COEI-2-VISCPE Determination of the ability of an enzymatic preparation to interrupt pectic chains by measuring viscosity

1. Principle

Here, it is proposed to measure the quantity of enzyme needed to halve the viscosity of a standard solution with a given pH, temperature and time.

This is a purely technological measurement designed to test the true clarifying efficiency of the enzyme. It essentially measures the pectinase activity, which cannot be directly deduced from the release of galacturonic acid in the medium.

Comment

To measure the enzyme's activity, there are two possible approaches:

- Either the time it takes a given concentration of the enzyme to halve the viscosity of the pectin solution,
- Or, the concentration of enzyme needed in order for the pectin solution's viscosity to be halved in a given period of time.

Tests show that, as long as the substrate is not limiting:

- In the first case, the viscosity logarithm (flow time) is inversely proportional to the reaction time and,
- In the second case, the viscosity logarithm is inversely proportional to the quantity of enzyme in the medium.
- In either case, it is easy to find either the time or the quantity of enzyme needed to halve the viscosity on the basis of a judiciously chosen spectrum.

2. Reagent conditions

70 mmol/l phosphate buffer medium 70 mmol/l and 30 mmol/l citrate

Substrate: 70-75 % esterified apple pectin (e.g. Sigma P 8471), diluted to 10 g/l in the buffer solution.

- pH = 3.5
- Temperature: 30 °C
- Reaction time: 15 minutes.
- Pectinase: spectrum of concentrations covering approximately 10 mg/l of enzyme dry weight in the sample; i.e., for example, 0.5 mg in 50 ml of substrate,

which corresponds to the quantity of enzyme that is liable to halve the substrate's viscosity in 15 minutes in the conditions described above.

3. Apparatus

3.1. Bath or water circulation thermostat (30 °C ± 1 °C)

3.2. Capillary flow viscometer (A.3.1: Fig. 2) with a water value (the time for water to flow between the two marks) of approximately 18 to 20 seconds (i.e. a capillary tube roughly 0.5 to 0.6 mm in diameter)

3.3. Timer

3.4. Analytical balance (sensitivity 0,001 g)

3.5. pH meter

3.6. Magnetic stirrer, conventional laboratory glassware

3.7. Rapid paper filters

3.8. Micro-pipettes or micro-syringes for dispensing volumes from 5 to 500 µl

4. Pure products

4.1. Pure citric acid (99,5 %)

4.2. Pure disodium hydrogenophosphate (Na_2 $HPO_4 \cdot 2H_2O$) (99,0 %)

4.3. 70-75 % esterified apple pectin with more than 90 % purity (e.g. Sigma P 8471)

4.4. Distilled or deionized water

4.5. Pure sodium hydroxide (98 %)

4.6. Pure hydrochloric acid (11.5 M) (33,5 %)

4.7. Pectinase the activity of which is to be measured.

5. Solutions

Each solution should be homogenised before using

5.1. M sodium hydroxide

Weigh out 80 g pure sodium hydroxide (4.5) in a 100-ml volumetric flask and dissolve in deionized water (4.4). Top up to the filler mark after complete dissolution and cooling.

5.2. 2M hydrochloric acid

In a 100-ml volumetric flask half-filled with deionized water, place enough pure hydrochloric acid (4.6) to obtain a 2 M solution, (after having topped up to the filler mark).

5.3. 47 mmol/l phosphate buffer, 53 mmol/l citrate, pH 3.5

5.3.1. Put 800 ml deionized water (4.4) in a 1,000-ml volumetric flask

5.3.2. Weigh out 11.22 g citric acid (4.1)

5.3.3. Weigh out 8.30 g pure disodium hydrogenophosphate ($Na_2 HPO_4 \cdot 2H_2O$) (4.2)

5.3.4. Transfer the quantitatively-weighed chemical products to the 1,000 ml volumetric flask, stirring all the time

5.3.5. Mix until completely dissolved

5.3.6. Adjust the pH to 3.50 ± 0.05, at ambient temperature, with 2 M sodium hydroxide (5.1) or 2M hydrochloric acid (5.2), depending on the initial pH

5.3.7. Top up to the filler mark with deionized water (4.4). Mix

Stability: 8 days at ambient temperature.

5.4. Substrate: Apple pectin (4.3),

5.4.1. Put a 400-ml cylindrical container into a bath of water with a temperature of 40° C ± 3° on a rotating stirrer

5.4.2. Add 250 ml of buffer with a pH of 3.5 (5.3), measured exactly, to the cylindrical container

5.4.3. Keep stirring gently at 40 °C

5.4.4. Weigh out 2,500 g ± 0,01 g of pectin (4.3)

5.4.5. Slowly add the pectin whilst stirring vigorously

5.4.6. Then stir slowly for 60 minutes, maintaining the temperature at 40° C

5.4.7. Stop stirring and cool to 30 °C ± 3 °C

5.4.8. Filter with rapid filter paper (3.8) if necessary (if lumpy)

Stability: 24 hours at ambient temperature.

5.5. 100 g/l dry weight pectinase solution (4.7)

5.5.1. Weigh out 2.50 g ± 0.01 g of powdered or granulated pectinase

5.5.2. Transfer to a 25-ml volumetric flask

5.5.3. Top up to the filler mark with buffer solution at pH 3.5 (5.3)

5.5.4. Dissolve by stirring for 20 minutes using a magnetic stirrer.

Filter through rapid filter paper if the enzyme is immobilised on an insoluble substance using a rapid filter (3.7)

5.5.5. In the case of a liquid enzymatic preparation, use it directly.

Stability: 4 hours at ambient temperature.

6. Measurements

6.1. Put the viscometer in the bath of water at 30 °C or use any device that makes it

possible to measure the viscosity at 30 °C.

6.2. Measure the viscosity (the flow time between the two marks on the viscometer) of the buffer solution at pH 3.5; that is, t_o. This time should be approximately 20 seconds for a capillary tube 0.5 to 0.6 mm in diameter.

6.3. Measure the flow time of the 10 g/l pectin solution, that is, $T_{p.}$ This time should be approximately 200 seconds or more.

6.4. Prepare a series of 4 volumetric flasks containing 50 ml of 10 g/l pectin and put them in the bath of water at 30 °C.

6.5. Add 5 µl of the 100 g/l enzyme solution to the first flask and homogenize.

Then, approximately every 15 minutes, successively add to the other flasks:

15 µl, 35 µl and 100 µl of the 100 g/l enzyme solution and homogenize.

Measure the time taken by the various solutions to flow between the two marks on the viscometer exactly 15 minutes after adding the enzyme.

7. Graphic representation of the measured valued

Deduct the t_o value corresponding to the buffer at pH 3.5 alone from the flow time.

Produce a graph to represent the flow time logarithm as a function of enzyme concentration.

There must be at least three points in a line corresponding to the strongest dilutions. If this is not the case, use a more diluted enzyme solution - 50 g/l or even 10 g/l, for example.

8. Interpretation of the results

Find the regression line equation passing through the three aligned points:

$$T = ax + b$$

Deduct from this the necessary concentration of enzyme C to halve the pectin solution's viscosi*ty* $(T_p - t_o)/2$; that is, $T_{0,5}$.

9. Examples

9.1. Determination of the necessary enzyme concentration to halve the viscosity of the pectin solution. (Table 1)

Flow time of the buffer alone t_o = 19.3 s

Table 1

Vol (µl) of 100 g/l enzyme /50 ml of pectin	Concentration (g/l)	Flow time(s)	Corrected time(s)	Corrected time log.
0	0	230 (Tp)	210.7 (Tp - to)	2.32
5	0.01	190	170.7	2.23
25	0.05	107	87.7	1.94
100	0.2	32.8	13.5	1.13
500	1	23.8	4.5	0.65*

Corrected time = flow time – flow time of buffer with a pH of 3.5

* value not taken into consideration regression line equation (Fig.1)

$$y = -5.8366x + 2.2844$$

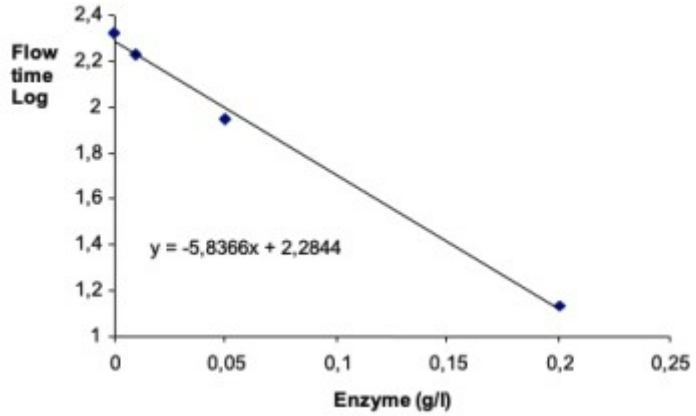
$$(T_p - t_o)/2 = 105s.$$

$$\log 105 = 2.02 \longrightarrow 2.28 - 2.02)/5.84 = 0.044$$

Therefore, 0.044 g/l of enzyme are needed to halve the viscosity of a 10 g/l apple pectin solution at 30 °C during 15 minutes.

It has been shown that 1 g/l of enzyme was sufficient to almost totally reduce the viscosity of the pectin solution in 15 minutes.

Fig.1 Reduction in the viscosity of a pectin solution as a function of enzyme concentration



9.2. Reduction in the viscosity of a 10 g/l pectin solution as a function of the reaction time at 30 °C of an enzyme with a concentration of 0.1 g/l. (Fig. 2) – *For information only*

The buffer flow time was 19.6 seconds.

Table 2:

Table 2:			
Reaction time (mn)	Flow time(s)	Corrected flow time(s)*	Flow time log.
0	170 (T_p)	150.7 (T_p - t_o)	2.18
11.5	101.4	82.1	1.91
15.6	86	66.7	1.82
21.08	72.8	53.5	1.73
29	59.83	40.53	1.61
40.31	48.79	29.49	1.47
57	40.08	20.78	1.32
90	32.25**	12.95	1.11

167	26.25**	6.95	0.84

* Corrected flow time

**Values not taken into consideration since the remaining quantity of pectin limits the reaction.

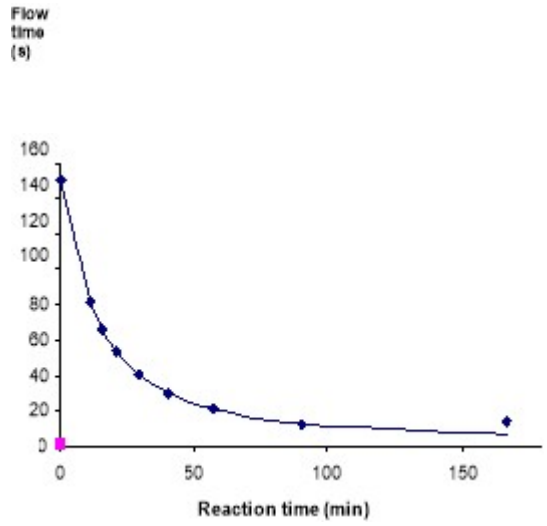


Fig. 2. Changes in the viscosity of a 10 g/l pectin solution as a function of the reaction time of a 0.1 g/l enzyme at 30 °C

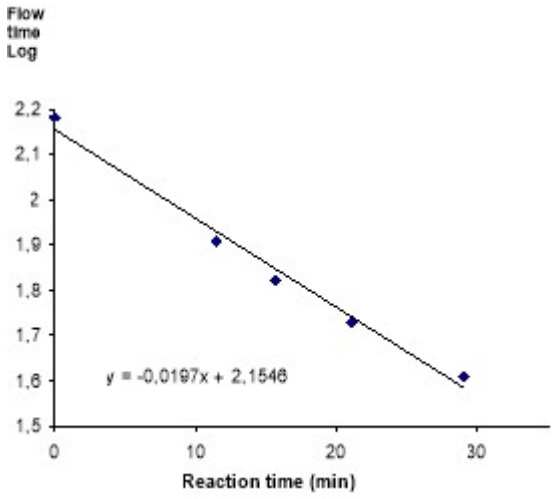


Fig.3. Changes in the viscosity of a 10 g/l pectin solution as a function of the reaction time logarithm of a 0.1 g/l enzyme at 30 °C.

Interpretation of the results

The values in table 4 show that a T/2 reaction time of 13.3 minutes is needed to halve the viscosity of the 10 g/l pectin solution at 30 °C.

For the calculation, on the basis of the regression line in Fig. 3:

$$\log 75.35 = 1.877$$

$$\text{Hence, } T_0/2 = (2.1545 - 1.877)/0.0197{:}14.1 \text{ minutes}$$

10. Bibliography

- Bertrand A. determination de la capacité d'une préparation enzymatique de type polygalacturonase a couper les chaines pectiques par la mesure de la viscosité OIV FV 1260