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

Experiment 2: Modeling of enzymatic reactors

Supporting Information

Students Handout

The experimental work described in the main manuscript as Experiment 2 comprises two laboratory sessions, of three hours each, aiming at the preparation, operation and modeling of continuous enzymatic reactors. The experimental protocol to be given to the students is described in the next pages.

A study with invertase from *Saccharomyces bayanus* cells immobilized in calcium alginate: Modeling of enzymatic reactors

Objective

The main goal of this laboratory activity is to evaluate the performances of a CSTR (Continuous Stirred Tank Reactor), a PFR (Plug Flow Reactor) and a FBR (Fluidized Bed Reactor) containing invertase from *Saccharomyces bayanus* cells immobilized in calcium alginate, operating in continuous mode at 45 °C and pH 4.5.

The effect of the volumetric flow rate of substrate solution fed to the reactor on the conversion degree (number of moles of substrate consumed divided by the number of initial moles of substrate) is evaluated and an experimental conversion profile as function of the feed flow rate is established. This profile is compared with predicted theoretical profiles, based upon kinetic parameters for free and immobilized enzyme obtained in previous laboratory classes.

Introduction

Assuming that the enzymatic reaction follows a Michaelis-Menten kinetic model, the conversion degree of the substrate solution fed to the reactor can be related to the volumetric flow rate, through equations that describe the behavior of each type of reactor, under steady state operation [1]:

$$\frac{v_{w\,int}\,W}{Q} = S_0\,x + K_{m,int}\,\frac{x}{1-x} \qquad \text{(CSTR)}$$

$$\frac{v_{w,int}W}{Q} = S_0 x - K_{M,int}\,ln(1-x) \qquad \text{(PFR and FBR)}$$

Where $K_{M,\,int}$ is the intrinsic Michaelis-Menten constant (free enzyme), $v_{w,\,int}$ is the intrinsic maximum specific velocity (free enzyme), W is the mass of biocatalyst, x is the conversion; S_0 is the initial concentration of substrate and Q is the feed rate. $v_{w,\,int} \times W$ is the total overall catalytic activity in the reactor.

When immobilized cells/enzymes are utilized, several factors (conformational, steric, partinioning, mass transfer or diffusional effects) can alter the access of substrate to the catalytic active center, thus affecting the apparent speed with which substrate is converted to products [12]. For enzyme-containing cells entrapped in calcium alginate beads such effects arise predominantly 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 [1]. Mass transfer resistance effects can be taken into account either by introducing a global effectiveness factor (η) in the left member of equation [1] or, alternatively, by replacing the intrinsic kinetic parameters by the apparent kinetic parameters, in the right member of the modeling equations. Accordingly, the equations that describe the behavior of the reactors can be written as:

$$\eta \cdot \frac{v_{w,int} W}{Q} = S_0 \, x + K_{M,\,int} \frac{x}{1 - x} \quad \Leftrightarrow \quad \frac{v_{w,app} W}{Q} = S_0 \, x + K_{M,app} \frac{x}{1 - x} \qquad \text{(CSTR)}$$

$$\eta \cdot \frac{v_{w,int} W}{Q} = S_0 \, x - K_{M,int} \, ln(1 - x) \quad \Leftrightarrow \quad \frac{v_{w,app} W}{Q} = S_0 \, x - K_{M,app} \, ln(1 - x) \qquad \text{(PFR and FBR)}$$

where $v_{w,\,app}$ and $K_{M,\,app}$ are the apparent kinetic constants, namely the apparent specific maximum reaction rate the apparent Michaelis-Menten constant. These constants are obtained in the kinetic study using the immobilized enzyme and, therefore, account for external and internal limitations on mass transfer [1].

Experimental Protocol

Each group of students studies one type of reactor. At the end of the two laboratory sessions, the groups share the results for overall discussion in the written report.

First Laboratorial Session (preparation)

1. Pump Calibration

(Distilled water can be used for pump calibration)

Establish 5 values of % of maximum rotation speed (corresponding to different rotation speeds) in the pump and, for each one, measure the flow rate using a measuring cylinder and a chronometer (measure the time required to drain a given volume). Correlate the % values

(abscissa) with the flow (ordinate). Based on this calibration, an estimate of the % value to be imposed on the pump to allow for the flow rates to be studied will be available. Check the flows, imposing the required % value and taking at least two consistent determinations for each flow rate. Register the data (required for the next session).

Flow rates to CSTR and PFR: 1, 2, 3, 4 and 5 $mL.min^{-1}$

Flow rates to FBR: ≈ 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100 $mL.min^{-1}$

2. Estimation of cross-sectional area for PFR and FBR

To estimate the cross-sectional area of PFR and FBR flow off a volume of buffer corresponding to a known column height (Ex. Δh = 3-5 cm marked on the wall of the column reactor) into a measuring cylinder. Two to three measures should be made and the average of ΔV/Δh calculated.

3. Enzyme immobilization

Immobilize the *S. baynus* cells in sodium alginate for each type of reactor according to the information in Table 1.

Table 1 – Immobilization of *S. bayanus* cells in calcium alginate for each type of reactor.

REACTOR	**Distilled water (mL)**	**Sodium Alginate (g)**	**Cell suspension* 5 % (w/v) (mL)**
CSTR	4.0	0.1	1.0
PFR	12	0.3	3.0
FBR	16	0.4	4.0

* In distilled water. Should be prepared 10-15 min before immobilization to allow complete cell suspension

Prepare the liquid matrices by weighting the amount of sodium alginate indicated for each type of reactor and adding the respective volume of distilled water. Warm the solutions in a water bath at 45 °C to facilitate the dissolution of alginate. Remove from the bath and cool down to room temperature. Add the respective volume of yeast cell suspension 5% (w/v).

Using a 1 mL micropipette, drip the alginate mixture containing the cells into 100 mL of a solution of 2% (w/v) calcium chloride ($CaCl_2$), under slight agitation. Leave the gel beads to harden for 5-10 minutes.

Store the immobilized cells at 4 °C in 2% (w/v) calcium chloride solution until the next session (operation).

Second Laboratorial Session (Operation and sample analysis)

All reactors are operated at 45 °C and pH 4.5.

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 re-dissolution of the spheres). Wipe them on absorbent paper.

For all reactors: impose the % value required for the first flow rate to be assessed (lower flow rate). Check the flow rate, using a measuring cylinder and a chronometer.

1. CSTR

Measure 10 ml of the substrate solution (sucrose 10 $g.L^{-1}$ in 20 mM acetate buffer pH 4.5 containing 1% (w/v) $CaCl_2$) into the reactor, add the biocatalyst gel beads, place the stopper and adjust the outlet tube to the height of the liquid in the reactor. Connect the pump. Allow for at least 3 renewals of the liquid in the reactor ($t_{waiting} = 3 \times V_{liquid}/Q$), so as to ensure that the reactor is in steady state before samples are taken. After having reached the steady state, take a 2 mL sample from outlet of the reactor for the quantification of reducing sugars by the DNS method (do triplicates for analysis). Reset the % in the pump and move to the following flow rate, repeating the procedure described above.

CAUTION: Do not change or stop the pump before taking the sample in steady state

2. PFR and FBR

Introduce the gel beads inside the reactor. Make a diluted mixture of gel beads and substrate solution or 20 mM acetate buffer pH 4.5, 1% (w/v) $CaCl_2$ and pour it carefully into the reactor

with the help of a funnel. Control the flux at the column outlet (using the Hoffman tweezer) to allow beads to settle and pack without drying.

PFR: Leave the volume of liquid ≈ 1 cm above the bed of alginate beads and make a label on the wall of the column reactor). Close the reactor with the inlet tube at the top.

FBR: Apply the outlet tube at the top of reactor and fill it with liquid at minimum flow rate to avoid bed fluidization. Mark the bed height corresponding to the resting bed (without fluidization).

Start the operation by turning on the pump, always ensuring that the reactor volume remains unchanged (constant). Allow for at least 3 renewals of the liquid inside the reactor ($t_{waiting} = 3 \times V_{liquid}/Q$), so as to ensure that the reactor is in steady state before samples are taken. Once the steady state is achieved, take a 2 mL sample from the outlet tube of the reactor for quantification of the reducing sugars by the DNS method (Do triplicates for analysis).

For the FBR: mark and measure the bed height at stationary state before changing the pump.

CAUTION: Do not change or stop the pump before taking the sample (or measuring the bed height).

Impose the stipulated % value in the pump for another flow rate and repeat the procedure described above.

3. For all reactors:

Collect a 2 mL sample from the sucrose feed solution (to be analyzed for the presence of total reducing sugars by the DNS method).

At the end of operation: measure/confirm the working volume (which is ≈10 mL in all cases) by draining the volume of liquid in the reactor into a measuring cylinder.

4. Quantification of reducing sugars by the DNS method

The dinitrosalicylic acid (DNS) reagent method for determination of reducing sugars is used for the detection of fructose and glucose (products of reaction, designated as total reducing

sugars). This method is based on the formation of a colorimetric complex resulting from the reduction of DNS, which absorbs at 540 nm [2, 3].

Determine the concentration of total reducing sugars (TRS) in the samples taken from the reactors and in the feed solution. For some flow rates, previous sample dilution is required, which shall be indicated by the Instructor.

- Measure 0.5 ml of DNS reagent into a test tube.
- Add 0.5 ml of the sample to be analyzed.
- Cover the tube with a loose cap and heat it in a bath at 100 °C (or in a dry bath).
- After 5 minutes, remove the tube and cool it down with tap water.
- Add 5 ml of distilled water and mix in a vortex.
- Read the absorbance of the solution at 540 nm against a blank that underwent the same procedure as the samples.
- Convert the absorbance values into TRS concentration (using a pre-determined calibration curve).

5. Estimation of the minimum fluidization velocity

Estimate the minimum fluidization velocity**,** U_{mf}**,** by applying the empirical correlation of Richardson and Zaki. This equation predicts that the superficial velocity of the fluid phase in a porous bed depends on its voidage, ε, and that the dependence of U on ε is linear in a *ln U vs ln ε* plot, where U_i and n are empirical parameters [4, 5]:

$$U = U_i \varepsilon^n \Leftrightarrow \ln U = \ln U_i + n \ln \varepsilon$$

The superficial velocity, *U,* is given by the ratio between the volumetric feed flow rate (*Q*) and the reactor cross-sectional area, *A (U=Q/A).* The void fraction (or voidage, ε_0) for the resting bed (before fluidization) is given by the ratio between the working volume and the total volume under these conditions:

$$\varepsilon_0 = \frac{V_0}{h_0 A}$$

where V_0 and h_0 represent the working volume and height of the resting bed, respectively. For high flow rates (Q_n), when bed fluidization occurs and the bed reaches a height h_n at stationary state, the void fraction, ε_n, is calculated by the following equation:

$$\varepsilon_n = \frac{h_n A - \left(h_0 A - V_0\right)}{h_n A}$$

where h_nA is the total volume of the bed for a given flow rate and ($h_oA - V_o$) represents the volume of solid-phase (alginate beads).

The minimum fluidization velocity**,** U_{mf}, corresponds to the superficial velocity obtained for ε_0 through the Richardson and Zaki correlation established with the experimental data.

Data treatment and analysis

1. Plot the substrate conversion degree as function of volumetric feed flow rate for all types of reactor.
2. Compare the results with those that would be expected by applying the model equations using the kinetic constants for free and immobilized enzymes. Interpret and discuss the overall results.
3. Estimate the minimum fluidization velocity for the FBR studied.

References

[1] Cabral, J.M.S.; Tramper, J. Bioreactor Design. In *Applied Biocatalysis*, Cabral, J.M.S., Best, D., Boross, L. Tramper, J. Eds.; Harwood Academic Publishers, Switzerland, 1994, pp 333-370.

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

[4] Richardson, J.F., Zaki W.N. Sedimentation and fluidisation. Part 1. Trans. Inst. Chem. Eng. **1954**, *32*, 35-53.

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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).