COEI-1-XYLANA Determination of endo-1,4- □-xylanase activity in enzymatic preparations

General specifications

Hemicellulases are generally present in enzymatic preparations among other activities within an enzymatic complex. Unless otherwise stated, the specifications must be compliant with Resolution OIV-OENO 365-2009 on the general specifications of enzymatic preparations that appear in the *International Oenological Codex*.

1. Origin and application

Hemicellulases catalyse the degradation of hemicelluloses. The hemicelluloses of the cell walls of grape berries are principally composed of xyloglucans and arabinoxylans; these two polysaccharides constitute almost 90% of grape hemicelluloses.

The hemicellulase activity of enzymatic preparations is evaluated by measuring the 1,4- □-xylanase activity.

Enzymatic preparations containing hemicellulase activities are used during grape maceration, and in the clarification and improvement of the filterability of musts and wines.

Enzymatic preparations containing these activities are derived from the managed fermentation of, for example, *Aspergillus* sp. or *Trichoderma* sp., or mixtures of enzymes thus obtained.

2. Scope of application

The method of determination was developed using commercial xylanase. The conditions and the method were developed for use with commercial enzymatic preparations such as those available on the market of oenological products.

3. Principle

Xylanases hydrolyse xylan chains and thus liberate the constitutive monosaccharides at the reducing ends. The measurement of the xylanase activity is estimated by measuring the reducing monosaccharides (xylose) liberated during the incubation period, according to the Nelson method (1944). In the alkaline environment the pseudo aldehyde groups of the sugars reduce the cupric Cu^{2+} ions. These ions react with the arsenomolybdate reagent, giving it a blue colouring, for which the absorbance – measured at 520 nm – varyies in a linear manner with the monosaccharide

concentration (between 0 and 400 µg/mL).

4. Apparatus

4.1. Magnetic-stirrer hotplate
4.2. Water bath at 40°C
4.3. Water bath at 100°C
4.4. 100-mL Cylindrical flask
4.5. Centrifuge compatible with 15-mL glass tubes
4.6. Stopwatch
4.7. 100-mL Calibrated flasks
4.7.1. 500-mL Calibrated flask
4.8. 200-µL Precision syringe
4.8.1. 1-mL Precision syringe
4.9. 10-mL Straight pipette calibrated with graduations at 0.1-mL intervals
4.10. Spectrophotometer
4.11. 15-mL Glass tubes
4.12. Vortex-type stirrer
4.13. 500-mL Brown-glass flask
4.14. Chamber at 4°C
4.15. Oven at 37°C
4.16. Cotton wool
4.17. Kraft paper
4.18. pH Meter
4.19. Metal tray for 15-mL tubes
4.20. Single-use spectrophotometer cuvettes with a 1-cm optical path, for measurement in the visible spectrum

5. Products

5.1. Sodium acetate (pure CH_3COONa at 99% - PM = 82 g/mol
5.2. Acetic acid (pure CH_3COOH at 96% - PM = 60 g/mol, density = 1.058)
5.3. Xylan (beechwood) P-XYLNBE-10G, Lot No. 171004a, Megazyme
5.4. Sodium sulphate anhydrous (pure Na_2SO_4 at 99.5% - PM = 142 g/mol)
5.5. Sodium carbonate anhydrous (pure Na_2CO_3 at 99,5% - PM = 105.99 g/mol)

5.6. Potassium sodium tartrate (pure $KNaC_4H_4O_6 \cdot 4H_2O$ at 99% - PM = 282.2 g/mol)

5.7. Sodium hydrogen carbonate anhydrous (pure $NaHCO_3$ at 98% - PM = 84.01 g/mol)

5.8. Copper sulphate pentahydrate (pure $CuSO_4 \cdot 5H_2O$ at 99% - PM = 249.68 g/mol)

5.9. Concentrated sulphuric acid (pure H_2SO_4 at 98%)

5.10. Ammonium heptamolybdate (pure $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ at 99% - PM = 1235.86 g/mol)

5.11. Sodium hydrogen arsenate (pure $Na_2HAsO_4 \cdot 7H_2O$ at 98.5% - PM = 312.02 g/mol)

5.12. D-xylose (pure $C_5H_{10}O_5$ at 99% - PM = 150 g/mol)

5.13. Distilled water

5.14. Commercial enzymatic preparation for analysis

6. Solutions

6.1. Reagents for the oxidising solution

These reagents should be prepared first, considering the 24-hour time limit for solution D.

6.1.1. Solution A

Successively place in a 100-mL cylindrical flask (4.4):

- 20 g sodium sulphate anhydrous (5.4),
- 2.5 g sodium carbonate anhydrous (5.5),
- 2.5 g potassium sodium tartrate (5.6),
- 2 g sodium hydrogen carbonate anhydrous (5.7).

Dissolve in 80-mL distilled water (5.13). Heat and mix (4.1) until dissolution and transfer to a 100-mL flask (4.7). Make up to the calibration mark with distilled water (5.13).

Store at 37 °C (4.15); if a deposit forms, filter through fluted filter.

6.1.2. Solution B

Dissolve 15 g copper sulphate pentahydrate (5.8) in 100 mL distilled water (5.13) and add a drop of concentrated sulphuric acid (5.9).

6.1.3. Solution C

This solution is prepared just before use in order to have good proportionality

between the colour density and quantity of glucose by mixing 1 mL solution B (6.1.2) with 24 mL solution A (6.1.1).

6.1.4. Solution D

In a 500-mL calibrated flask (4.7.1), dissolve 25 g ammonium heptamolybdate (5.10) in 400 mL water (5.13). Add 25 mL concentrated sulphuric acid (5.9) (cooled under a flow of cold water).

In a 100-mL cylindrical flask (4.4), dissolve 3 g sodium hydrogen arsenate (5.11) in 25 mL water (5.13) and quantitatively transfer to a 500-mL calibrated flask (4.7.1) containing ammonium molbydate (5.10).

Make up to the mark with water (5.13) to obtain a final volume of 500 mL.

Place at 37 °C (4.15) for 24 hours then store at 4°C (4.14) in a 500-mL brown-glass flask (4.13).

6.2. Sodium acetate buffer (pH 4.2, 100 mmol/L)

It is made up of solutions A and B.

6.2.1. Solution A (0.1 M sodium acetate): dissolve 0.5 g sodium acetate (5.1) in 60 mL distilled water (5.13).

6.2.2. Solution B (0.1 M acetic acid): dilute 1 mL acetic acid (5.2) with 175 mL distilled water (5.13)

6.2.3. Preparation of sodium acetate buffer: mix 23.9 mL solution A (6.2.1) + 76.1 mL solution B (6.2.2).

Verify the pH o the buffer using a pH meter (4.18).

The solution must be stored at 4 °C (4.14).

6.3. 2% Oat-spelt xylan solution (p/v)

In a 100-mL calibrated flask (4.7), dissolve 1 g oat-spelt xylan (5.3) in 100 mL sodium acetate buffer (6.2).

6.4. Xylose stock solution at 400 µg/mL

Dissolve 0.040 g D-xylose (5.12) in 100 mL distilled water (5.13).

7. Preparation of xylose calibration range

Prepare the calibration range (from 0 to 400 µg/mL) based on the xylose stock solution (6.4) as presented in Table 1.

Table 1: Xylose calibration range

Xylose (µg/mL)	0	50	100	150	200	250	300	400

Xylose (µmol/mL)	0	0.232	0.462	0.694	0.925	1.156	1.387	1.890
Vol. stock solution (µL) (6.4.)	0	125	250	375	500	625	750	1000
Vol. distilled water (µL) (5.13)	1000	875	750	625	500	375	250	0

8. Sample preparation

It is important to mix the enzymatic preparation before sampling, by inverting the container, for example. The enzymatic solution and the blanks should be prepared just before use.

8.1. Enzymatic solution 2 g/L

Place 200 mg of enzymatic preparation (5.14) in a 100-mL calibrated flask (4.7), make up to the mark with distilled water (5.13) and stir in order to obtain a homogenous mixture.

8.2. Heat-denatured blank

Place 10 mL enzymatic solution at 2 g/L (8.1) in a 15-mL tube (4.11) stoppered with cotton wool (4.16) covered with Kraft paper (4.17) and immerse the tube in the water bath at 100 °C for 5 min (4.3).

9. Procedure

9.1. Enzymatic reaction

Prepare the tubes in duplicate at the minimum.

In 5 x 15-mL tubes (4.11) numbered from 1 to 5 and placed in a tray (4.19),

use the 200- µL precision syringe (4.8) to add 200 µL enzymatic solution at 2 g/L (8.1), then

use the 1-mL precision syringe (4.8.1) to add 400 µL sodium acetate buffer (6.2) and

600 µL 2% oat-spelt xylan (6.3), and start the stopwatch (4.6)

After stirring (4.12), place the tubes stoppered with cotton wool (4.16) and Kraft paper (4.17) in the water bath at 40 °C (4.2):

- for 1 min for tube 1,
- for 2 min for tube 2,
- for 5 min for tube 3,
- for 10 min for tube 4,

- for 20 min for tube 5.

The reaction is stopped by placing each of the tubes numbered from 1 to 5 immediately in the water bath at 100 °C (4.3) for 10 min after they have been removed from the water bath at 40 °C.

Cool the tubes under a current of cold water.

9.2. Determination of liberated reducing substances (xylose in this case)

In a 15-mL tube (4.11),

place 1 mL reaction medium (9.1),

add 1 mL solution C (6.1.3),

after stirring (4.12), place the tube in a water bath at 100°C (4.3) for 10 min.

Then cool the tube under a current of cold water.

Add 1 mL solution D (6.1.4),

add 9.5 mL water (5.14) using the10-mL straight pipette (4.9)

wait 10 min for colour stabilisation.

Centrifuge (4.5) each of the tubes at 5000 rpm for 10 min.

Place the supernatant in a cuvette (4.20).

Immediately measure the absorbance at 520 nm, using a spectrophotometer (4.10).

9.3. Blanks

Proceed as described in 9.1, replacing the enzymatic solution at 2 g/L (8.1) by the heat-denatured blank (8.2). Ideally perform the enzymatic reaction of the blanks at the same time as that of the enzymatic solution.

9.4. Calibration range

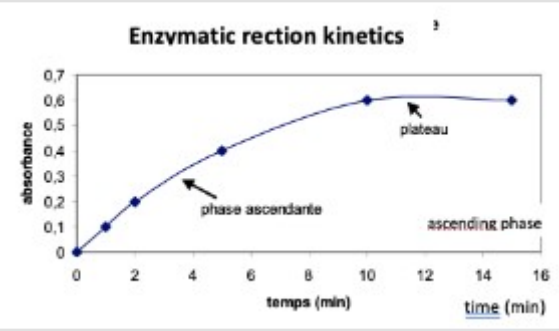
Proceed as described in 9.2, replacing the reaction medium (9.1) by the different media of the xylose calibration range from 0 to 400 µg/mL (7).

10. Calculation

10.1. Kinetics

Generally, calculation of enzymatic activity may only be carried out when the substrate and the enzyme are not in limited quantities. This therefore refers to the ascending phase of the representation of kinetics: the enzymatic activity is linear over time. Otherwise, the activity would be underestimated (Figure 1).

Figure 1: Enzmatic reaction kinetics



Determine the kinetics over 15 min. Measure the activity concerned at T=1 min T=2 min, T=5 min, T=10 min and T=15 min.

After determining the enzymatic reaction kinetics, plot the curve for the absorbance variation in relation to the reaction time. The absorbance corresponds to the difference between the absorbance at time T of the enzymatic preparation and of the corresponding blank.

Then calculate the equation (1) of the regression line, considering the points of the ascending phase (see Figure 1).

10.2. Calibration line

For the calibration line, plot a graph showing the different concentrations of the xylose calibration range (0-1,89 µmol/mL) as the abscissa and the corresponding optical density values as the ordinates, obtained in 9.4. Then calculate the slope (Q/T) of the regression line (2) resulting from the linearity of the graph data.

10.3. Calculation of enzymatic activity

Based on the regression line (1), calculate the absorbance for a mean time, T (e.g. 4 min in the case of Figure 1), by deducing from it quantity Q of xylose released (in micromoles) for this intermediary time using equation (2).

The enzymatic activity in U/g of preparation is calculated as follows:

$$\text{Activity in U/g} = 1000 \times (Q/T)/(V \times C)$$

Where

- Q: quantity of xylose released in µmols during time T (min),
- V: quantity of enzymatic solution introduced (mL) – 0.2 mL in this case,
- C: concentration of enzymatic solution (g/L) – 2 g/L in this case.

The enzymatic activity in nanokatals:

$$\text{Activity in nkat/g} = (\text{acitivity in U/g}) \times (1000/60)$$

This unit corresponds to the number of nanomoles of product formed per second.

11. Method characteristics

r= 0.056

R= 0.056

S_r= 0.02

S_R= 0.02

The repeatability of the method is estimated using the mean standard deviation of the absorbance values derived from the same sampling of the enzymatic preparation, determined 5 times. Therefore, for the determination of xylanase the mean standard deviation of the values is 0.02 with a percentage of error of 9.7%. The % error corresponds to the following:

$$\frac{\text{mean standard deviation of values} \times 100}{\text{mean test value}}$$

As such, the method of determination as presented is deemed repeatable.

The reproducibility tests were carried out using 2 enzymatic preparations with 5 samplings for each one.

There were 2 tests used in order to determine the satisfactory reproducibility of the method:

Variance analysis (study of the probability of the occurrence of differences between samplings). Variance analysis is a statistical method that makes it possible to test the hypothesis of homogenity of a series of k-means. Carrying out variance analysis consists of determining whether the 'treatment' effect is 'significant or not'.

The power of the test for type I □-risk (5%). The type I □ risk is the risk of concluding that the identical treatments are in fact different.

If the power is low (≅ 20%), this means that no difference has been detected between treatments, yet there is little chance of seeing a difference if one really does exist.

If the power is high (≅ 80%), this means that no difference has been detected between treatments, however, if one does exist, the means are available to see it.

The results are given in Table 2.

Table 2: Variance analysis – study of the sampling effect					
Determination	Variance analysis hypotheses	Probability	Test power (□ = 5%)	Newman-Keuls test (*)	Bonferroni test (**)
Xylanase	Respected	0.00087	93%	Significant	Significant

* Newman-Keuls test: this comparison test of means makes it possible to constitute homogenous treatment groups: those belonging to the same group are considered as not being different to the given type I □-risk.

** Bonferroni test: also called the 'corrected t-test', the Bonferroni test makes it possible to carry out all comparisons of pairs of means, i.e. (t (t-1))/2 comparisons before treatments, respecting the given type I □-risk.

Therefore, the tests put in place make it possible to see a difference if there really is one (high test power); in addition, the method of determination shows a probability of occurrence of differences in activity (between samplings) of less than 5%, strengthened by membership of the same group (Newman-Keuls test non-significant) and considered as not being different to the given type I □-risk (Bonferroni test non-significant).

12. Bibliographic references

- Nelson, N., 'A photometric adaptation of the SOMOGYI method for the determination of glucose', *Journal of Biological Chemistry,* May Institute for Medical Research of the Jewish Hospital, vol. 153, 1944, pp. 375-380.
- Doco, T., *et al.*, 'Polysaccharides from grape berry cell walls. Part II. Structural characterization of the xyloglucan polysaccharides', *Carbohydrate Polymers*, vol. 53, Issue 3, 15 August 2003, pp. 253-261.