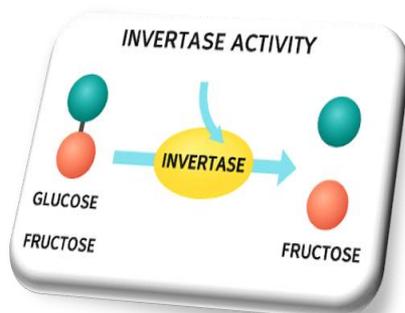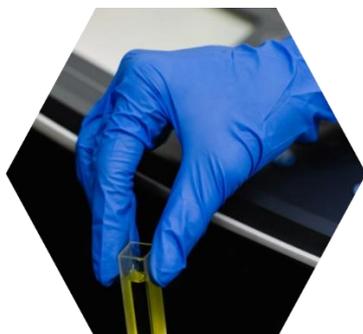# Practical work 3

KINETIC STUDY OF INVERTASE

INTRODUCTION

1) Study of the kinetics of enzymes (Michaelis-Menten theory)

Michaelian enzymes catalyze the transformation of the substrate (S) according to reactions that follow the Michaelis-Menten theory and that take place in two steps with a passage through an intermediate complex:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \overset{k_{+2}}{\rightarrow} E + P$$

To study the kinetic properties of these enzymes experimentally, one must always use a substrate concentration in excess of the enzyme concentration, in order to be in accordance with one of the simplifying assumptions of the Michaelis-Menten theory.

The kinetic study can be done by following the variation of the substrate concentration as a function of time or the variation of the concentration of the formed product (P) as a function of time.

$$d\,[S] \qquad d\,[P]$$

$$V = \frac{\text{-------}}{dt} = \frac{\text{------}}{dt}$$

On peut Also, when possible, follow the variation in concentration as a function of time of one of the forms of the coenzyme (see details in the course).

[P] ... Tangente à l'origine $= V_i$ ... Temps
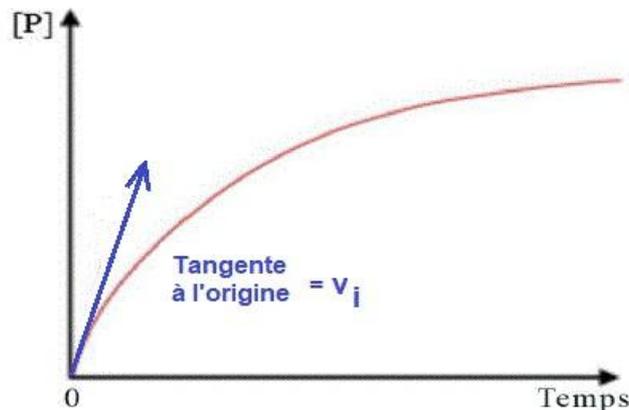
If a technique is available to monitor the increase in the concentration of the product formed as a function of time, the curve [P] = f(t) for different concentrations of substrate [S] is first drawn.

For each concentration of fixed S, we obtain a curve [P] = f (time) with two distinct parts:

1. A **linear part** : only in the first moments of the reaction. In this 'initial velocity period' phase, the reaction behaves as a zero-order reaction, the change in the concentration of S being negligible compared to the initial concentration $[S]_o$ less than 10 %.

    - Une partie non linéaire qui suit l'équation de la vitesse des réactions d'ordre 1, observée quand la quantité de S transformé devient importante (supérieur à 10 %).

1. In the first linear phase of this curve, the velocity **V = d[P]/dt** is constant and corresponds to the **initial velocity** Vo of the reaction. To determine Vo precisely, it must be done on the tangent at the origin of the curve [P] = f (time).

For a given concentration of enzyme [E]o, the initial rate of the reaction depends on the concentration of substrate [S].

It increases with the increase of [S], until it reaches a constant maximum value called Vmax. In addition, the larger [S], the shorter the linear part of the curve (constant speed).

The reaction rate, expressed in µg of P per min or in µmole/min, allows the inference of enzyme activity expressed in international units (IU).
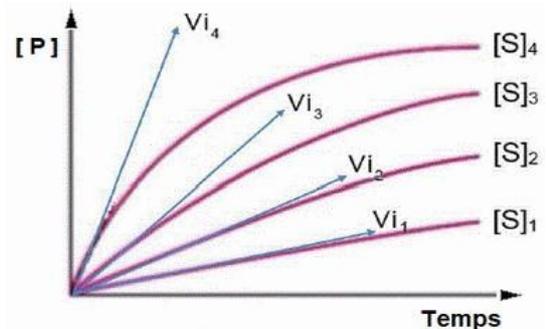
The most widely used unit of enzyme activity to quantify the rates of enzymatic reactions is the international unit (IU). **1 IU corresponds to the transformation of 1 micromole of substrate per minute** at 25°C and under optimal conditions of enzymatic activity (pH, ionic strength).
 Once the initial velocity has been precisely determined for different initial concentrations of substrate, the experimental curve Vo = f ([S]o) can then be drawn, which should follow the shape of the curve of the Michaelis-Menten equation if all the simplifying assumptions have been respected.

$$V = \frac{K_2 \ [E]_o}{1 + K_m / [S]_o} \quad \text{with k+2: catalytic constant}$$

K+2 $[E]_o$ = Vmax, the maximum speed of the reaction which corresponds to the horizontal asymptote of the curve Vo = f ([S]o). Km = Michaelis constant, it corresponds to the concentration of substrate for which the initial rate of the reaction is equal to half of the maximum rate.

$$Km = \frac{[E] \ [S]}{[ES]} = \frac{k_{-1} + k_2}{k_1}$$

In the case of simple reactions to a single intermediate complex [ES], the first k-1 step being the fastest; the constant of **Km = ------ = KD**

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Michaelis Km can be likened to the **k1** dissociation constant of the ES complex.

(For more details see enzymology course).

Therefore, **Km can be used to quantify the bond strength between the enzyme and the substrate** or the affinity of the enzyme for the substrate. The lower the Km, the stronger the affinity and *vice versa*.

$$V = \frac{Vmax}{1 + (K_m / [S])}$$
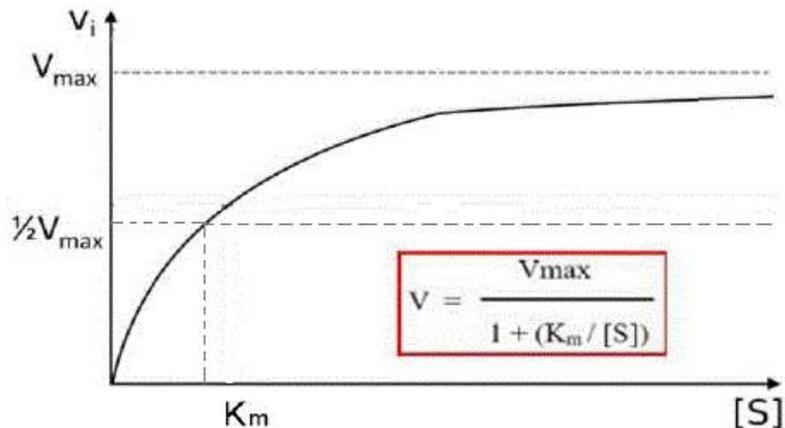
To determine the kinetic constants more precisely, it is preferable to use the so-called "double inverses" representation, the Lineweaver-Burk representation. Other curves can be drawn, including those of Eadie-Hofstee and Hanes-Woolf (see figure below).

1 / Vi = f (1 / [S]) which is a straight line of equation:
1/v = [Km/Vmax] x 1/(S) + 1/Vmax , of form y = ax + b

$$\text{Pente} = \frac{K_m}{V_{max}}$$

$$\frac{1}{V_i} = \frac{1}{Vmax} + \frac{Km}{Vmax} \times \frac{1}{[S]}$$
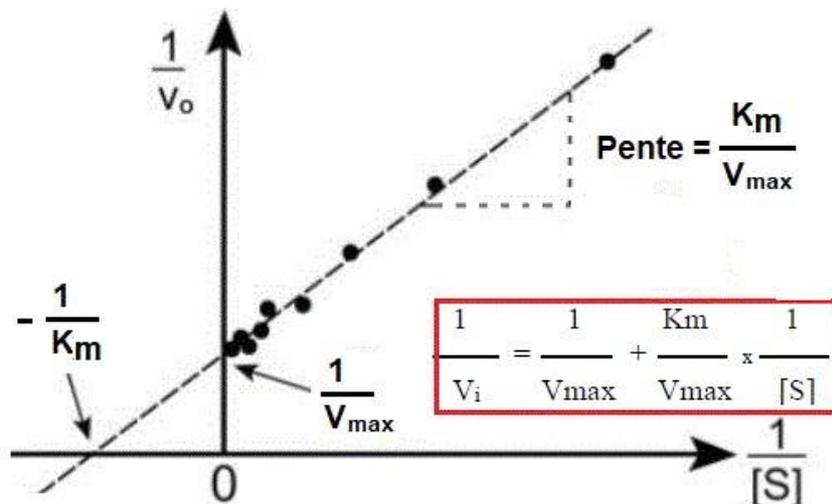
2). Invertase enzyme activity assays

The measurement of the hydrolysis activity of sucrose by invertase can be carried out by several methods using different techniques, e.g.

1. Polarimetry: by following the variation of the rotational power of the solution using a polarimeter.
2. the determination of the reducing sugars released by the reaction through the reduction of DNS(3,5-dinitrosalicylate) followed by spectrophotometry.

3 Glucose + 3,5-DiNitroSalicylate + 3 OH⁻ —Heat→ 3 Gluconate + 3-amino-5-nitrosalicylate + 2 H₂O

Réduction du DNS par les sucres - إختزال DNS بواسطة السكريات - DNS reduction by sugars

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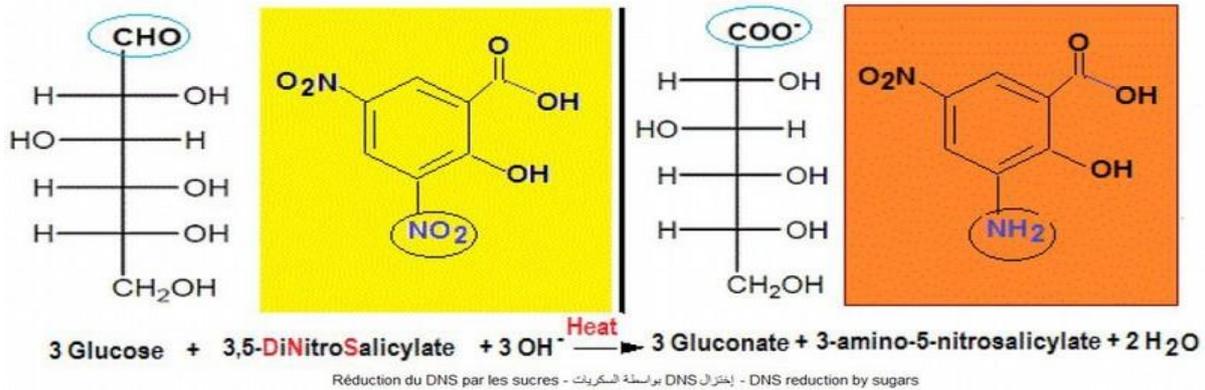
EXPERIMENTAL PART.

P W 3 : STUDY OF THE KINETICS OF BAKER'S YEAST INVERTASE

Preparation of the standard range for the determination of reducing sugars

The hydrolysis of sucrose (non-reducing sugar) releases two reducing sugars (glucose and fructose). By incubating the sucrose solution with the DNS, the two reducing sugars produced will reduce it. The result is the reduced DNS that absorbs light at 540 nm. This absorbance is therefore proportional to the amount of glucose and fructose released and therefore proportional to the amount of hydrolyzed sucrose.

A standard solution of 20 mM hydrolyzed sucrose (equimolar solution of Glucose 10 mM + Fructose 10 mM) is used as a reference in the determination of reducing sugars. The realization of this standard range is done according to the following table.

Tube 1 (without sugar reducers) acts as a control. The tubes are topped up to 3 ml with distilled water.

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Préparation de la gamme étalon de sucres réducteurs

Tubes	1	2	3	4	5
Saccharose hydrolysé 10 mM (ml)	0	0,1	0,3	0,5	1
Eau distillée (ml)	3	2,9	2,7	2,5	2
DNS (ml)	1	1	1	1	1
Incubation à 100℃			5 minutes		
Refroidissement à + 4℃			4 minutes		
Eau distillée (ml)	10	10	10	10	10
Absorbance à **540** nm	0	.....	.....	.....	.....

- Calculate the amount of micromole reducing sugars in each tube - Graph the OD at 540 nm on graph paper as a function of the amount of micromole reducing sugars in each tube.

The resulting standard line will be used to determine the amount of sucrose hydrolyzed by invertase in any subsequent enzymatic reaction.

PW3 : Study of the kinetics of sucrose hydrolysis as a function of time

The experiment to study the kinetics of sucrose hydrolysis by yeast invertase is carried out according to the following table. We use 6 tubes numbered 6 to 11.

Cinétique d'hydrolyse du saccharose en fonction du temps par l'invertase de l'extrait brut F

Tubes	6	7	8	9	10	11
Saccharose 0,3 M (ml)	1	1	1	1	1	1
Tampon acétate 0,1 M pH 4,7 (ml)	1	1	1	1	1	1
Invertase (extrait F au 1/25) (ml) (Ne rajouter l'enzyme qu'une fois les tubes sont à 30°C)	1	1	1	1	1	1
Temps d'incubation à 30°C (min)	0	2	4	6	8	10
DNS (ml)	1	1	1	1	1	1
Incubation à 100°C	5 minutes					
Refroidissement à + 4°C	4 minutes					
Eau distillée (ml)	10	10	10	10	10	10
Absorbance à 540 nm	0	....	....	....	....	....
Sucres réducteurs (µmoles)	0	....	....	....	....	....

- At first, add 1 ml of DNS only in tube 6 (control of the reaction). Due to its very basic pH, the DNS solution acts as an inhibitor of the enzymatic reaction. The reaction cannot start in tube 6, containing the DNS from the start.

- At time 0 min, quickly add 1 ml of the diluted enzymatic extract F to tubes 6 to 11 and shake.

- Incubate in a water bath at 30°C, tubes 7 to 11 (keep the control tube on the bench).

- After 2 minutes of reaction, remove tube 7, add 1 ml of DNS and shake with the vortex to stop the reaction. Place the tube on the bench. After 4, 6, 8, and 10 minutes, add 1 mL of DNS to tubes 8, 9, 10, and 11, respectively, and then shake.

- At the end of the experiment, put all the tubes at 100°C for 5 minutes, cool and add 10 ml of distilled water to each tube.

- After setting the OD datum of the spectrophotometer with the solution in tube 6 (control), measure the ODs at 540 nm of the other tubes (7 to 11).

Determination of the initial rate of the reaction

- Relate the ODs obtained to the different reaction times on the right of the standard range of reducing sugars to obtain the quantity of reducing sugars (in µmoles) at the different reaction times.

- Draw the curve representing the quantity of reducing sugars (in μmoles) as a function of the reaction time.
- Determine from this curve the **initial velocity (vi)** in μmoles of reducing sugars released per minute, for the concentration of sucrose equal to 0.3 M. - Calculate the initial velocity in μmoles of hydrolyzed sucrose per minute (IU) found for the concentration of sucrose equal to 0.3 M.
- Determine the number of IU contained in 1 mL of undiluted F extract.

The experiment was a failure

- Justifying failure with logical reasons.

- Draw the curves properly according to the data in front of you.

Raw absorbance data (DNS reagent, $\lambda = 540$ nm)

(Time min)	Absorbance 540nm
0	0.020
2	0.145
4	0.280
8	0.520
10	0.610

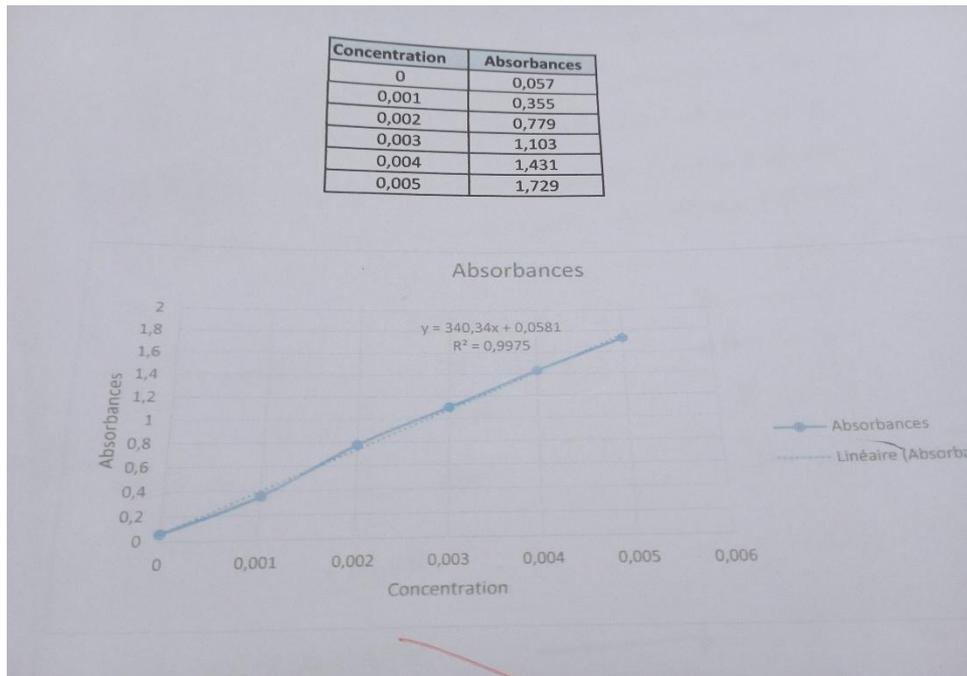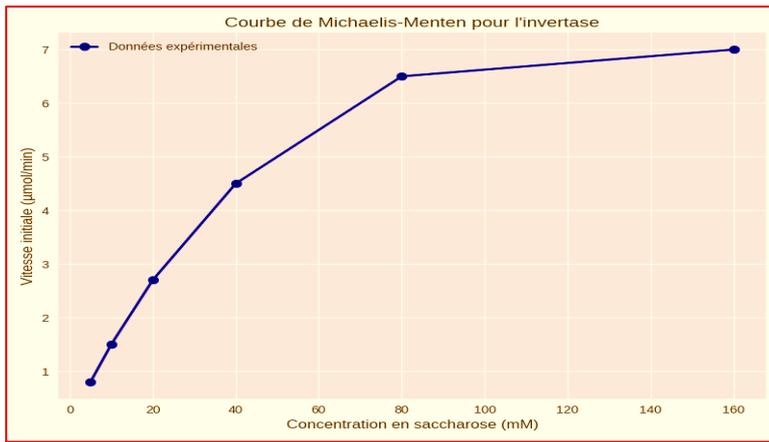## 🔍 Interpretation

Time 0 min : almost zero absorbance → no reducing sugars formed yet.

Time 2–4 min : rapid increase → initial high enzyme activity.

Time 8–10 min : The curve tends to a plateau → the enzyme approaches saturation or the substrate begins to run out.

Courbe de Michaelis-Menten pour l'invertase

Concentration	Absorbances
0	0,057
0,001	0,355
0,002	0,779
0,003	1,103
0,004	1,431
0,005	1,729

$y = 340,34x + 0,0581$
$R^2 = 0,9975$

Calibration curve for the determination of reducing sugars by the DNS method

• Ensuite, nous avons ajouté 6 ml d'eau distillée dans tous les tubes (à chaque tube nous avons ajouté 6 ml d'eau distillée).