Student Collaboration in a Series of Integrated Experiments to Study Enzyme Reactor Modeling with Immobilized Cell-Based Invertase

Experiment 1: Determination of kinetic parameters

Supporting Information

Students Handout

The experimental work described in the main manuscript as Experiment 1 comprises two laboratory sessions of three hours each aiming at the determination and comparison of the kinetic parameters of invertase from *Saccharomyces bayanus* cells, free and immobilized in calcium alginate beads.

The experimental protocol to be given to the students is described in the next pages.

Determination of the kinetic constants of invertase contained in *Saccharomyces bayanus* cells

Objective

The goal of this work is the determination of the kinetic parameters (maximum reaction rate, v_{max} and Michaelis-Menten constant, K_M) of the hydrolysis of sucrose by biologically active invertase contained in *Saccharomyces bayanus* cells.

$$\text{Sucrose} + H_2O \xrightarrow{invertase} \text{Glucose} + \text{Fructose}$$

Introduction

Enzymes are biocatalysts of protein nature that participate in most of the chemical reactions that occur in living organisms. They are highly specific and able to change their state of activity (regulated). Enzymes have been exploited since ancient times, and since the nature and mechanisms of their action have been discovered, they have been increasingly used in the food, textile and pharmaceutical industries.

The kinetics of enzymatic reactions can be described by the Michaelis-Menten equation:

$$v_0 = \frac{v_{\max} \times S}{K_M + S} \quad \Leftrightarrow \quad \frac{1}{v_0} = \frac{K_M}{v_{\max}} \frac{1}{S} + \frac{1}{v_{\max}}$$

where v_0 is initial reaction rate, S is the initial substrate concentration, v_{max} is the maximum reaction rate and K_M is the Michaelis-Menten constant. The kinetic constants (v_{max} and K_M) can be determined by measuring the initial reaction rate of the reaction at different initial concentrations of substrate.

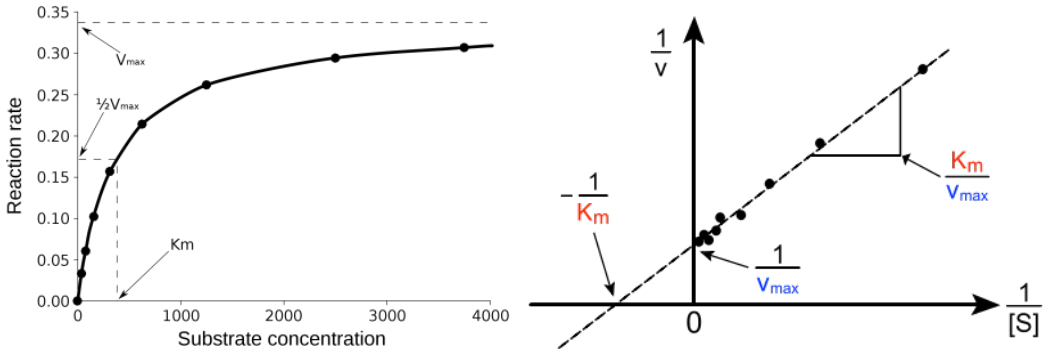

Figure 1. Graphic representation of the Michaelis-Menten equation (left) and Lineweaver-Burk double reciprocal linearization (right).

Experimental Protocol

The hydrolysis of sucrose will be studied at 45 °C. Substrate (sucrose) solutions prepared in 20 mM acetate buffer, pH 4.5 with 1% (w/v) calcium chloride ($CaCl_2$) with different concentrations will be assayed.

Solutions with the following sucrose concentration are supplied:

10, 15, 25, 35, 45, 55, 70, 100 g Suc.L^{-1}

1. Cell suspension

Prepare a 1% (p/v) suspension of *S. bayanus* in distilled water.

(Allow to ressuspend for 10-15 minutes before use)

2. Enzymatic assays

2.1 For each substrate concentration, add 25 mL of sucrose (in 20 mM acetate buffer, pH 4.5, 1% $CaCl_2$) to the reactor and allow at least 5-10 minutes of thermostabilization at 45 °C, under magnetic stirring. Perform the assays in increasing order of concentration.

2.2 Take a sample of 0.5 mL, corresponding to time zero.

2.3 Add 0.5 mL cell suspension and begin counting the reaction time.

2.4 Take samples of 0.5 mL every 1 minute until completing 9 minutes of reaction.

3. Quantification of reducing sugars

Determine the concentration of reaction products (glucose and fructose) designated as 'total reducing sugars' or TRS in the samples by the DNS method:

3.1 Measure 0.5 mL of DNS reagent into a test tube.

3.2 Add 0.5 mL of the sample to be analyzed.

3.3 Cover the tube with a loose cap and heat in a bath at 100 °C.

3.4 After 5minutes, remove the tube and cool it down to room temperature with tap water.

3.5 Add 5 mL of distilled water and mix in a vortex.

3.6 Read the absorbance of the solution at 540 nm against a blank that underwent the same procedure as the samples (time zero sample).

(Note: Samples taken from the reaction vessel (0.5 mL) are added to tubes containing 0.5 mL of DNS reagent, which stops the hydrolysis reaction at each point. Preferentially, process all tubes to the heating step (at 100 °C) at the end of each reaction to minimize error.)

Data Treatment and Analysis

1. Calculate the initial reaction velocity (v_0) for each sucrose solution.

2. Determine the kinetic constants using the Lineweaver-Burk double reciprocal plot and the least squares method (using the Solver tool of Microsoft Excel). Analyse and compare the values of K_M and v_{max} obtained by the two methodologies.

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Determination of the kinetic constants of invertase contained in *Saccharomyces bayanus* cells immobilized in calcium alginate

Objectives

The goal of this work is the determination of the kinetic parameters (maximum reaction rate and Michaelis-Menten constant) of the hydrolysis of sucrose by invertase from *Saccharomyces bayanus* whole cells immobilized in calcium alginate. In this immobilization method, the biocatalyst is incorporated into a sodium alginate solution that is then dripped into a solution containing calcium ions. Gelation occurs by ion exchange between sodium and calcium, being the cells retained within the gel matrix (beads).

Introduction

The use of biocatalysts in industrial applications is limited not only by the relative instability of most enzymes, but also by the difficulties in product separation and recovery of the enzymes from reaction mixtures. The immobilization of biocatalysts on solid supports can eliminate some of the drawbacks encountered with soluble enzymes, and in some cases, the catalytic activity and stability may be altered favourably. Methods for enzyme immobilization include adsorption or covalent bonding to different supports and occlusion in gel matrices. One of the most used hydrogels is alginate, which complexes with Ca^{2+} ions forming a reticulated matrix with porosity adequate to retain cells. Whole cell immobilization can take advantage of biologically active multi-enzymatic systems and avoid the process of enzyme extraction from the cells, which is expensive and time-consuming.

In many cases, the kinetics of reactions with immobilized cells/enzymes follows the Michaelis-Menten model:

$$v_0 = \frac{v_{\max,app} \times S}{K_{M,app} + S}$$

where v_0 is initial reaction rate, S is the initial substrate concentration, $v_{max,\ app}$ and $K_{M,\ app}$ are the apparent kinetic constants, namely the apparent maximum reaction rate the apparent

Michaelis-Menten constant. These constants take into account external and internal limitations on mass transfer due to enzyme immobilization, which arise from diffusional resistance to the transport of substrate from the bulk solution into the catalytic sites and from the diffusion of products back into the bulk medium.

Mass transfer resistance for each substrate concentration can be quantified by the global effectiveness factor (η) that is estimated by dividing the initial reaction rate (v_o) of the immobilized enzyme by the initial reaction rate of the free enzyme:

$$\eta = \frac{v_{0,immobilized}}{v_{0,free}}$$

The effectiveness factor varies between 0 and 1 in the absence of substrate inhibition.

Experimental Protocol

The hydrolysis of sucrose will be studied at 45 °C. Substrate (sucrose) solutions prepared in 20 mM acetate buffer pH 4.5 with 1% calcium chloride ($CaCl_2$) with different concentrations will be assayed.

Solutions with the following sucrose concentration are supplied:

10, 15, 25, 35, 45, 55, 70, 100 g Suc.L^{-1}

1. Cell suspension

Prepare a 1% (p/v) suspension of *S. bayanus* in distilled water.

(Allow to ressuspend for 10-15 minutes before use).

2. Immobilization in calcium alginate

2.1. Prepare the liquid matrix by weighting:

1 g of sodium alginate

34 mL of distilled water

Warm this solution in a water bath at 45 °C to facilitate the dissolution of alginate. Remove from the bath and cool down to room temperature. Add 5 mL of cell suspension 1% (p/v).

2.2. Using a 1 mL micropipette, drip the matrix containing the cells into 200-250 mL of a solution of 2% (w/v) $CaCl_2$, under slight agitation. Wait for 5 minutes to allow for complete gelation.

2.3. Collect the gel beads in a sieve and wash with 20 mM acetate buffer pH 4.5 containing 1% (w/v) $CaCl_2$ (to prevent redissolution of the spheres). Wipe the gel beads on absorbent paper.

2.4. Weigh the gel beads and divide the total weight into 10 equal portions. Each portion is equivalent to 0.5 mL of cell suspension.

3. Enzymatic assays

3.1 For each substrate concentration, add 25 mL of sucrose (in 20 mM acetate buffer, pH 4.5, 1% $CaCl_2$) to the reactor and allow at least 5-10 minutes of thermostabilization at 45 °C, under magnetic stirring. Perform the assays in increasing order of concentration.

3.2 Take a sample of 0.5 mL, corresponding to time zero.

3.3 Add one portion of gel beads (equivalent to 0.5 mL of cell suspension) and begin counting the reaction time.

3.4 Take samples of 0.5 mL every 3 minutes until completing 15 minutes of reaction.

4. Quantification of reducing sugars

Determine the concentration of reaction products (glucose and fructose) designated as 'total reducing sugars' or TRS by the DNS method:

4.1 Measure 0.5 mL of DNS reagent into a test tube.

4.2 Add 0.5 mL of the sample to be analyzed.

4.3 Cover the tube with a loose cap and heat it in a bath at 100 °C.

4.4 After 5minutes, remove the tube and cool it down to room temperature with tap water.

4.5 Add 5 mL of distilled water and mix in a vortex.

4.6 Read the absorbance of the solution at 540 nm against a blank that underwent the same procedure as the samples (time zero sample).

(Note: Samples taken from the reaction vessel (0.5 mL) are added to tubes containing 0.5 mL of DNS reagent. Preferentially, process all tubes to the heating step (at 100 °C) at the end of each reaction to minimize error.)

Data treatment and analysis

1. Calculate the initial reaction velocity (v_o) for each sucrose solution.

2. Determine the kinetic constants using the Lineweaver-Burk double reciprocal plot and the least squares method (using the Solver tool of Microsoft Excel). Analyse and compare the values of K_M and v_{max} obtained by the two methodologies.

3. Analyse and compare the kinetic parameters obtained for the free and immobilized enzyme.

4. Determine the global effectiveness factor for each substrate concentration. Analyse and discuss the incidence of mass transfer limitations as function of the bulk substrate concentration.

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APPENDIX 1

Table A1 – CAS-numbers and link to safety data of reagents used in the laboratory activities.

Compound	Producer	CAS number	Material Safety Data Sheet	Classification
Saccharomyces bayanus	Lallemand Inc. (Montreal, Canada)*			
D-Sucrose	BDH-Prolabo (VWR International, Radnar, PA/USA)	57-50-1	MSDS	Not hazardous
Sodium alginate	BDH-Prolabo (VWR International, Radnar, PA/USA)	9005-32-7	MSDS	Not hazardous
Sodium-potassium tartrate	Sigma-Aldrich (St. Louis, MO)	304-59-6	MSDS	Not hazardous
D-Glucose	Merck (Darmstadt, Germany)	50-99-7	MSDS	Not hazardous
3,5-Dinitrosalicylic acid	Merck (Darmstadt, Germany)	609-99-4	MSDS	Acute Tox. 4; Skin Irrit. 2; STOT SE 3; H302, H315, H335
Sodium hydroxide	Merck (Darmstadt, Germany)	1310-73-2	MSDS	Met. Corr. 1; Skin Corr. 1A; H290, H314
Sodium acetate	Panreac (Castellar del Vallès, Spain	127-09-3	MSDS	Not hazardous
Calcium chloride	Panreac (Castellar del Vallès, Spain	10043-52-4	MSDS	Eye Irrit. 2; H319
Acetic acid	Panreac (Castellar del Vallès, Spain	64-19-7	MSDS	Flam. Liq. 3; Skin Corr. 1A; H226, H314

* Common baker yeast available at the supermarket can also be used.

(To see the safety data click simultaneously in MSDS and CRTL key)

Disposable of all reagents should be made according to recommended/common laboratory safety rules. Alginate beads containing yeast cells are processed as “solid residues” (go for incineration).