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

Instructors Notes

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.

Materials

Lyophilized *Saccharomyces bayanus* yeast cells for champagne production were purchased from Lallemand Inc. (Montreal, Canada); D-Sucrose, sodium alginate and sodium-potassium tartrate were obtained from BDH-Prolabo (VWR International, Radnar, PA/USA). D-Glucose, 3,5-dinitrosalicylic acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany) and acetic acid, sodium acetate and calcium chloride were from Panreac (Castellar del Vallès, Spain). All reagents needed are commercially available products of analytical grade, from the above specified or alternative suppliers.

The following solutions must be prepared beforehand:

- 20 mM acetate buffer pH 4.5 with 1% (w/v) $CaCl_2$
- 2% (w/v) $CaCl_2$
- Sucrose solutions at different concentrations (10, 15, 25, 35, 45, 55, 70 and 100 g/L) in 20 mM acetate buffer pH 4.5 with 1% (w/v) $CaCl_2$
- Glucose at different concentration (0.2, 0.4, 0.6, 0.8 and 1 g/L) in 20 mM acetate buffer pH 4.5 with 1% (w/v) $CaCl_2$
- DNS reagent.

Table A1 in Appendix 1 summarizes the CAS-numbers for all the reagents used as well as links to safety data information.

Equipment

Instrumentation required includes four jacketed glass vessel reactors ($V_T \approx 100$ mL), one thermo circulator (*e.g.,* BBraun Thermomix M), automatic pipettes, four magnetic stirrers, four vortex mixers, four chronometers, and one dry bath (or a boiling water bath). For absorbance readings, an UV/Vis spectrophotometer is required (*e.g.,* T80 from PG Instruments Ltd).

Immobilization of cells in calcium-alginate

Encapsulation in calcium alginate is a simple and effective method for cell or cell-based enzyme immobilization with application in different areas. Alginate bead-containing cells (of variable diameters) are easily obtained by extruding (with a pipette or a syringe) a cell-sodium alginate mixture into a calcium chloride solution. Gelation occurs by exchange between sodium and calcium ions with formation of a gel-matrix with porosity adequate to retain cells. The pore size can be adjusted by the concentration of sodium alginate in the initial mixture. A typical protocol for calcium-alginate cell immobilization is described in Students Handout (1 and 2). Further information about this immobilization procedure in different applications can be found in references [1-4].

[1] Li H., Yang, T., Gong, J.-S., Xiong, L., Lu, Z.-M., Li, H. Shi, J.-S., Xu, Z.-H. Improving the catalytic potential and substrate tolerance of *Gibberella intermedia* nitrilase by whole-cell immobilization. *Bioprocess Biosyst. Eng.* **2015,** *38*, 189-197.

[2] Wynant, I., Duriex, A., Bohets, H., Lavrijsen, K., Horvath, G. Simon, J.-P. Recombinant *Escherichia coli* cells immobilized in Ca-alginate for metabolite production. *Biocatal. Biotransf*. **2009**, *27*, 348-359.

[3] Ha, J., Endler, C.R., Wild, J.R. Biodegradation of coumaphos, chlorferon, and diethylthiophosphate using bacteria immobilized in Ca-alginate gel beads. *Bioresource Technol*. **2009**, *100*, 1138-1142

[4] D'Souza S.F. Immobilized enzymes in bioprocess. *Curr. Sci*. **1999**, *77*, 69-79

Determination of total reducing sugars

The dinitrosalicylic acid (DNS) reagent method for determination of reducing sugars is used for the detection of fructose and glucose (reaction products, designated as total reducing sugars or TRS). This method is based on the formation of a colorimetric complex resulting from the reduction of DNS, which absorbs at 540 nm [5,6].

Preparation of the DNS reagent

Dissolve 5 g of 3,5-dinitrosalicilic acid in 100 mL of 2N sodium hydroxide, at room temperature; Then add 500 mL of distilled water and 300 g of sodium potassium tartrate. Fill the solution with distilled water up to 1 L and let it dissolve to clear orange solution.

Calibration curve

To convert absorbance units into total reducing sugars (TRS) concentration it is necessary to construct a calibration curve, which can be done preparing several stock solutions of glucose (or another reducing sugar) that undergo the same procedure of the samples.

Table S1. Absorbance at 540 nm of different glucose standard solutions.

Glucose (g/L)	Abs 540 nm
0.0	0.000
0.2	0.112
0.4	0.241
0.6	0.352
0.8	0.491
1.0	0.592

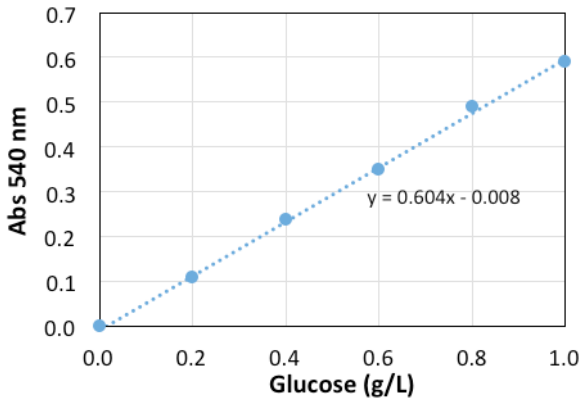

Figure S1. Calibration curve for the DNS method

[5] Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*. **1959**, *31*, 426–428.

[6] Genç R.; Rodriguez-Couto. Production of a biopolymer at reactor scale: A laboratory experience. S. *J. Chem Educ*. **2011**, *88*, 1175-1177

Determination of enzymatic activity

In order to determine the kinetic parameters (*i.e.* the maximum reaction rate, v_{max} and the Michaelis constant, K_M), students need to determine the enzymatic activity of invertase at different initial substrate concentrations (S_0), which is done experimentally by measuring the initial reaction rate (v_0) of sucrose hydrolysis. Replicates of values of v_0 for each S_0 are obtained from different laboratory sessions (or shifts) working with 3-4 groups of students (3-4 students/group). In each shift, each group determines the v_0 for 2-3 out of 8 initial sucrose concentrations (depending on the number of groups at the bench), with a invertase-containing yeast cell concentration of 0.2 mg/mL per assay (*see* Students Handout 1 and 2). The data obtained are registered in an Excel file in the Lab computer. Results are analysed (in terms of 'eye-guided' fitting to a Michaelis-Menten profile) and assays repeated when necessary (*outliers*). At the end of each lab shift, a whole set of coherent results of v_0 *vs* S_0 is obtained. After several lab shifts (minimum of three; in the present case four lab shifts) all groups in the course share the whole set of data for statistic analysis.

The sucrose (substrate) solutions, with different concentration, are prepared in the beginning of the week and the same solutions are used along the week for the enzymatic assays (replicates) by the different groups/lab shifts. The solutions are stable and kept at 4ºC between classes.

Table S1 illustrates absorbance measurements at 540 nm in the enzymatic assay with free yeast cells containing biologically active invertase (during 9 minutes) and an initial sucrose concentration of 55 g/L, and the conversion of Abs 540 nm into total reducing sugars concentration (g TRS/L) using the calibration curve given in equation 1.

$$Abs_{540nm} = 0.604\ [TRS]\ (g/L) - 0.008 \qquad (1)$$

Table S1. Conversion of Absorbance units into concentration of total reducing sugars (TRS).

Time (min)	Abs 540 nm	[TRS] (g/L)
0	0.000	0.000
1	0.067	0.124
2	0.143	0.250
3	0.178	0.308
4	0.284	0.483
5	0.322	0.546
6	0.400	0.675
7	0.408	0.755
8	0.509	0.922
9	0.622	1.033

By plotting the TRS concentration as function of time a linear variation is obtained and the slope corresponds to the initial reaction velocity. The determination of v_0 for an initial sucrose concentration of 55 g/L is illustrated in Table S2 and Figure S2.

Table S2. Example of calculation of the initial reaction rate (v_0), for S_0= 55 g Sucrose/L (with free cells). Each experiment in was performed in a different lab session (shift).

Time	**Concentration of Total Reducing Sugars (gTRS/L)**			
(min)	**Experiment 1**	**Experiment 2**	**Experiment 3**	**Experiment 4**
0	0.000	0.000	0.000	0.000
1	0.124	0.121	0.119	0.161
2	0.250	0.248	0.214	0.273
3	0.308	0.343	0.310	0.377
4	0.483	0.490	0.394	0.495
5	0.546	0.555	0.454	0.639
6	0.675	0.661	0.586	0.760
7	0.689	0.755	0.684	0.889
8	0.856	0.922	0.833	0.975
9	1.043	1.033	0.892	1.056
***v_0* (g/L.min)**	***0.108***	***0.112***	***0.099***	***0.119***

The average initial reaction rate (v_0) is calculated from the average slope of the plot of total reducing sugars concentration *versus* time (Figure 2S) for each group of data obtained in the different laboratory shifts. For the example given in Table S2 and illustrated in Figure S2, the initial velocity is 0.109 ± 0.008 g TRS/L.min.

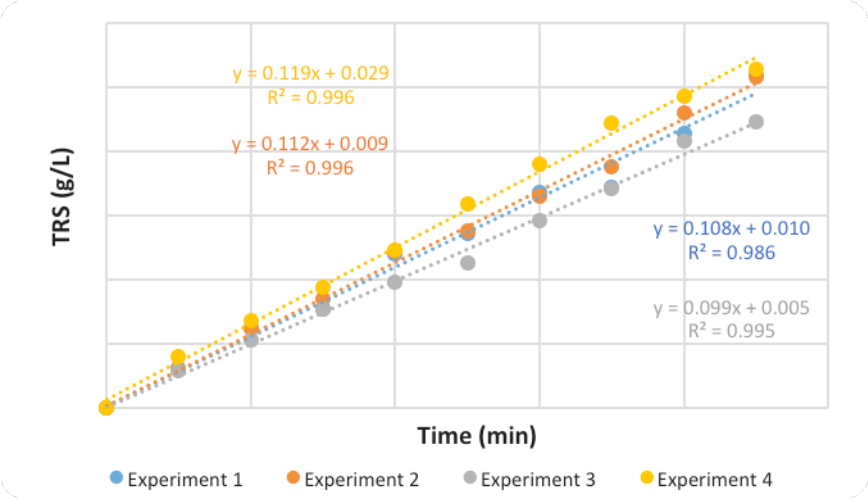

Figure S2. Increase of the total reducing sugars concentration with time during sucrose hydrolysis by invertase from *S. bayanus* cells. Replicates were obtained in 4 different laboratory sessions (or shifts) using the same substrate solution (55 g/L).

At the end of the different lab shifts, an average value of v_0 for a given S_0 and the respective standard deviation are determined for both free and immobilized enzyme-containing cells (Table S3). The effectiveness factor (η) is calculated by dividing the initial velocity obtained for the immobilized cells by the initial velocity obtained for the free cells for the same S_0 using equation 2. The effectiveness factor lies between 0 and 1 (in the absence of substrate inhibition) and is dependent on the bulk substrate concentration, among other factors.

$$\eta = \frac{V_0\big|_{\text{immobilized}}}{V_0\big|_{\text{free}}} \qquad (2)$$

This parameter compares, for each S_0, the reaction rate observed in the immobilized system with that obtained with free cells (which are in the absence of mass transfer resistance), and thus accounts for the mass transfer limitations when the catalyst (enzyme) is immobilized.

Table S3. Effectiveness factor and enzymatic activity of invertase for free and immobilized cells determined by the average initial reaction rate, v_0, and respective standard deviation (STDEV), obtained for different initial substrate concentrations, S_0.

S_0 (g Suc/l)	***Activity of free Cells***		***Activity of immobilized cells***		***Effectiveness factor η***
	Mean v_0 (gTRS/L.min)	*STDEV v_0 (gTRS/L.min)*	*Mean v_0 (gTRS/L.min)*	*STDEV v_0 (gTRS/L.min)*	
0	0	0	0	0	
10	0.059	0.008	0.029	0.004	0.496
15	0.072	0.010	0.038	0.007	0.524
25	0.090	0.011	0.048	0.008	0.536
35	0.097	0.011	0.055	0.009	0.567
45	0.103	0.006	0.059	0.006	0.571
55	0.109	0.008	0.064	0.009	0.583
70	0.111	0.009	0.067	0.007	0.606
100	0.115	0.008	0.071	0.012	0.619

Determination of Michaelis-Menten kinetic parameters

The Michaelis-Menten model is described by a hyperbola (Figure 1A) and the kinetic parameters (K_M, v_{max}) are determined with precision by direct fitting of experimental data (v_0 and S_0) to the model equation (1), using appropriate software. Alternatively, several rearrangements of the Michaelis-Menten equation describing linear variations between experimental-related values can be used to determine the kinetic parameters. One of the most popular and widely used linearization is the Lineweaver and Burk derived in equation (1), which

describes a linear variation of the reciprocal of initial reaction rate with the reciprocal of substrate concentration. In the Lineweaver-Burk plot, the y-intercept is the inverse of v_{max}, the slope is equal to K_M/v_{max} and the x-intercept represents $-1/K_M$.

$$\frac{1}{v_0} = \frac{K_M}{v_{\max}} \frac{1}{S_0} + \frac{1}{v_{\max}} \quad (1)$$

The Michaelis-Menten kinetic parameters for the free and immobilized systems are first estimated by linear regression using the Lineweaver-Burk plot (eq. 2), as illustrated in Figure 2B of the manuscript. Although this is a current and convenient way to estimate the kinetic parameters, Lineweaver-Burk linearization is based on a double reciprocal plot that favours error propagation. The points obtained at high concentrations of substrate are tightly clustered, thus placing too much weight on the points obtained at low concentrations of substrate, which are the ones more likely to have a higher percentage of error. Hence, the Lineweaver-Burk plot is used in as a first approach to estimate values of K_M and v_{max}. The kinetic parameters are then further tuned by adjusting the experimental values to the Michaelis-Menten model (eq. 1). This adjustment is easily made using the *Solver* tool of *Microsoft Excel*, through the least squares method applied to sum of the square differences between predicted and experimental values and using the parameters obtained by the Lineweaver-Burk plot as the base-values for iteration (*see* Figure 2A of the manuscript). Typical values obtained using both methodologies are presented in Table 1 (manuscript).

COMMON PROBLEMS

Second-year/Upper-Division Undergraduate students with former laboratory experience easily undertake these laboratory activities. Main problems can advent from common errors in pippetting, for example, in the DNS method for reducing sugar determination, which involves several steps. Such errors are, however, surpassed by the collaborative work between groups/students in several laboratory sessions and statistic data analysis.

VERSATILITY

Other dried yeast cells with invertase activity may also be used instead of S. *bayanus* cells (e.g., common baker's yeast). Also, although yeast cells and invertase are particularly suitable for

teaching laboratory experiments, this laboratory activity can easily be adapted to other cell-contained enzymes immobilized in calcium alginate.

FURTHER READING

(Suggestions. Alternative books/papers may also be used/found by the instructors)

Brady, D.; Jordaan, J. Advances in enzyme immobilization. *Biotecnol. Lett*. **2009**, *31*, 1639-1650.

Doran, P.M. *Bioprocess Engineering Processes*, 1st Ed, Academic Press, London, UK, 1995.

Experimental Biochemistry: Theory and Exercises in Fundamental Methods, 3rd Ed., Switzer R., Garrity, L. Eds., WH Freeman and Company, NY, USA, 1999.

Garcia-Galan, C.; Berenguer-Murcia, A.; Fernandez-Lafuente, R.; Rodrigues, R.C. Potential of different enzyme immobilization strategies to improve enzyme performance. *Adv. Synth. Catal*. **2011**, *353*, 2885–2904.

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		9005-32-7	MSDS	Not hazardous
sodium-potassium tartrate		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).