# RESOLUTION OIV/OENO 340/2010

# DETERMINATION OF BETA-GLUCANASE (ß 1-3, ß 1-6) ACTIVITY IN ENZYME PREPARATIONS

THE GENERAL ASSEMBLY,

IN VIEW of article 2, paragraph 2 iv of the agreement dated 3rd April 2001 by which the Inter-national Organization of Vine and Wine was founded,

HAVING LEARNED of the work carried out by the "Specifications of oenological products" group of experts,

HAS HEREBY DECIDED, following a proposal made by Commission II "Oenology", to add the following analytical and control techniques to Section II of the International Oenological Codex:

# DETERMINATION OF BETA-GLUCANASE (ß 1-3, ß 1-6) ACTIVITY IN ENZYME PREPARATIONS

## 1. SCOPE

Raw materials containing enzymes characterised by □-glucanase activity, as well as commer-cial preparations.

## 2. PRINCIPLE

The analytical method is based on measuring the glucose released by the enzyme, the activity of which is to be measured, using a standardised solution of Schizophyllum sp. glucan.

### 2.1. Definition of units

A unit of □-glucanase (□-Glu-U) is defined as the quantity of reducing sugars, expressed as glucose, released in test conditions by 1 g (or 1 ml) of enzyme per minute.

### 2.2. Role of the enzyme

As it grows on infected grapes (as noble or grey rot) Botrytis cinerea excretes a beta-1,3-glucan which, at every third unit of glucose, possesses a □-1,6 glycosylated



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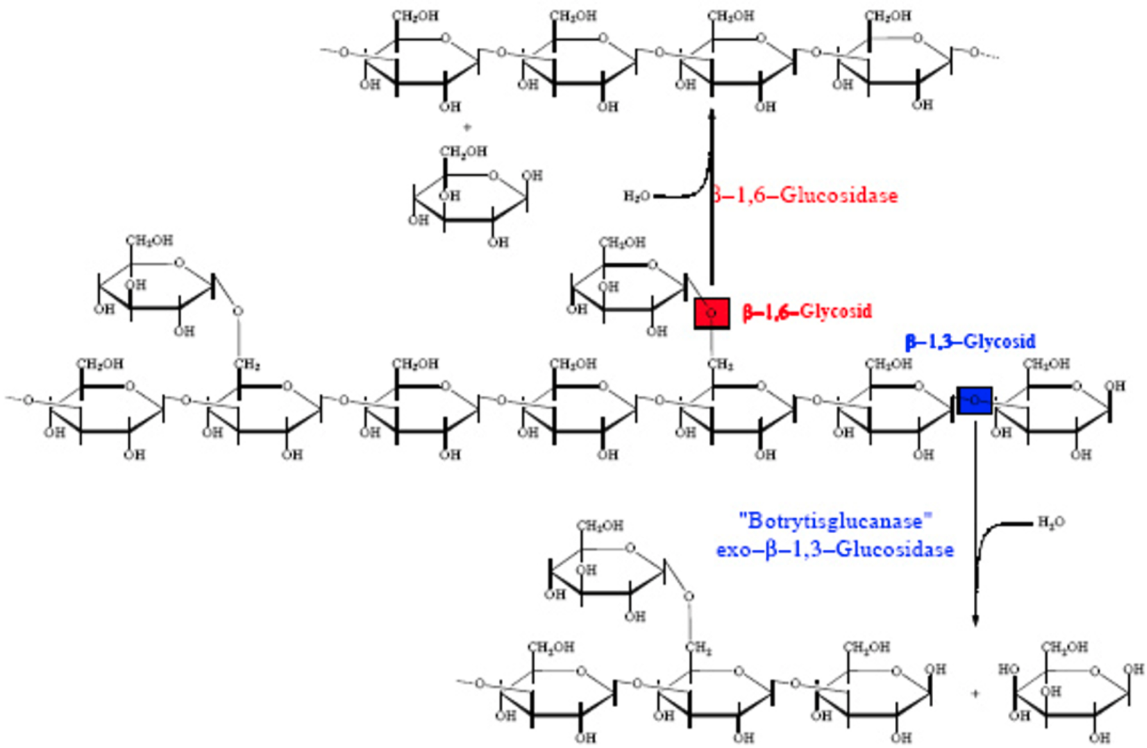
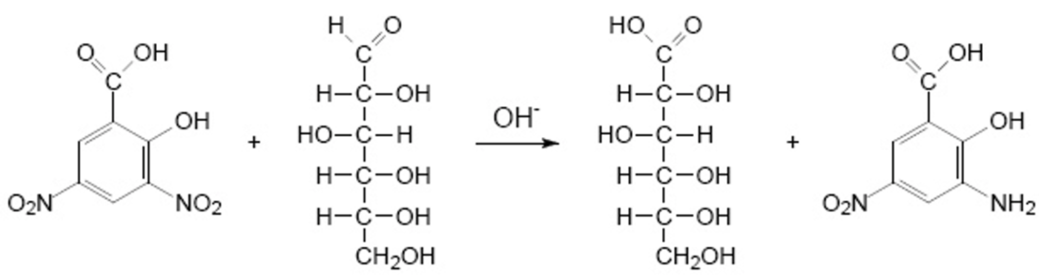
residue of glucose. (Fig. 1) This glucan is very similar to glucan synthetised by Schizophyllum sp.




## 2.3. Principle of measurement

The enzymatic activity releases glucose which, in an alkaline salt solution, reduces 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The addition of phenol increases the sensi-tivity of the reaction. Sodium bisulphite serves to stabilise colour.



# 3. APPARATUS

3.1. Spectrophotometer and cuvettes with an optical path length of 1 cm
3.2. 40°C, 100°C water bath
3.3. Standard magnetic stirrer
3.4. Submersible multi-point magnetic stirrer set at 300 rpm
3.5. Measuring containers (volumetric flasks, beakers, conical flasks, etc.)
3.6. Beaker
3.7. Micro-pipettes
3.8. Timer
3.9. Ultrasonic bath
3.10. pH meter

# 4. REAGENTS AND PRODUCTS

## 4.1. Substrate

Glucan stock solution supplied by the University of Braunschweig[1] , the glucan content of which has been determined by the University of Braunschweig.

## 4.2. Pure products

4.2.1. Citric acid monohydrate (CAS N° 5949-29-1)
4.2.2. Sodium hydroxide (CAS N° 1310-73-2)
4.2.3. Potassium sodium tartrate (CAS N° 304-59-6)
4.2.4.. Sodium metabisulphite $Na_2S_2O_5$ (CAS N° 7681-57-4)
4.2.5. Phenol (CAS N° 108-95-2)
4.2.6. Anhydrous glucose
4.2.7. 3,5-dinitro-2-hydroxybenzoic (3,5-dinitrosalicylic) acid (CAS N° 609-99-4)
4.2.8. Distilled water

## 4.3. Solutions

4.3.1. IM sodium hydroxide solution
In a 100-ml volumetric flask, dissolve 4.0 g of sodium hydroxide (4.2.2) in distilled water (4.2.8) and make up to the required volume.



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4.3.2. Citrate buffer solution (pH 4.0) - 0.2 mol/l

In a 500-ml volumetric flask, dissolve 21.0 g of citric acid monohydrate (4.2.1) in 400 ml of distilled water, then adjust the pH to 4.0 with a molar solution of sodium hydroxide (4.3.1) and make up to the required volume with distilled water (4.2.8).

4.3.3.Citrate buffer solution (pH 4.0) - 0.1 mol/l

In a 1,000-ml volumetric flask, dissolve 21.0 g of citric acid monohydrate (4.2.1) in 900 ml of distilled water (4.2.8), then adjust the pH to 4.0 with a molar solution of sodium hydroxide (4.3.1) and make up to the required volume with distilled water (4.2.8).

4.3.4. Titrating solution: DNS (dinitrosalicylic) acid colour reagent with phenol

This is prepared from solutions A, B and C below:

4.3.4.1. Solution A:

Weigh out 154.2 g of potassium sodium tartrate (4.2.3) in an 800-ml beaker and dissolve completely in 500 ml of distilled water (4.2.8). Add 9.7 g of sodium hydroxide (4.2.2).

4.3.4.2. Solution B:

In a 2,000-ml beaker, completely dissolve 5.3 g of 3,5-dinitrosalicylic acid (4.2.7) in 500 ml of distilled water (4.2.8). The best results are obtained using an ultrasonic bath.

4.3.4.3. Solution C:

In a 100-ml beaker, dissolve 4.2 g of phenol (4.2.5) in 50 ml of distilled water (4.2.8). Then add 1g of sodium hydroxide (4.2.2) and, when completely dissolved, 4.2 g of sodium metabi-sulphite (4.2.4) and dissolve again.

4.3.4.4. 0.3% glucose solution

In a 100-ml volumetric flask, put exactly 300 mg of glucose (4.2.6), dissolve in distilled water (4.2.8) and make up to the required volume with distilled water.

4.3.4.5. DNS acid colour reagent with phenol

Solutions A and C are mixed with solution B in a 2,000-ml beaker, which is then covered with aluminium foil.

Before using, keep in the dark for at least 3 days.

Transfer the reagent to a brown glass container.

If stored in a dark place at 15-20° C, this solution can be kept for a month.

For each newly-prepared reagent and before each measurement, a new calibration is carried out prior to each enzyme analysis.

Before every use, 3 ml of 0.3% glucose solution (4.3.3.4) should be added to 200 ml of the DNS acid colour reagent with phenol.

4.3.5. Glucan in solution at 0.1%, pH 4.0

Weigh out the exact quantity of glucan stock solution (4.1) to obtain a final concentration of 1 g /l.

The final substrate solution should contain 50% of the citrate buffer solution (pH 4.0) - 0.2 mol/l (4.3.2).

To obtain 100 ml of substrate solution from the glucan stock solution (4.1) (actually contain-ing 5.2 g/l), weigh out 19.2 g in a 100-ml beaker. Add 50 ml of the citrate buffer solution (pH 4,0) - 0.2 mol/l (4.3.2). Homogenize the glucan mixture by stirring for at least 15 minutes. When well-mixed, adjust the pH to 4.0 with a sodium hydroxide molar solution (4.3.1). Then transfer the solution to a 100-ml volumetric flask and make up to the required volume later with distilled water (4.2.8).

Store all glucan stock solutions at ambient temperature. If a new glucan stock solution is used, a glucan substrate factor (Gf or G-factor) should be determined by means of the stand-ard enzyme. The "Gf" is essential for comparing the results of previous glucan stock solutions with the new ones. The G-factor is calculated with the values measured by considering that standard enzymatic activity is 10,000 ▯-Glu U/g in the formula (See: Calculation of enzymatic activity).

## 4.4. Enzyme preparations

### 4.4.1. Glucanase standard enzyme solution:

Dissolve 0.5 g of glucanase standard enzyme preparation in 25 ml of the citrate buffer solu-tion (pH 4.0; 0.1 mol/l) (4.3.3) and make up to 100 ml with distilled water (4.2.8).

### 4.4.2. For all other enzyme preparations:

Dissolve 1 ml of enzyme preparation or 0.5 g of solid powdered or granulated enzyme prepa-ration in 25 ml of the citrate buffer solution (pH 4.0; 0.1 mol/l) (4.3.3) and make up to 100 ml with distilled water (4.2.8). If the absorption values are too high or too low, appropriate dilution is necessary. The enzyme dilution should contain 25% of citrate buffer solution (4.3.3).

# 5. PROCEDURE

## 5.1. Reagent “blank” test

Add 7 ml of DNS acid colour reagent with phenol (4.3.4) to 3 ml of distilled water (4.2.8) in a 50-ml volumetric flask and heat for exactly 10 minutes over a bath of boiling water. Cool for 5 minutes in an ice bath, then transfer the flask into a bath of water at 20° C and add distilled water (4.2.8) to just below the mark. After 10 minutes at 20° C, make up to the required vol-ume.

## 5.2. Glucose calibration curve with DNS acid colour reagent with phenol

Dissolve 2.00 g of glucose (4.2.6) in a 200-ml volumetric flask and make up to volume with distilled water (4.2.8). Using this solution, prepare the following dilutions:

| N° | V standard solution / 100 ml | glucose/100 ml | glucose (µg) in the trial (= 0.5 ml) |
|---|---|---|---|
| 1 | 2 ml | 20 mg | 100 µg |
| 2 | 5 ml | 50 mg | 250 µg |
| 3 | 10 ml | 100 mg | 500 µg |
| 4 | 15 ml | 150 mg | 750 µg |
| 5 | 20 ml | 200 mg | 1,000 µg |
| 6 | 30 ml | 300 mg | 1,500 µg |
| 7 | 40 ml | 400 mg | 2,000 µg |

Use a pipette to put 0.5 ml of each glucose dilution into a 50-ml volumetric flask and add 7 ml of DNS colour reagent with phenol (4.3.4) and 2.5 ml of distilled water (4.2.8). Heat the measuring containers for exactly 10 minutes in a bath of boiling water. Cool for 5 minutes in a bath of ice, then transfer the flask to a bath of water at 20° C and add distilled water (4.2.8) to just below the mark. After 10 minutes at 20° C, make up to volume. Measure the absorb-ance of the solutions within the next 15 minutes, using a spectrophotometer with a wave-length of 515 nm against the "blank" (reagent alone).

On a diagram, plot the quantity of glucose released in the test against the absorbance at 515 nm (Fig. 2).

The calibration curve is produced the same day before every enzyme analysis.

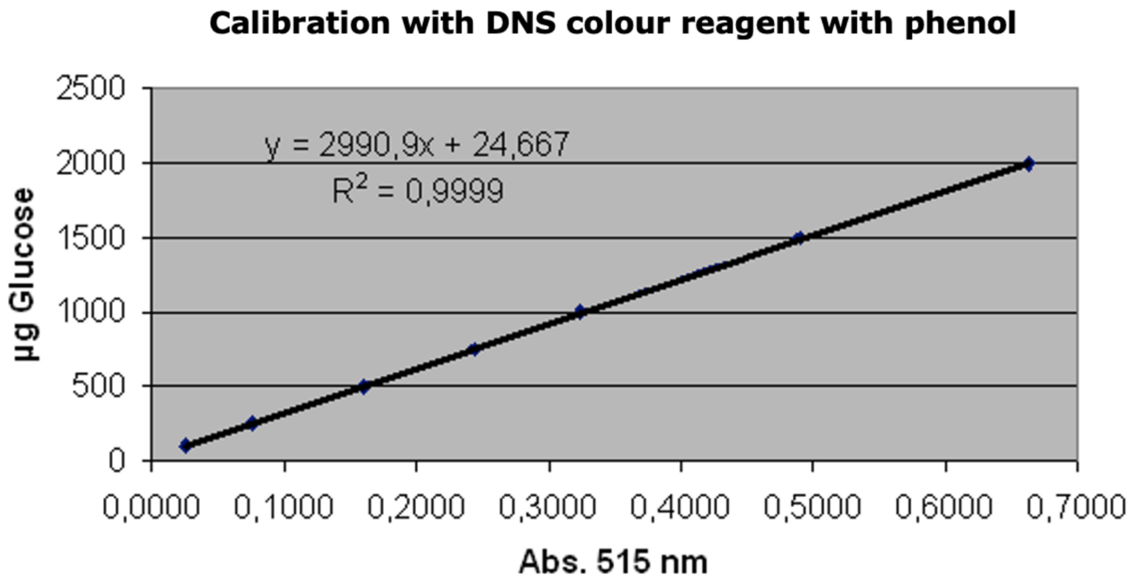


*Figure 2*

## 5.3. "Blank" testing of enzymes

Use a pipette to put 0.5 ml of each enzyme solution (4.4.1 or 4.4.2) into a 50-ml volumetric flask and add 7 ml of DNS acid colour reagent with phenol (4.3.4). Mix carefully and add 2.5 ml of substrate solution (4.3.5). Stir well by hand. Then heat all samples over a bath of boil-ing water for exactly 10 minutes, cool for 5 minutes in a bath of ice and transfer the flask to a bath of water at 20° C and add distilled water (4.2.8) to just below the mark. After 10 minutes at 20° C, make up to volume. Measure the absorbance of the solutions within the next 15 minutes, using a spectrophotometer with a wavelength of 515 nm against the "blank" (reagent alone).

## 5.4. Measuring the activity of enzyme preparations

For each sample of enzymes, put 10 ml of substrate (4.3.5) into a conical flask for 5 minutes in a bath of water at 40° C. Samples should be homogenized using a submersible multi-point magnetic stirrer set at 300 rpm.

After 5 minutes, 2 ml of the enzyme solution (4.4.1 or 4.4.2) are added to the first sample and a timer started just after adding the first enzyme solution.

Then add the following enzyme solutions to all the other samples with an interval of

30 sec-onds between samples.

Samples should then be stirred at 300 rpm throughout the entire reaction time.

After exactly 15 minutes, remove 3 ml of the first mixture, followed by all the other samples, at intervals of 30 seconds.

Using a pipette, put each 3-ml mixture into as many 50-ml volumetric flasks as required, each of which contains 7 ml of DNS acid colour reagent with phenol (4.3.4).

Then heat all the samples, at 30-second intervals, for exactly 10 minutes over a bath of boil-ing water.

Cool for 5 minutes in a bath of ice, transfer the flask to a bath of water at 20° C and add dis-tilled water (4.2.8) to just below the mark.

After 10 minutes at 20° C, make up to volume. Measure the absorbance of the solutions with-in the next 15 minutes, using a spectrophotometer with a wavelength of 515 nm against the "blank" (reagent alone).

The difference in the absorbance between the "blank" reading of enzymes and the value after reaction should be between 0.1 and 0.6 absorbance units.

If the values are over the measuring range of the calibration curve, repeat the experiment with dilutions adapted to the enzymes.

For all enzymes, always prepare 1 "blank" enzyme reading and 2 values after reaction. The two post-reaction values should be similar.

## 6. Calculations

To calculate the enzyme activity, use the mean value of the two readings.

The enzymatic activity of an enzyme preparation is calculated according to the following for-mula:

□-Glu-Unit activity/g or ml = (G X 200)/(15 x E) X 1/Gf

Nkat/g or ml = (Activity □-Glu-Unit/g or ml) X (1000/60)

Where

- G = Quantity of reducing sugars released during the test (reducing sugars released by Δ = the mean of 2 repetitions of the absorbance after reaction – the absorbance of the "blank" en-zyme, calculated in glucose from the glucose calibration curve in µg).

- E = Quantity of enzyme diluted to 100 ml in g or ml
- 200 = Dilution factor
- 15 = Reaction time
- Gf = Glucan factor (to be calculated)

Example of a calculation:

| Enzyme | Measured value 1 -2 | | "Blank" enzyme | E | µg glucose | ß-Glu units/g or ml |
|---|---|---|---|---|---|---|
| Standard enzyme | 0.621 | 0.618 | 0.415 | 0.503 | 662 | 10325 |
| *Penicillium funiculosum* ß-Glucanase | 0.417 | 0.416 | 0.023 | 1 | 1249 | 9799 |

Gf calculation:

- 1 Measure using old substrate and standard enzyme (Value 1)
- 2 Measure using new substrate and standard enzyme (Value 2)

Calculation: Value 1 / Value 2

## 7. Bibliography

1. Bertrand A. Determination de l'activite β-glucanase de Botrytis des preparations enzymatiques OIV FV 1263

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