

**DETERMINATION OF GALACTANASE ACTIVITY
IN ENZYMATIC PREPARATIONS
ENDO – (1.4) – B-GALACTANASE
(EC 3.2.1.89 – CAS no. 58182-40-4)
(OIV-Oeno 313-2009, Oeno 490-2012)**

These enzymes are generally present among other activities, within an enzyme complex. Unless otherwise stipulated, the specifications must comply with resolution Oeno 385-2012 concerning the general specifications for enzymatic preparations" included in the International Oenological Codex.

1. Origin

Reference is made to paragraph 5 "Source of enzyme and fermentation environment" of the general monography on enzymatic preparation

The enzymatic preparations containing galactanase activities are produced by directed fermentations, for example, of *Aspergillus niger*, *Disporotrichum dimorphosporum*, *Penicillium sp.* or *Talaromyces emersonii*.

2. Scope/Applications

Reference is made to the International Code of Oenological Practices, OENO 11/04; 12/04; 13/04; 14/04 and 15/04.

Galactanases are catalysing the hydrolysis of rhamnogalacturonan I galactan type-I (RG-I galactan). They are useful for the maceration of grapes. This activity can be estimated by the hydrolysis of potato galactan.

3. Principle

The galactanases cut the galactan chains (eg. potato galactan), thereby releasing the reducing ends of the constitutive sugars. Measurement of the galactanase activity is based on determination the galactose according to the NELSON method (1994). In an alkaline medium, the

pseudoaldehydic group of sugars reduces the cupric ions Cu^{2+} . The latter react with the arsenomolybdic reagent producing a blue colour, whose absorbance, measured at 520 nm, varies linearly with the concentration in starch hydrolysates (between 0 and 400 $\mu\text{g}/\text{mL}$). The determination method was developed using a commercially available endo-(1.4)- β -galactanase.

4. Apparatus

- 4.1 Heating magnetic stirrer
- 4.2 water bath at 40 °C
- 4.3 water bath at 100 °C
- 4.4 100-mL cylindrical flask
- 4.5 centrifuge capable of housing 15-mL glass test tubes
- 4.6 chronometer
- 4.7 100-mL graduated flask
- 4.7.1 500-mL graduated flask
- 4.8. 200- μL precision syringe
 - 4.8.1 1-mL precision syringe
- 4.9 10-mL straight pipette graduated to 1/10 mL
- 4.10 spectrophotometer
- 4.11 15-mL glass test tubes
- 4.12 shaker of the vortex type
- 4.13 500-mL amber glass bottle
- 4.14 chamber at 4 °C
- 4.15 drying oven at 37 °C
- 4.16 carded cotton
- 4.17 Kraft paper
- 4.18 pH-meter
- 4.19 metal rack for 15-mL test tubes
- 4.20 cuvetts with a 1-cm optical path length, for single use, for spectrophotometer, for measurement in the visible spectrum

5. Reagents and products

- 5.1 Sodium acetate (CH_3COONa 99 % pure - PM = 82g/mole)
- 5.2 acetic acid (CH_3COOH 96 % pure - PM = 60 g/mole, density= 1.058)
- 5.3 potato galactan (Megazyme, batch 71201) as an example. If this substrate is not available alternative substrates must be validated for this essay.
- 5.4 anhydrous sodium sulphate (Na_2SO_4 99.5 % pure - PM = 142

g/mole)

5.5 anhydrous sodium carbonate (Na_2CO_3 99.5 % pure - PM = 105.99 g/mole)

5.6 sodium and potassium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ 99 % pure - PM = 282.2 g/mole) 5.7 anhydrous sodium hydrogenocarbonate (NaHCO_3 98 % pure - PM = 84.01 g/mole)

5.8 penta-hydrated copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 99 % pure - PM = 249.68 g/mole)

5.9 concentrated sulphuric acid (H_2SO_4 98 % pure)

5.10 ammonium heptamolybdate ($(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 99 % pure - PM = 1235.86 g/mole) 5.11 sodium hydrogenarsenate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ 98.5 % pure - PM = 312.02 g/mole) g/mole). Given the toxicity of this product, special attention must be paid during manipulation. Waste material must be treated in an appropriate manner.

5.12 D-galactose ($\text{C}_6\text{H}_{12}\text{O}_6$ 99 % pure - PM = 180.16 g/mole)

5.13 distilled water

5.14 commercial enzymatic preparation for analysis.

6. Solutions

6.1 Reagents of the oxidizing solution

These reagents must be prepared first, taking into account the 24 hours lead-time for solution D.

6.1.1 Solution A: place in a 100-mL cylindrical flask (4.4) successively

20 g of anhydrous sodium sulphate (5.4)

2.5 g of anhydrous sodium carbonate (5.5)

2.5 g of sodium and potassium tartrate (5.6)

2 g of anhydrous sodium hydrogenocarbonate (5.7)

Dissolve in 80 mL of distilled water (5.13). Heat (4.1) until dissolution and decant into a 100-mL flask (4.7). Make up to the gauge line with distilled water (5.13). Maintain at 37 °C (4.15); if a deposit is formed, filter using a folded filter.

6.1.2 Solution B:

Dissolve 15 g of penta-hydrated copper sulfate (5.8) in 100 mL of distilled water (5.13) and add a drop of concentrated sulphuric acid (5.9).

6.1.3 Solution C:

This solution is prepared extemporaneously in order to have a

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

6.1.4 Solution D:

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

In a 100-mL cylindrical flask (4.4), dissolve 3 g of sodium hydrogenarsenate (5.11) in 25 mL of water (5.13) and quantitatively transfer into the 500-mL graduated flask (4.7.1) containing ammonium molybdate (5.10).

Make up with water (5.13) to have a final volume of 500 mL.

Place at 37°C (4.15) for 24 hours then maintain at 4 °C (4.14) in a 500 mL amber glass bottle (4.13).

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

It consists of solutions A and B.

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

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

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

Check the pH of the buffer using a pH-meter (4.18).

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

6.3 Solution of potato galactan at 1 % (p/v)

In a 100-mL graduated flask of (4.7) dissolve 1 g of potato galactan (5.3) in 100 mL of sodium acetate buffer (6.2).

6.4 Stock solution of Galactose with 400 µg/mL

Dissolve 0.040 g of galactose (5.12) in 100 mL of distilled water (5.13).

7. Preparation of the standard range of galactose

A standard range is produced using the stock solution of galactose (from 0 to 400 µg/mL) (6.4) as indicated in table 1.

Galactose ($\mu\text{g/mL}$)	0	50	100	150	200	250	300	400
Galactose ($\mu\text{mole/mL}$)	0	0.278	0.555	0.833	1.110	1.388	1.665	2.220
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

Table 1. Standard range of galactose

8. Preparation of the sample

It is important to homogenise the enzymatic preparation before sampling, by upturning the container for example. The enzymatic solution and the blanks will have to be prepared at time of use.

8.1 Enzymatic solution with 2 g/L to be prepared just before use.

Place 200 mg of commercial preparation (5.14) in a 100-mL graduated flask (4.7), make up with distilled water (5.13), and stir in order to obtain a homogeneous mixture.

8.2. Blank denatured by heating to be prepared just before use

Place 10 mL of the enzymatic solution at 2 g/L (8.1) in a 15 mL test tube (4.11), plug with carded cotton (4.16) covered with Kraft paper (4.17) and immerse the test tube for 5 minutes in the water bath at 100 °C (4.3). Chill and centrifuge for 5 min at 6500 g.

9. Procedure

9.1 Enzymatic kinetics: The test tubes are produced at least in duplicate.

In 5 x 15-mL test tubes (4.11) numbered from 1 to 5, placed in a rack (4.19) in a water bath at 40 °C, introduce

200 μL of the enzymatic solution at 2 g/L (8.1), using the precision syringe (4.8),

400 μL of sodium acetate buffer (6.2), using the precision syringe

(4.8.1),

600 µL of potato galactan (6.3) beforehand warmed at 40 °C in water bath, start the chronometer (4.6)

After shaking (4.12), the test tubes plugged with carded cotton (4.16) and Kraft paper (4.17) are replaced in the water bath at 40 °C (4.2) for 1 min for test tube no.1
for 2 min for test tube no.2
for 5 min for test tube no.3
for 10 min for test tube no.4
for 15 min for test tube no.5.

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

The test tubes are then cooled under running cold water.

Note: Kinetic point at 10 min enables the evaluation of sought after enzymatic activity

9.2 Determination of the reducing substances released

In a 15-mL test tube (4.11)

Place 1 mL of the reaction medium (9.1)

Add 1 mL of solution C (6.1.3)

After shaking (4.12), the test tube is placed in the water bath at 100 °C (4.3) for 10 min. The test tube is then cooled under running cold water.

Add 1 mL of solution D (6.1.4)

Add 9.5 mL of water (5.14) using the straight 10 mL pipette (4.9)

Wait 10 min for the colour to stabilise.

Centrifuge (4.5) each test tube at 2430 g for 10 min.

Place the supernatant liquid in a tank (4.20).

Zero the spectrophotometer using distilled water.

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 blank denatured by heat (8.2). For each kinetic point, the enzymatic reaction of each blank is carried out at the same time as that of the enzymatic solution.

9.4 Standard range

Proceed as described in 9.2, replacing the reaction medium (9.1) by the various mediums of the standard range of galactose from 0 to 400 µg/mL (7).

10. Calculations

10.1 Determining the kinetics

In general, calculating the enzymatic activity can only be done when the substrate and the enzyme are not in limiting quantities. This therefore refers to the ascending phase of the kinetic representation: the enzymatic activity is linear in time. Otherwise, the activity would be underestimated (Figure 1).

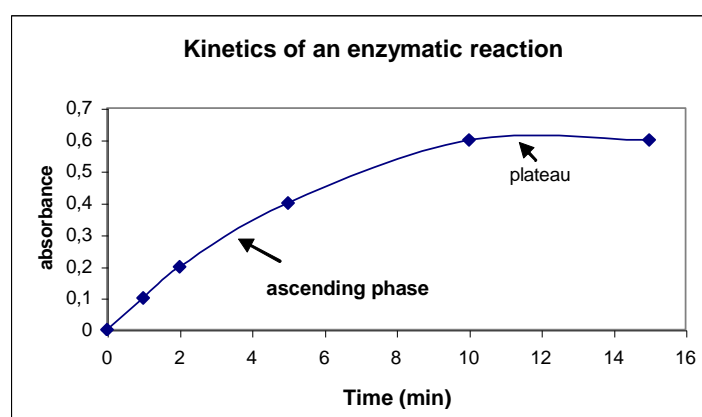


Figure 1. Kinetics of an enzymatic reaction

The kinetics are determined over 15 minutes. The activity concerned is measured at T=1 min, T=2 min, T=5 min, T=10 min, T=15 min.

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

Then calculate the equation (1) of the straight regression line, taking

into account only the points of the ascending phase (see figure 1).

10.2 Producing the calibration line

The calibration line corresponds to plotting a graph whose X-coordinates are the various concentrations of the standard range of galactose (from 0 to 0.693 $\mu\text{mol/mL}$) and whose Y-coordinates are the corresponding values of optical densities, obtained in 9.4. Then calculate the straight regression line (2) resulting from the linearity of the data of the graph.

10.3 Calculating the enzymatic activity

Based on the straight regression line (1) calculate the absorbance for an average time T (for example 4 min. in the case of figure 1) deduct from it the quantity Q of galactose released (in μmoles) for this intermediate time using equation (2).

The formula used to calculate the enzymatic activity in U/g of the preparation is as follows

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

Where Q: quantity of galactose released in μmol during time T (min)

V: quantity of enzymatic solution introduced (mL)
here 0.2 mL

C: concentration of the enzymatic solution (g/L)
here 2 g/L

It is then possible to express the enzymatic activity in nanokatals. This unit corresponds to the number of nanomoles of product formed per second under the conditions defined by the determination protocols and therefore:

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

11. Characteristics of the method

$$r= 0,056$$

$$R= 0,056$$

$$Sr= 0,02$$

$$SR= 0,02$$

The repeatability of the method is estimated using the mean standard deviation of the absorbance values resulting from the same sampling of the enzymatic preparation, proportioned 5 times. In this way, to proportion the galactanase the mean standard deviation of the values is 0.02 with a percentage error of 9.7, in which the % error corresponds to:

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

In this way, the determination method as presented is considered repeatable.

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

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

- variance analysis (the study of the probability of the occurrence of differences between samplings). Variance analysis is a statistical method used to test the homogeneity hypothesis of a series of K averages. Performing the variance analysis consists in determining if the "treatment" effect is "significant or not". The standard deviation of reproducibility given by this variance analysis is 0.02.
- the power of the test for the first species of risk α (5 %) – first species of risk α is the risk of deciding that identical treatments are in fact different.

If the power is low (\cong 20 %), this means that no difference has been detected between treatments, but there is little chance of seeing a difference if one did in fact exist.

If the power is high ($\cong 80\%$), this means that no difference has been detected between the treatments, but, if there was one, we have the means of seeing it.

The results are given in table 2.

Determination	Variance analysis hypotheses	Probability	Power of test ($\alpha= 5\%$)	Newman-Keuls test (*)	Bonferroni test (**)
Galactanase	Adhered to	0.00087	93 %	Significant	Significant

Table 2: Variance analysis – study of the sampling effect

* Newmann-Keuls test: this comparison test of means is used to constitute homogeneous groups of treatments: those belonging to the same group are regarded as not being different to risk α of the first species selected

** Bonferroni test: also referred to as the "corrected T test", the Bonferroni test is used to carry out all the comparisons of pairs of means, i.e., $(t(t-1))/2$ comparisons before treatments, respecting the risk α of the first species selected.

In this way, the tests set up are used to see a difference if there really is one (high power test); in addition, the determination method involves a probability of occurrence of a discrepancy in activity (between samplings) lower than 5%, reinforced by belonging to the same group (Newmann-Keuls test not significant) and considered not to be different to the first species of risk α (Bonferroni test not significant).

12. Bibliography

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