RESOLUTION OENO 8/2008

CODEX-CELLULASES

THE GENERAL ASSEMBLY

IN VIEW of article 2, paragraph 2 IV of the Agreement of 3 April 2001, by which the International Organisation of Vine and Wine was founded,

Following a proposal made by the Sub-Commission of Methods of Analysis and Appraisal of Wine and the Specifications of Oenological Procedures expert group,

HAS HEREBY DECIDED to add the following monograph to the international Oenological Codex:

CELLULASES

endo-(1 →4)-β-D- glucanase

(EC 3.2.1.4 – CAS N° 9012-54-8)

General specifications

These enzymes are not found in a pure state but they are present within an enzyme complex. Unless otherwise stipulated, the specifications must comply with resolution Oeno 14/2003 concerning the general specifications for enzyme preparations included in the International Oenological Codex.

1. Origin and scope

Enzymes catalysing the decomposition of cellulose-type polysaccharides of the cellular walls of the grape berry, including beta glucanases (in this case endo-(1 □4)-□-D-glucanase), are used to clarify wines, to improve the filterability of musts and wines, and to increase diffusion phenomena during maceration.

The enzyme preparations demonstrating this activity come from directed fermentations of Aspergillus Niger and/or from a mixture of Aspergillus Niger – Trichoderma reesi.

Secondary activity: proteases, beta-glucosidases. In this case, the 50% clause can be applied (resolution Oeno 14/2003 4.1).

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2. Scope

The method of determination was developed using a commercially available glucanase (5.4). The conditions and the method were developed for application to the commercial enzyme preparations such as those found on the oenological market.

3. Principle

The endo-(1 →4)-β -D-glucanase catalyses the hydrolysis of the oside bonds within cellulose in a random way. Its activity can therefore be assessed by determination of the reducing sugars (expressed in glucose), released during incubation, by the NELSON method (1944).

Only the activities of the "endo-" type are measured because of the presence of carboxymethyl groups that block the action of the exo-glucanases. The endo-glucanases act inside the chains in non-carboxymethylated regions. In an alkaline environment, the pseudo-aldehydic group of sugars reduces the cupric ions Cu^{2+}. The latter react with the arsenomolybdate reagent to produce a blue colour, whose absorbance, measured at 520 nm, varies linearly with the concentration in monosaccharides (between 0 and 250 μg/ml).

4. Apparatus

4.1. magnetic stirrer with hot-plate

4.2. water bath at 40°C

4.3. water bath at 100°C

4.4. 100-mL beaker

4.5. centrifuge capable of housing 15-mL glass test tubes

4.6. stop-watch

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

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4.12. Vortex-type mixer

4.13. 500-mL amber glass bottle

4.14. room at 4°C

4.15. oven at 37°C

4.16. cotton-wool

4.17. brown paper

4.18. pH-meter

4.19. metal rack for 15-mL test tubes

4.20. disposable spectrophotometer cuvettes with a 1-cm optical path length, for measurement in the visible spectrum

4.21. ultrasonic probe

5. Reagents

5.1. Sodium acetate (CH_3COONa 99% pure - MW = 82g/mole)

5.2. Acetic acid (CH_3COOH 96% pure - MW = 60 g/mole, density = 1.058)

5.3. Carboxy-methyl-cellulose (CMC) with a degree of substitution from 65 to 95%.

5.4. Cellulase of Trichoderma reesei (Fluka, 4U/mg, ref: 22173 as an example). One unit releases 1 µmole of glucose from carboxy-methyl-cellulose per minute.

5.5. Anhydrous sodium sulphate (Na_2SO_4 99.5% pure - MW = 142 g/mole)

5.6. Anhydrous sodium carbonate (Na_2CO_3 99.5% pure - MW = 105.99 g/mole)

5.7. Sodium potassium tartrate ($KNaC_4H_4O_6.4H_2O$ 99% pure - MW = 282.2 g/mole)

5.8. Anhydrous sodium bicarbonate ($NaHCO_3$ 98% pure - MW = 84.0 1 g/mole)

5.9. Copper sulfate penta-hydrate ($CuSO_4.5H_2O$ 99% pure - MW = 249.68 g/mole)

5.10. Concentrated sulphuric acid (H_2SO_4 98% pure)

5.11. Ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$ 99% pure - MW = 1235.86 g/mole)

5.12. Sodium hydrogenoarsenate ($Na_2HAsO_4.7H_2O$ 98.5% pure - MW = 312.02 g/mole). Given the toxicity of this product, special attention must be paid during manipulation. Waste material must be treated in an appropriate manner.

5.13. Anhydrous D-glucose ($C_6H_{12}O_6$ 99% pure - MW = 180.16 g/mole)

5.14. Distilled water

5.15. Commercial enzyme preparation for analysis

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6. Solutions

6.1. Reagents of the oxidizing solution

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

6.1.1. Solution A:

Place successively in a 100-mL beaker (4.4):

20 g of anhydrous sodium sulphate (5.5)

2.5 g of anhydrous sodium carbonate (5.6)

2.5 g of sodium potassium tartrate (5.7)

2 g of anhydrous sodium bicarbonate (5.8)

Dissolve in 80 mL of distilled water (5.14). Heat with stirring (4.1) until dissolution and decant into a 100-mL graduated flask (4.7). Make up to the mark with distilled water (5.14). 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 copper sulfate pentahydrate (5.9) in 100 mL of distilled water (5.14) and add a drop of concentrated sulphuric acid (5.10). Maintain at 4°C.

6.1.3. Solution C:

This solution is prepared just before use in order to have a satisfactory proportionality between the depth 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 molybdate (5.11) in 400 mL of water (5.14). Add 25 mL of concentrated sulphuric acid (5.10) (cooled under cold running water).

In a 100-mL beaker (4.4), dissolve 3 g of sodium arsenate (5.12) in 25 mL of water (5.14) and quantitatively transfer into the 500-mL graduated flask (4.7.1) containing ammonium molybdate (5.11).

Make up to the mark with water (5.14) .

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).

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6.2. Sodium acetate buffer (pH 4.2, 100 mM)

This consists of solutions A and B below.

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 (5.14)

6.2.2. Solution B

Acetic acid 0.1 M: dilute 1 mL of acetic acid (5.2) with 175 mL of distilled water (5.14)

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. Carboxy-methyl-cellulose solution (CMC) at 2% (p/v) to be prepared just before use

Into a 100-mL graduated flask (4.7) introduce 2 g of CMC (5.3) and 100 mL of distilled water (5.14)

Given the high viscosity and in order to have a homogeneous solution, it must be subject to ultrasonic treatment (4.21), stirred without heating (4.1) and kept in suspension while constantly stirring.

6.4. Stock glucose solution at 250 µg/mL

In a 100-mL graduated flask (4.7), dissolve 0.0250g of glucose (5.13) in distilled water (5.14), and make up to 100 ml.

7. Preparing the standard solutions of glucose

This is produced using the stock solution of glucose at 250 µg/mL (6.4.), as indicated in Table 1.

Table 1: standard solutions of glucose based on the stock solution

Glucose (µg/ml) 0	25	50	100	150	200	250

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Glucose (µmole/ml) 0	0.139	0.278	0.555	0.833	1.110	1.388
Vol. (µl) stock solution (6.4) 0	100	200	400	600	800	1000
Vol. (µl) distilled water (5.14) 1000	900	800	600	400	200	0

8. Preparation of the sample

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

8.1. Enzyme solution at 2 g/l to be prepared just before use

Place 200 mg of commercial preparation (5.15) in a 100-mL graduated flask (4.7), make up to the mark with distilled water (5.14), and shake in order to obtain a homogeneous mixture.

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

Place 10 mL of the enzyme solution at 2 g/l (8.1) in a 15-mL test tube (4.11), plug with cotton-wool (4.16) covered with brown paper (4.17) and immerse the test tube for 5 minutes in the water bath at 100°C (4.3). Then chill and centrifuge 5 min at 6500 g

9. Procedure

9.1. Enzyme kinetics:

The test tubes are prepared 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 enzyme 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 the carboxy-methyl-cellulose solution (6.3) previously warmed at 40°C in a water bath, start the stop-watch (4.6).

After mixing (4.12), the test tubes plugged with cotton-wool (4.16) and borwn paper (4.17) are replaced in the water bath at 40°C (4.2)

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- for 1 min. for test tube N°1
- for 2 min. for test tube N°2
- for 5 min. for test tube N°3
- for 10 min. for test tube N°4
- for 15 min. for test tube N°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: the kinetic point at 10 min pemits the evaluation of the enzymatic activity

9.2. Determination of the reducing substances released

In a 15-mL test tube (4.11):

Place 1 mL of the reaction mixture (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 graduated 10-mL pipette (4.9)

Wait 10 min. for the colour to stabilise.

Centrifuge (4.5) each test tube at 2340 g for 10 min.

Place the supernatant liquid in a cuvette (4.20).

Zero the spectrophotometer using distilled water.

Immediately measure the absorbance at 520 nm (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 solutions

Proceed as described in 9.2, replacing the reaction mixture (9.1) by the various

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mixtures of the standard solutions of glucose from 0 to 250µg/mL (7).

10. Calculations

10.1. Determining the reaction 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 curve: the enzymatic activity is linear in time. Otherwise, the activity would be underestimated (Figure 1).

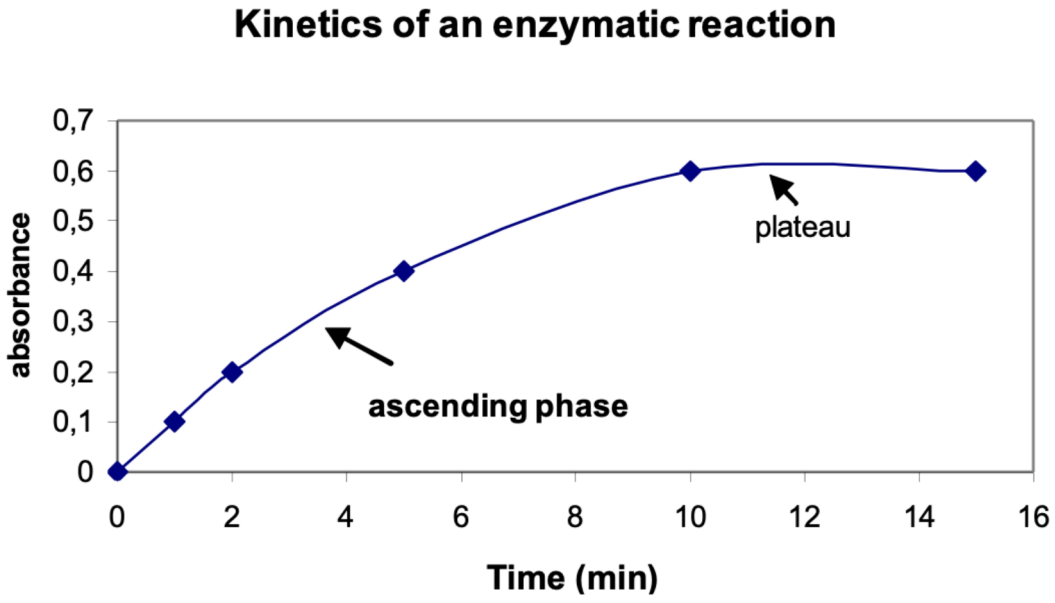


Figure 1: Kinetics of an enzymatic reaction

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

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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 glucose (from 0 to 0.693 µmole/ml) and whose Y-coordinates are the corresponding values of optical densities, obtained in 9.4. Then calculate the (Q/T) slope of 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 glucose 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

- Activity in U/g = 1000 x (Q/T)/(VxC)

Where Q: quantity of glucose released in µmoles during time T (min)

V: quantity of enzyme solution introduced (ml), in this case 0.2 ml

C: concentration of the enzyme solution (g/l) in this case 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:

- Activity in nkat/g = (activity in U/g) x (1000/60)

11. Characteristics of the method

r = 0.084

R = 0.056

Sr = 0.03

SR = 0.02

The intralaboratory repeatability of the method is estimated using the mean standard deviation of the absorbance values resulting from the same sampling of the enzyme preparation, determined 5 times. In this way, for the determination with carboxy-

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methyl-cellulose the mean standard deviation of the values is 0.03 with a percentage error of 13.56, in which the % error corresponds to:

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

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

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

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

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

If the power is low (≅ 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 (≅ 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 (□= 5%)	**Newman-** Keuls test (*)	Bonferroni test (**)
Endo-(1 □4)-□-D-glucanase	Adhered to	0.00011	95%	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

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as not being different to risk □ 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 □ 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 method of determination involves a probability of occurrence of a discrepancy in activity (between samplings) lower than 5%.

12. Bibliography

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