RESOLUTION OIV/OENO 314/2009

CODEX – PECTINLYASE

The GENERAL ASSEMBLY

IN VIEW of article 2, paragraph 2 iv of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

FOLLOWING a proposal made by the Sub-Commission of Methods of Analysis and the Specifications of Oenological Products expert group,

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

PECTINLYASE

(PECTINLYASE activity)

EC. 4.2.2.10. – CAS no. 9033-35-6)

General specifications

These enzymes are not found in a pure state but they are present within an enzymatic complex. Unless otherwise stipulated, the specifications must comply with the resolution concerning the general specifications for enzymatic preparations included in the international oenological Codex.

1. Origin and oenological scope

These activities are used to support the maceration of grapes, the clarification of musts and wines, to improve the filterability of musts and wines and the pressing of grapes.

The enzymatic preparations containing these activities come from directed fermentations of Aspergillus niger.

Principle activities accompanying lyase pectin activity include:

- Polygalacturonase
- Pectin methyl esterase

Secondary activities: Various hemicellulases such as xylanases as well as cellulases

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can be considered as secondary activities, but are also extremely useful for the hydrolysis of pectic substances. In this case, given their utility, it is not appropriate to apply the clause of the resolution on enzymatic preparationsto these activities requiring that the sum of secondary activities should not be higher than 50 % of the sum of activities needed by the required function since they usefully contribute to reaching that objective.

On the other hand, the 50 % clause can be applied for the following secondary activities:

Protease, beta glucosidase

2. Scope

The determination method was developed using a commercially available pectinlyase (5.5). The conditions and the method were developed for application to the commercial enzymatic preparations such as those found on the oenological market.

3. Principle

This enzymatic activity results in the decomposition of highly methylated pectins by the β-elimination of methylated galacturonic acids. In so doing, a system of highly delocalised conjugated double bonds is created, absorbing in the ultraviolet range.

4. Apparatus

4.1. magnetic stirrer

4.2. water bath at 25 °C

4.3. water bath at 100 °C

4.4. 1000-mL graduated flask

4.4.1. 100-mL graduated flask

4.5. chronometer

4.6. quartz cuvets with a 1-cm optical path length, for spectrophotometer, for measurement in the UV spectrum

4.7. pH-meter

4.8. 100-µL precision syringes

4.8.1. 1000-µL precision syringes

4.9. spectrophotometer

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4.10. 15-mL test tubes

4.11. shaker of the vortex type

4.12. metal rack for 15-mL test tubes

4.13. chamber at 4 °C

4.14. carded cotton

4.15. Kraft paper

5. Products

5.1. Citrus fruit pectin with a 63-66 % degree of esterification (Pectin from citrus peel, Fluka, Ref. 76280), as an example.

5.2. Sodium hydroxide (NaOH, 99 % pure - PM = 40 g/mole)

5.3. Citric acid ($C_6H_8O_7.H_2O$, 99.5 % pure - PM = 210.14 g/mole)

5.4. Sodium dihydrogenophosphate ($NaH_2PO_4.2H_2O$, 99 % pure - PM = 156.01 g/mole)

5.5. Distilled water

5.6. Commercial enzymatic preparation for analysis

6. Solutions

6.1. Solution of sodium hydroxide 1M

Introduce 40 g of sodium hydroxide (5.2) into a 1000-mL graduated flask (4.4) and make up with distilled water (5.5).

6.2. Mc Ilvaine buffer (Devries et al).

It consists of solutions A and B.

6.2.1. Solution A: acid citric at 100 mM: dissolve 4.596 g of citric acid (5.3) in 200 mL of distilled water (5.5)

6.2.2. Solution B: sodium dihydrogenophosphate at 200 mM: dissolve 6.25 g of sodium dihydrogenophosphate (5.4) in 200 mL of distilled water (5.5).

6.2.3. Preparation of the Mac Ilvaine buffer

Mix 50% of solution A (6.2.1) + 50 % of solution B (6.2.2) and adjust pH to 6 using the solution of sodium hydroxide (6.1).

The solution must be maintained at 4 °C (4.13). Check the pH of the buffer using a pH-meter (4.7)

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6.3. Solution of citrus fruit pectin at 1 % (p/v)

Dissolve 0.5 g of pectin (5.1) in 50 mL of Mc Ilvaine buffer (6.2).

7. Preparation of the sample

It is important to homogenise the enzymatic preparation before taking a sample by turning over the recipient, for example. The enzymatic solutions and blanks should be prepared at time of use.

7.1. Enzymatic solution at 10 g/L to be prepared just before use.

Place 1g of commercial preparation (5.6) in a 100-mL graduated flask (4.4.1), make up with distilled water (5.5), stir (4.1) in order to obtain a homogeneous mixture.

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

Place 10 mL of the enzymatic solution at 10 g/L (7.1) in a 15-mL test tube (4.10), plug with carded cotton (4.14) covered with Kraft paper (4.15) and immerse the tube for 5 minutes in the water bath at 100°C (4.3). Then chill and centrifuge 5 min at 6500 g.

8. Procedure

8.1. Enzymatic reaction:

The test tubes are produced at least in duplicate.

In 5 x 15-mL test tubes (4.10) numbered from 1 to 5, placed in a rack (4.12) in a water bath at 25°C, introduce

400 µL of Mc Ilvaine buffer (6.2) using a 1000-µL precision syringe (4.8.1)

100 µL of the enzymatic solution at 10 g/L (7.1) using a 100-µL precision syringe (4.8)

500 µL of citrus fruit pectin solution (6.3) beforehand warmed at 25°C in water bath; start the chronometer (4.5)

After stirring (4.11), the tubes plugged with carded cotton (4.14) and Kraft paper (4.15), are placed in the water bath at 25 °C (4.2)

- for 1 min for tube no.1
- for 2 min for tube no.2
- for 5 min for tube no.3
- for 10 min for tube no.4

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- for 15 min for tube no.5

The reaction is stopped by rapid (30 seconds max) heating by placing each tube numbered from 1 to 5 in the water bath at 100 °C (4.3) and adding acid or basic concentrated solutions as stop reagent. The tubes are then cooled under running cold water.

8.2. Determination of released substances

The reactional medium (8.1) is diluted to one tenth with distilled water (5.5). The dilution is placed in a cuvet (4.6) with an optical path of 1 cm.

Zero spectrophotometer using distilled water.

Immediately measure the absorbance at 235 nm, using a spectrophometer (4.9).

8.3. Blank

Proceed as described in 8.1, replacing the enzymatic solution by the blank denatured by heating (7.2). For each kinetic point, the enzymatic reaction of each blank is carried out at the same time as that of the enzymatic solution.

9. Calculations

9.1. 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).

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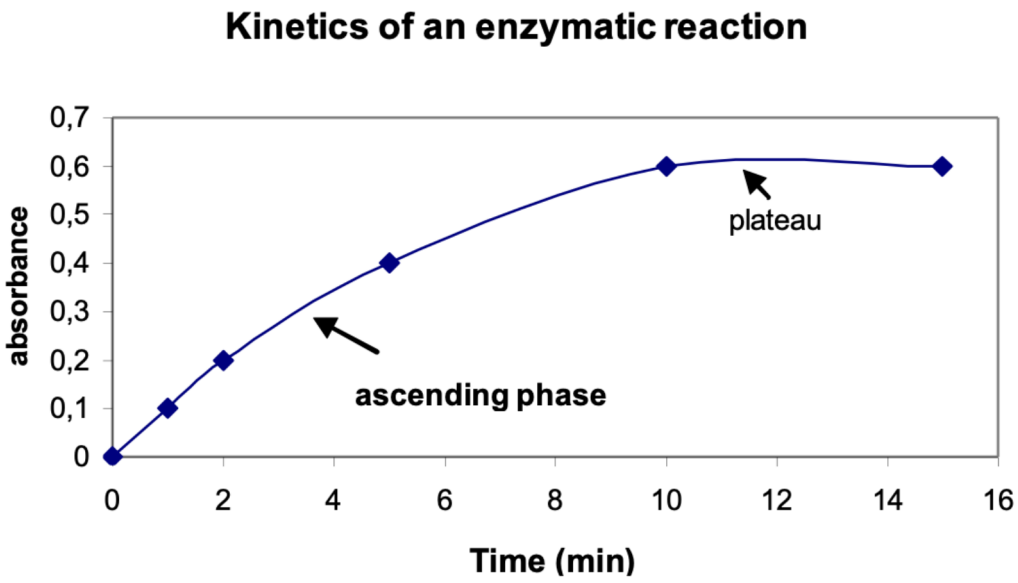


Figure 1: kinetics of enzymatic reaction

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 DO/T slope (1) of the straight regression line, taking into account only the points of the ascending phase (see figure 1).

9.2. Calculating the enzymatic activity

The enzymatic activity of the pectinlyase is calculated using the molar extinction coefficient of the molecule formed (ε = 5500 M-1cm-1). The formula to be applied is as follows:

- Activity in U/g = (DOT / T) /(0.1/ V) x (1000/(5.5/C))

Where DOT: absorbance value at time T (min)

V: quantity of enzymatic solution introduced (mL): in this case, 0.1 mL

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C: concentration of the enzymatic solution (g/L): in this case 10 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)

10. Characteristics of the method

- r= 0,028
- R= 0,112
- Sr= 0,01
- SR= 0,04

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 pectinlyase the mean standard deviation of the values is 0.01 with a percentage error of 4.66, in which the % error corresponds to:

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

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 reproductibility given by this variance analysis is 0,04.

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- the power of the test for the first species of risk α (5 %) – first species 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 1.

Determination	Variance analysis hypotheses	Probability	Power of test (= 5 %)	Newman-Keuls test (*)	Bonferroni test (**)
PL	Adhered to	0.00725	87 %	Significant	Significant

Table 1: 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 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 determination method involves a probability of occurrence of a discrepancy in activity (between samplings) lower than 5 %.

11. Bibliography

1. DE VRIES J.A., F. M. ROMBOUTS F.M., VORAGEN A.g.J., PILNIK W. Enzymic degradation of apple pectins. Carbohydrate Polymers, 2, 1982, 25-33.

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