COEI-1-GLYCOS Determination of glycosidase activity in enzymatic preparations

Introduction

Enzymes of the glycosidase type are used to reveal the flavours of wines based on their glycosylated precursors.

Aromatic molecules are partially in the form of heterosides; they are for the main part associated with glucose; the measurement of enzymatic activity sufficient to break this specific bond has been described under " □-D-glycosidase activity". However, this activity is not really functional if the glucose is itself bound to another type of sugar (which is the case for most aromatic precursors). These are essentially apiose, arabinose, rhamnose and yxlose.

In order to measure the true efficiency of an enzymatic preparation so as to obtain the aromatic potential of the grape or wine, the measurement concerning □-D-glucosidase activity should include the measurement of apiofuranosidase, arabinofuranosidase, □-D-galactosidase, rhamnosidase, and xylosidase activities.

Determination of glucosidase activity in enzymatic preparations

(activity □-D-glucosidase)

(EC 3.2.1.21 – CAS no. 9001-22-3)

(OENO 5/2007; OIV-OENO 489-2012)

General specifications

These enzymes are usually present among other activities, within an enzymatic complex. Unless otherwise stipulated, the specifications must comply with the resolution OIV/OENO 365/2009 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 monograph on Enzymatic preparation.

The enzymatic preparations containing these activities are produced by directed fermentations of Aspergillus niger.

2. Scope/Applications

Reference is made to the International Code of Oenological Practices, OENO 16/2004 and OENO 17/2004.

Enzymes belonging to the glycosidase type are used to reveal and enhance the

flavours of wines. This is realized through the hydrolysis of the glycosylated aroma precursors. The enzymes can also be added to the wine before the end of alcoholic fermentation but they will become active only after completion of the alcoholic fermentation

3. Principle

The enzymatic hydrolysis of *p*-nitrophenyl- □-D-Glucopyranoside, which is colourless, releases glucose and *para*-Nitrophenol *(*□-Np); the latter turns yellow in the presence of sodium carbonate, the absorbance of which is measured at 400 nm.

4. Apparatus

1. magnetic stirrer
2. water bath at 30°C 4.3 water bath at 100°C
3. cuvets with a 1-cm optical path length, for single use, for spectrophotometer, for measurement in the visible spectrum
4. crushed ice

precision syringe 500 – 5000 µl
precision syringe 100 µl
precision syringe 1000 µl
4.5. spectrophotometer
4.6. Eppendorf tubes
4.7. 100-mL graduated flask
4.8. pH-meter
4.9. cold room at 4°C
4.10. metal rack for Eppendorf tubes
4.11. carded cotton
4.12. Kraft paper
4.13. agitator of the vortex 4.18 chronometer
4.14. 15-mL glass tubes

5. Products

1. Sodium carbonate (Na_2CO_3 99.5% pure - PM:105.99 g/mole)
2. Sodium acetate (CH_3COONa 99% pure - PM: 82g/mole)

3. Acetic acid (CH_3COOH 96% pure - PM: 60g/mole)

□-*nitrophenyl*- □-D-Glucopyranoside (Fluka, ref. 73676) as an example

5.4. □-D-glucosidase (Fluka; 250 mg; 6.3 U/mg, ref. 49290) as an example. One unit corresponds to the quantity of enzyme required to release 1 µmole of glucose per minute with pH 5 and 35°C.

5.5. □-*nitrophenol* (□ - Np) ($C_6H_5NO_3$ 99.5% pure - PM: 139.11 g/mole)

5.6. Distilled water

5.7. Commercial enzymatic preparation for analysis

6. Solutions

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

It consists of solutions A and B.

6.1.1. Solution A: introduce 0.5 g of sodium acetate (5.2) into 60 ml of distilled water (5.7)

6.1.2. Solution B: introduce 1 ml of acetic acid (5.3) into 175 mL of distilled water (5.7) 6.1.3

Preparation of the sodium acetate buffer: mix 47.8 ml of solution A (6.1.1) + 152 ml of solution B (6.1.2).

Check the pH of the buffer using a pH-meter (4.12).

Maintain at 4°C

6.2. Solution of *p* -nitrophenyl - □-D-Glucopyranoside 4mM

Place 0.096 g of *p -nitrophenyl* - □-D-Glucopyranoside (5.4) in 80 mL of sodium acetate buffer (6.1.).

6.3. Sodium carbonate solution 1M

Dissolve 10.6 g of sodium carbonate (5.1) in 100 mL of water distilled (5.7) in a 100-ml graduated flask (4.11). The solution can be maintained at 4°C (4.13).

6.4. Stock solution of □ -nitrophenol (*p*-Np) at 125 µg/ml

Dissolve 0.01 g of p-Np (5.6) in 80 mL of distilled water (5.7). The stock solution must be prepared extemporaneously.

6.5. **Preparation of the standard range of *p-nitrophenol* (*p*-Np) from 0 to 50 µg/ml**

It is made up using the stock solution of *p-nitrophenol* (*p*-Np) (6.4.) as indicated in table 1.

Table 1: Standard range of para-Nitrophenol

Quantity of p-Np (µg)	0	2	4	6	8	10
P-Np concentration (µg/mL)	0	10	20	30	40	50
P-Np concentration (µmol/mL)	0	.07222	0.14	0.22	0.29	0.36
Volume of stock solution (6.4) (µl)	0	16	32	48	64	80
Distilled water (5.7) (µl)	200	184	168	152	136	120

7. 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 extemporaneously.

7.1. Place 1 g of commercial preparation (5.8) in a 100-mL graduated flask (4.11), make up with distilled water (5.7), and stir (4.1) in order to obtain a homogeneous mixture.

7.2. Blank denatured by heating

Place 10 mL of the enzymatic solution at 10 g/l (8.1) in a 15 mL tube (4.19), plug with carded cotton (4.15) covered with Kraft paper (4.16) and immerse the tube for 5 minutes in the water bath to 100°C (4.3).

8. Procedure

8.1. Enzymatic reaction: The tubes are produced at least in duplicate..

In 5 Eppendorf tubes (4.10) numbered 1 to 5, placed in a rack (4.14) in ice crushed (4.5) introduce

100 µl of the solution of *p*-nitrophenyl- □-D-Glucopyranoside (6.2), using a precision syringe (4.7),

- 100 µl of the enzymatic solution with 2 g/l (8.1), start the chronometer (4.18)

After stirring (4.17), the Eppendorf tubes are placed in the water bath at 30°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
- for 15 min. for tube no.5

The reaction is stopped by placing each of the tubes numbered from 1 to 5 immediately after they have been removed from the water bath at 30°C, in a bath of crushed ice (4.5)

8.2. Determination of *p*-nitrophenol released

From the Eppendorf tubes containing the various reactional mediums (9.1)

Add 600 µl of sodium carbonate solution (6.3), using a precision syringe (4.8),

1.7 ml of distilled water (5.7), using a precision syringe (4.6),

Place the resulting mixture in a tank (4.4).

Immediately measure the absorbance at 400 nm, using a spectrophotometer (4.9)

8.3. Blanks

Proceed as described in 9.1 by replacing the enzymatic solution with 2 g/l (8.1) by the blank denatured by heat (8.2). The ideal situation is to carry out the enzymatic reaction of the blank at the same time as that of the enzymatic solution.

8.4. Standard range

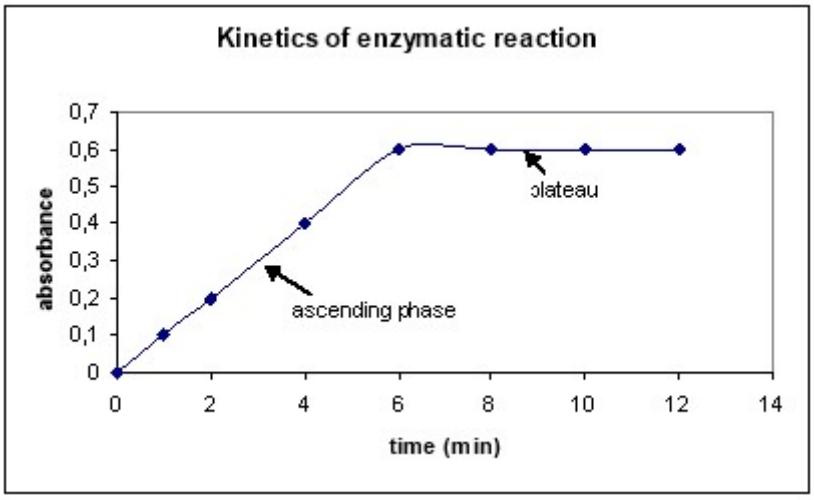
Proceed as described in 9.2 by replacing the reactional medium (9.1) by the various mediums of the standard range of *p -nitrophenol* from 0 to 50 µg/mL (7).

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).

Figure 1: Kinetics of an enzymatic reaction



The kinetics are determined over 12 minutes. The activity concerned is measured at

- T=1 min
- T=2 min
- T=4 min
- T=6 min
- T=8 min
- T=10 min
- T=12 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).

9.2. Producing the calibration line

The calibration line corresponds to plotting a graph whose X-coordinates are the various concentrations of the standard range of *p.nitrophenol* (from 0 to 0.36 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.

9.3. 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 *p.nitrophenol* 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

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

Where

- Q: quantity of *p.nitrophenol* formed in µmoles during time T (min)
- V: quantity of enzymatic solution introduced (ml) here 0.1 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:

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

10. Characteristics

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 □-D-glucosidase the mean standard deviation of the values is 0.01 with a percentage error of 8.43, 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 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 (≅ 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.

Table 2: Variance analysis – study of the sampling effect

Determination	Variance analysis hypotheses	Probability	Power of test (□= 5%)	Newman-Keuls test(*)	Bonferroni test (**)
□-D-glucosidase	Adhered to	0.0285	42%	Non Significant	Non Significant

* 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%, reinforced by belonging to the same group (Newmann-Keuls test not significant) and considered not to be different to the first species of risk □ (Bonferroni test not significant).

Determination of various glycosidase activities in enzyme preparations

□-D-galactosidase (EC 3.2.1.23 – CAS n° 9031-11-2)

□-L-arabinofuranosidase (EC 3.2.1.55 – CAS n° 9067-74-7)

□-L-rhamnosidase (EC 3.2.1.40 – CAS n° 37288-35-0)

□-D-xylosidase (EC 3.2.1.34 – CAS n° 9025-53-0)

(OIV-OENO 451-2012)

General specifications

These enzymatic activities are usually present among other activities within an

enzymatic complex. Unless otherwise stipulated, the specifications must comply with the resolution OIV/OENO 365/2009 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 monograph on Enzymatic preparation

The enzymatic preparations containing these activities are produced by directed fermentations of *Aspergillus niger* for example.

2. Scope/ Applications

Reference is made to the International Code of Oenological Practices, OENO 16/2004 and OENO 17/2004.

The glycosidase activities are used to reveal and enhance the flavours of wines based on hydrolysis of the sugar part of their glycosylated precursors. The enzymes can also be added to the must but their technological efficiencies will become active only after completion of the alcoholic fermentation.

3. Principle

Available enzymatic preparations with glycosidase activity contain enzymes that are able to hydrolyse the glycosidic bonds between glucose and other types of sugar, and in particular: apiose, galactose, arabinose, rhamnose and xylose- which then liberate the aromatic compounds contained in glucose by means of glycosidase activity. Similarly, the enzymes are capable of hydrolysing the bond of synthetic compounds that includes these various types of osidic compounds and *p*-nitrophenol. This enables to measure

these different activities.

Determination of □-D-galactosidase activity

The enzymatic hydrolysis of □-D- galactopyranoside of p-nitrophenyl, which is colourless, liberates galactose and para-nitrophenol (p-Np); the latter takes on a yellow colour when mixed with sodium carbonate, the absorbance of which is measured at 400nm.

Determination of □-L-arabinofuranosidase activity

The enzymatic hydrolysis of □-L-arabinofuranoside of *p*-nitrophenyl, which is colourless, liberates arabinose and *p*-nitrophenol *(p*-Np); the latter takes on a yellow colour when mixed with sodium carbonate, the absorbance of which is measured at 400nm.

Determination of □-L-rhamnosidase activity

The enzymatic hydrolysis of □-L-rhamnopyranoside of *p*-nitrophenyl, which is colourless, liberates rhamnose and *p*-nitrophenol (*p*-Np); the latter takes on a yellow colour when mixed with sodium carbonate, the absorbance of which is measured at 400nm.

Determination of □-D-xylosidase activity

The enzymatic hydrolysis of □-D-xylopyranoside of *p*-nitrophenyl, which is colourless, liberates xylose and *p*-nitrophenol (*p*-Np); the latter takes on a yellow colour when mixed with sodium carbonate, the absorbance of which is measured at 400nm.

4. Apparatus

1. magnetic stirrer
2. 40°C water bath
3. 100°C water bath
4. single-use 1 cm optical path vats for spectrophotometer measurement in the visible range
5. crushed ice
6. precision syringes 500 – 5000 µl
7. precision syringe 100 µl
8. precision syringe 1000 µl
9. spectrophotometer
10. eppendorf tube
11. 100 ml volumetric flask
12. pH meter
13. 4°C cold room
14. metal tray for eppendorf tubes
15. absorbent cotton
16. Kraft paper
17. vortex type stirrer
18. timer

19. 15 ml glass tubes

5. Products

1. Sodium carbonate (pure Na2CO3 at 99.5% - PM: 105.99 g/mole)
2. Sodium acetate (pure NaCH3COO at 99% - PM: 82g/mole)
3. Acetic acid (pure CH3COOH at 96% - PM: 60g/mole)
4. *p- nitrophenol (p-Np)* (pure C6H5NO3 at 99.5% - PM: 139.11 g/mole)
5. Distilled water
6. Commercial enzymatic preparation to be analysed, and depending on the measurement of the considered activity:
7. a β -D-galactopyranoside de *p*-nitrophenyl (Sigma ref. N1252, 250 mg) as an example
8. b α -L-arabinofuranoside de *p*-nitrophenyl (Sigma ref. N3641, 10 mg) as an example
9. α -L-rhamnopyranoside de *p*-nitrophenyl (Sigma ref. N7763, 100 mg) as an example
10. β -D-xylopyranoside de *p*-nitrophenyl (Sigma ref. N2132, 500 mg) as an example

6. Solutions

For the determination of □-L-arabinofuranosidase or □-L- rhamnosidase

6.1. Sodium acetate buffer (100 mM, pH 4.4) It is made of solutions A and B.

6.1.1. Solution A: add 0.984 g of sodium acetate (5.2) in 60 ml of distilled water (5.6)

6.1.2. Solution B: add 2 ml of acetic acid (5.3) in 175 ml of distilled water (5.6)

6.1.3. Preparation of the sodium acetate buffer: Add 78 ml of solution A (6.1.1) + 122 ml of solution B (6.1.2).

Control the pH of the buffer with the pH meter (4.12).

Keep at 4°C

For the determination of □-D-galactosidase or □-D-xylosidase activity

6.2. Sodium acetate buffer (100 mM, pH 4.0) It is made of solutions A and B.

6.2.1. Solution A: add 0.984 g of sodium acetate (5.2) in 60 ml of distilled water (5.6)

6.2.2. Solution B: add 2 ml of acetic acid (5.3) in 175 ml of distilled water (5.6)

6.2.3. Preparation of the sodium acetate buffer: Add 36 ml of solution A (6.1.1) + 164 ml of solution B (6.1.2).

Control the pH of the buffer with the pH meter (4.12).

Keep at 4°C

6.3. Reagent solution (depending on the measurement of the considered enzymatic activity)

6.3.1. Solution of *p*-nitrophenyl □ -L-arabinofuranoside 4 mM

Add 0.086 g of *p*-nitrophenyl □ -L-arabinofuranoside (5.4) in 80 ml of sodium acetate buffer (6.1.).

6.3.2. Solution of *p*-nitrophenyl □-D-galactopyranoside 4 mM

Add 0.096 g of p-nitrophenyl □-D-galactopyranoside (5.4) in 80 ml of sodium acetate buffer (6.1).

6.3.3. Solution of *p*-nitrophenyl □ -L-rhamnopyranoside 4 mM

Add 0.091 g of *p*-nitrophenyl □ -L-rhamnopyranoside (5.4) in 80 ml of sodium acetate buffer (6.1.).&

6.3.4. Solution of *p*-n i t rop hen yl □-D-xylopyranoside 4 mM

Add 0.0868 g of p-nitrophenyl □-D-xylopyranoside (5.4) in 80 ml of sodium acetate buffer (6.1).

6.4. Solution of sodium carbonate 1M

Dissolve 10.6 g of sodium carbonate (5.1) in 100 ml of distilled water (5.6) in a 100 ml volumetric flask (4.11). The solution may be kept at 4°C (4.13).

6.5. Stock solution of *p*-nitrophenol at 125 µg/ml

Dissolve 0.01 g of p-nitrophenol (5.5) in 80 ml of distilled water (5.6). The stock solution must be prepared extemporaneously.

7. Preparation of the standard range of *p*-nitrophenol from 0 to 100 µg/ml

It is made of the stock solution of *p*-nitrophenol (6.4.) as indicated in table 1.

Table 1: Standard range of p-nitrophenol (p.Np)

Quantity of *p*-Np (µg)	0	4	8	12	16	20
Concentration of *p*-Np (µg/ml)	0	20	40	60	80	100
Concentration of *p*-Np (µmol/ml)	0	0.14	0.2	0.43	0.5	0.72
		9		8		

Volume of stock solution (6.4) (µl)	0	16	32	48	64	80
Distilled water (5.5) (µl)	200	184	168	152	136	120

8. Preparation of the sample

It is important that the enzymatic preparation be homogeneous before sampling, by shaking it for example. The enzymatic solution and whites are to be prepared extemporaneously.

8.1. Enzymatic solutions

For the determination of □-L-rhamnosidase or □-D- xylosidase activity

10 g/l enzymatic solution

Put 1 g of commercially available preparation (5.6) in a 100 ml volumetric flask (4.11), add distilled water (5.5), and stir (4.1) in order to achieve a homogeneous solution.

For the determination of □-L-arabinofuranosidase activity

1 g/l enzymatic solution

Put 100 mg of commercially available preparation (5.6) in a 100 ml volumetric flask (4.11), add distilled water (5.5), and stir (4.1) in order to achieve a homogeneous solution.

For the determination of □-D-galactosidase activity

2 g/l enzymatic solution

Put 100 mg of commercially available preparation (5.6) in a 100 ml volumetric flask (4.11), add distilled water (5.5), and stir (4.1) in order to achieve a homogeneous solution.

8.2. Denatured white through heating

Put 10 ml of the enzymatic solution (8.1) in a 15 ml tube (4.19), plug with absorbent cotton (4.15) covered with Kraft paper (4.16) and immerse the tube for 5 minutes in the 100°C water bath (4.3).

9. Procedure

1. Enzymatic reaction: The tubes must be at least doubled.

In 6 eppendorf tubes (4.10) numbered from 1 to 6 and placed in a tray (4.14) of crushed ice (4.5), introduce

- 100 µl of the considered reagent solution (6.2), with a precision syringe (4.7),
- 100 µl of the corresponding enzymatic solution (8.1), start the timer (4.18)

After stirring (4.17), the eppendorf tubes are placed in the 40°C water bath (4.2)

- for 2 mn in tube n° 1 for 5 mn in tube n° 2
- for 10 mn in tube n° 3 for 15 mn in tube n° 4 for 20 mn in tube n° 5 for 30 mn in tube n° 6

The reaction is stopped by placing each numbered (1-6) tube immediately after extraction from the 40°C water bath in the tray of crushed ice (4.5).

9.2. Determination of liberated *p*-nitrophenol

With the eppendorf tubes containing the various reactive media (9.1)

add 600 µl of the considered reagent solution (6.3), with a precision syringe (4.8), and

1.7 ml of distilled water (5.5) with a precision syringe (4.6), Place the resulting mixture in a vat (4.4).

Immediately measure the absorbance at 400 nm with a spectrophotometer (4.9)

(This can also be simplified by indicating: See point 8.2 pertaining to the measurement of

□-D-glycosidase activity)

9.3. Blank

Proceed as per indications given in point 9.1 by replacing the enzymatic solution (8.1) with whites denatured by heating (8.2). Ideally, the enzymatic reaction of whites should be carried out at the same time as the reaction of the enzymatic solution.

9.4. Standard range

Proceed as described for point 9.2 by replacing the reactive medium (9.1) with various media of the standard range of *p*-nitrophenol from 0 to 100 µg/ml (7).

10. Calculations

1. Chemical kinetics

Generally, the calculation of the enzymatic activity can only be carried out when the substrate and the enzyme are not in limiting quantities. This corresponds to the ascending phase of the kinetic representation: the enzymatic activity is linear in time. If this were not to be the case, the activity would be underestimated (Illustration 1).

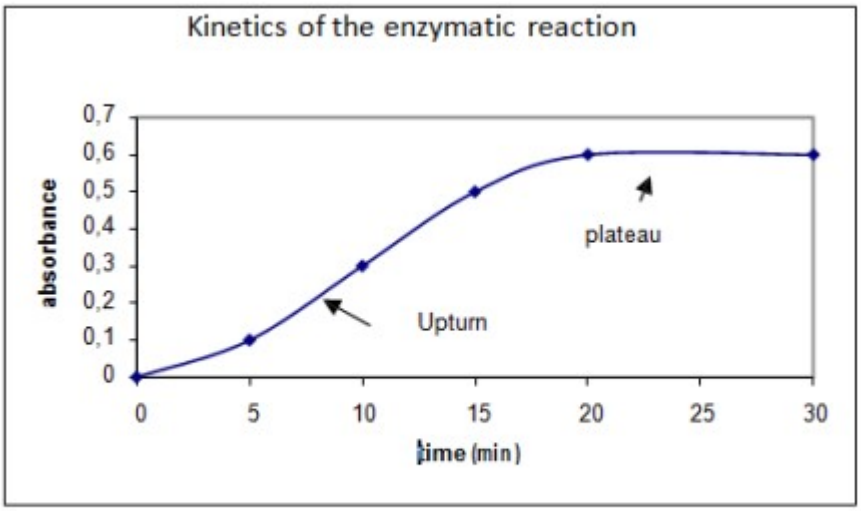


Illustration 1: Kinetic rate of an enzymatic rate

A kinetic calculation is performed for 30 minutes. The activity under consideration is measured at T=2 min, T=5 min, T=10 min, T=15 min, T=20 min, T=30 min.

After having calculated the kinetic rate of the enzymatic reaction, establish the variation curve of absorbance according to reaction times. Absorbance is the difference between absorbance at time T of the enzymatic preparation and the corresponding white.

Then calculate the equation (1) of the regression curve by considering only the points of the ascending phase (see illustration 1).

10.2. Establishing the standard line

The standard calibration line is established in a graph where the x-axis represents the various concentrations of the standard range of the *p*-nitrophenol (0 to 0.72 µmole/ml) and the y-axis represents the various corresponding optical densities established in 8.4. Then calculate the regression curve (2) that results from the linearity of the graph's data.

10.3. Calculation of enzymatic activities

Based on the regression curve (1), calculate the absorbance for an average time of T (for example 4 mn in the case of illustration 1) and deduce the Q quantity of liberated *p*- nitrophenol (in µmoles) for this intermediate time with equation (2).

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

$$Activity\ at\ U/g = Q/T/V/C * 1000$$

Where

- Q: quantity of *p*-nitrophenol formed in µmoles during time T (min)
- V: quantity of introduced enzymatic solution (ml), in this instance 0.1 ml
- C: concentration of the enzymatic solution (g/l), in this instance 10 g/l

It then becomes possible to represent the enzymatic activity in nanokatal. This unit corresponds to the number of nanomoles of the amount of product created per second in the conditions defined in determination protocols, and therefore:

$$Activity\ in\ nkat/g = activity\ in\ U/g * 1000/60$$

11. Reproducibility

The reproducibility of the method is estimated with the average of standard deviations of absorbance values resulting from a sample taken from the same enzymatic preparation, determined five times.

The table below summarises the results:

Activity	Average of values' standard deviations	Error percentage (%)
□-L-arabinofuranosidase	0	5
□-D-galactosidase	0.03	3.78
□-L-rhamnosidase	0.001	4.66
□-D-xylosidase	0.03	3.78

The % of error corresponds to:

$$\frac{\text{average of standard deviations of the values} \times 100}{\text{average of trial values}}$$

Hence, the determination method as presented herein is deemed to be reproducible.

The reproducibility trails were carried out with 2 enzymatic preparations and 5 samplings for each.

Two tests were used to determine the proper reproducibility of the method:

- the analysis of variance (the study of the probability of deviations between samples). The variance analysis is a statistical method that enables to test the

homogeneity hypothesis of a set of average k values. The variance analysis consists in determining whether the "treatment" effect is "significant or not"

- the strength of the trial with type I error (5%) – type I error is the risk of deciding that identical treatments are different

If the strength is feeble (≅ 20%), this means that no difference has been detected between treatments, but there is little chance of seeing a difference if there actually were one.

If the strength is high (≅ 80%), this means that no difference has been detected between treatments, but we would have the means of seeing it if such a difference were present.

The results are given in table 2.

Table 2: Variance analysis – stuffy of the sampling effect

Determinations	Hypothses of variance analysis	Probability	Strength of the trial (□ = 5%)	Newman-Keuls test (*)	Bonferroni test (**)
□-L-arabinofuranosidase	Satisfied	0.0125	45%	Not significant	Not significant
□-D-galactosidase	Satisfied	0.01	75%	Not significant	Not significant
□-L-rhamnosidase	Satisfied	0.006	65%	Not significant	Not significant
□-D-xylosidase	Satisfied	0.0253	73%	Not significant	Not significant

* Newman-Keuls test: this test is used to compare averages and enables to establish homogeneous treatment groups: those that belong to a same group are considered as not different to the chosen type I error

** Bonferroni test: also known as the “Bonferroni correction” the Bonferroni test enables to carry out all 2 on 2 average comparisons. i.e. (t(t-1))/2 comparisons before treatments. respecting the chosen type I error.

Therefore. the tests conducted enable to identify a difference if such a difference exists (high trial strength); furthermore the determination method presents the

probability of activity deviations (from one sampling to the next) of less than 5% reinforced by belonging to the same group (non-significant Newmann-Keuls test) and considered to be not different from type I error (non-significant Bonferroni test).

12. Bibliography

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