVn type ANTON PAAR viscosimeter:

The enzyme has to be diluted 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

HAAKE or RHOVISC type viscosimeter:

Every day an analysis has to be performed, the syringe and the golden ball have to be calibrated against water (**water blank**) and against the substrate (**substrate blank**), prior to starting any enzymatic analyses serie. At the end of each enzymatic analyses serie, an additional substrate blank can be performed if time allows it.

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

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

AMVn type ANTON PAAR viscosimeter:

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 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, then ethanol then acetone and dry with a flux of nitrogen.

10.2.1. Calibration against water - Water blank determination

HAAKE or RHOVISC type viscosimeter:

Place 5 mL of water in a disposable sterile tube. Sonicate for 5 seconds. Place at 30 °C for 5 minutes.
Using a pinch, place a calibrated ball (0. 1 - 2 mPa.s) in the syringe and insert the syringe piston.
Fill up the syringe with 30 °C water. Remove all air bubbles using the magnet and adjust the volume to 300 µL. Place the syringe in the viscosimeter.
The viscosimeter will perform measurements every 30 seconds. After 15 minutes, or 30 measurements, remove the syringe. The drop time average for the *20 last measurements* for water, labelled Tw, is given by the software for the 5 - 15 minutes time interval (value equal to about 4 s).

Perform two water blanks and use the average of the two obtained values.

AMVn type ANTON PAAR viscosimeter:

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, and 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 (7.1.) - Substrate blank determination

HAAKE or RHOVISC type viscosimeter:

Place 4.8 mL of 0.25 % arabinoxylan (7.1.) in a disposable sterile tube. Sonicate for 5 seconds. Place at 30 °C for 5 minutes.
Add 0.2 mL of 30 °C water to the substrate (final concentration = 0.24 % w/V). Mix using a vortex.
Using a pinch, place a calibrated ball (0. 1 - 2 mPa.s) in the syringe and insert the syringe piston.
Fill up the syringe with the arabinoxylan solution (final concentration = 0.24 % w/V) at 30 °C. Remove all air bubbles using the magnet and adjust the volume to 300 µL. Place the syringe in the viscosimeter.
The viscosimeter will perform measurements every 30 seconds. After 15 minutes, or 30 measurements, remove the syringe. The drop time average for the 20 last measurements for the substrate, labelled Ts, is given by the software for the 5 - 15 minutes time interval. This value should be within the range 8800 - 9800 ms (*Note: depending upon the syringe used, this value can be outside the given range*). If Ts is too low, discard the substrate and prepare a new arabinoxylan solution (7.1.).

Note: The blank substrate should give a linear regression with a viscosity reduction maximum of - 5 msec / min. A lower value may indicate a contamination of the substrate or of the measurement equipment by an enzyme.

AMVn type ANTON PAAR viscosimeter:

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

HAAKE or RHOVISC type viscosimeter:

Place 4.8 mL of 0.25 % wheat arabinoxylan solution (7.1.) in a disposable sterile tube. Sonicate for 5 seconds. Place in a thermostated water bath at 30 °C and leave to equilibrate for 5 minutes.
Using a pinch, place a calibrated ball (0. 1 - 2 mPa.s) in the syringe and insert the syringe piston.
Add 0.2 mL of the enzymatic preparation to the 30 °C incubated substrate. Start the stopwatch. Mix using a vortex and collect the mixture using the syringe. Remove all air bubbles using the magnet and adjust the volume to 300 µL. Place the syringe in the viscosimeter after exactly 1 minute.
The viscosimeter will perform a measurement every 30 seconds. After 15 minutes, or 30 measurements, remove the syringe.
At the end of the analysis, clean thoroughly the syringe and the ball.

In order for the relative fluidity slope to be comprised between 0.01 and 0.02, the outflow reduction between 5 and 15 minutes should be within the 450 to 550 msec range.

AMVn type ANTON PAAR viscosimeter:

Place 4.8 mL of 0.25 % wheat arabinoxylan solution (7.1.) in a disposable sterile tube. Sonicate for 5 seconds then 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, and then dry it.

11. Calculation

11.1. Determination of the enzymatic activity

Fr	=	relative fluidity
Tw	=	drop time average for the water (msec)
Ts	=	drop time average 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, find out the activity: the obtained slope is proportional to the enzymatic activity present in the sample.

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

⑤ Determine the final activity

$$Activity\ (U/g) = \text{Raw output activity}(U/g) \times CF$$

11.2. Determination example

HAAKE or RHOVISC type viscosimeter:

Density	=	1.110 g/mL
Calibrated ball	=	3.175 mm (0.1 - 2 mPa.s)
Enzyme dilution	=	0.6808 g in 100 mL, then 1 in 100 mL (D = 14689)
Tw	=	4158 msec
Ts	=	9184 msec
Slope Fr	=	0.0149
CF	=	1.334
Activity	=	0.0149 x 14689 x (5/0.2) x 1.334
	=	**7301 U / g**
	=	**8104 U / mL**

Remark: this calculation is automatically performed by the viscosimeter software

AMVn type ANTON PAAR viscosimeter:

Calibrated ball	=	1.5 mm (steel 1.4125)
Enzyme dilution	=	0.6669 g in 250 mL then 0.77 in 200 mL (D = 97369)
Tw	=	12354 msec
Ts	=	26815 msec
Slope Fr	=	0.0105
CF	=	1.334
Activity	=	0.0105 x 97369 x (5/0.2) x 1.334
	=	**34103 U / g**

11.3. Results interpretation

HAAKE or RHOVISC or AMVn type ANTON PAAR viscosimeter:
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

HAAKE or RHOVISC type viscosimeter:

Substrate blank: the viscosity reduction maximum that can be obtained during the calibration is - 5 msec / min. Lower values could indicate a contamination of the substrate or of the syringe by an enzyme. Clean thoroughly the syringe and repeat the calibration. If the problem remains, prepare a new substrate solution.

AMVn type ANTON PAAR viscosimeter:

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 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

- 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 for example

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