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Laboratory Experiment

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

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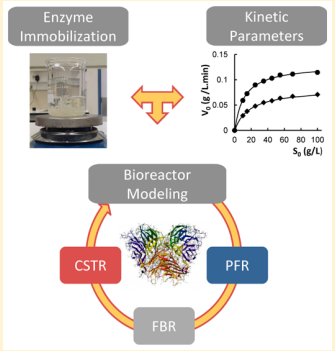
Supporting Information

ABSTRACT: An integrative laboratory study addressing fundamentals of enzyme catalysis and their application to reactors operation and modeling is presented. Invertase, a β-fructofuranosidase that catalyses the hydrolysis of sucrose, is used as the model enzyme at optimal conditions (pH 4.5 and 45 °C). The experimental work involves 3 h of laboratory time for each student per week over four weeks. Students, organized in laboratory sessions of three groups with 3 or 4 students per group, work in a collaborative manner to obtain a set of replicates of initial reaction rate for different substrate concentrations, using both free and calcium alginate-immobilized *Saccharomyces bayanus* cells containing biologically active invertase. The results are shared by all of the groups for statistical data treatment, calculation of Michaelis−Menten kinetic parameters, and modeling of three types of classical reactors, a Continuous Stirred Tank Reactor (CSTR), a Plug Flow Reactor (PFR), and a Fluidized Bed Reactor (FBR), operating in continuous mode with immobilized invertase. For each reactor, the experimental conversion profile as a function of the feed flow rate is compared to predicted profiles based upon the kinetic parameters obtained.



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Enzymes can advantageously replace chemical catalysts in many processes for the synthesis of products of interest in the areas of food and pharmaceutical chemistry.[1−3] Invertase (E.C. 3.2.1.26) catalyzes the hydrolysis of sucrose to glucose and fructose[4] and has an important role in the food industry. Immobilization on a variety of supports has been widely employed to make the application of enzymes as catalysts in industrial processes easier and economically viable. Immobilization methods allow the conversion of large quantities of substrate while favoring biocatalyst reutilization over multiple cycles, and make the product purification process simpler.[5−8] Among the several known methods, the entrapment in hydrogels is cheap, nontoxic, and safe to biocatalysts. One of the most used hydrogels is alginate, which complexes with Ca^{2+} ions forming a reticulated matrix with porosity adequate to retain cells.[9,10]

■ ENZYMATIC REACTORS

Continuous flow reactors are common in chemical industries. However, such flow processes are often not taught in undergraduate chemistry/biochemistry laboratory classes.[11] Specifically, the application of immobilized enzymes as biocatalysts at a manufacturing scale requires the use of reactors such as the Continuous Stirred Tank Reactor (CSTR), the Plug Flow Reactor (PFR) or the Fluidized Bed Reactor (FBR).[12−14] It is, therefore, important to ensure that students in Chemistry, Biochemistry, Pharmacy, and Chemical and Biological Engineering have a theoretical background in enzyme kinetics/immobilization and reactor modeling, complemented with laboratory courses where they can put into practice such knowledge.

Modeling equations for different reactors can be derived from a mass balance to substrate(s)/product(s) and considering the enzyme kinetics follows the Michaelis−Menten model[12−14] (eq 1)

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

where v_0 is the initial reaction rate, v_{max} is the maximum reaction rate, K_M is the Michaelis constant, and S_0 is the initial substrate concentration.

The stirred tank is the most widely used geometry for industrial enzyme reactors. Agitation homogenizes suspensions, contributing to effective heat and mass transfer,[13] being very close to ideal mixing, meaning that, at steady state, the reaction rate and product/substrate concentration are the same in every given position in the reactor. The outlet stream composition is, therefore, assumed equal to that of the reaction medium. This makes CSTR inappropriate in situations where the product is toxic, but very useful when the substrate has an adverse effect on kinetics and stability.[12] The modeling of an ideal CSTR is described by eq 2

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$$\frac{v_w W}{Q} = S_0 x + K_M \frac{x}{1 - x} \quad (2)$$

where W is the total catalyst mass, Q is the volumetric flow rate, S_0 is the substrate concentration in the feeding stream, x is the conversion (number of moles of substrate consumed divided by the initial number of moles of substrate), and K_M and v_W are the Michaelis−Menten constant and maximum specific reaction rate, respectively, for the free enzyme in solution (intrinsic or inherent enzyme kinetic parameters).

In a packed-bed reactor with a plug flow regime, the substrate solution passes through a settled bed of particles held in a column and the product emerges continuously at the far end. The degree of conversion is determined by the time the fluid remains in the bed and depends on the length of the column. Fluid can pass upward or downward through a vertically mounted bed, although horizontal cylinders may also be used.[12] In an ideal PFR, it is assumed that conversion and species concentration vary axially, but are constant along the radial axis. This type of reactor has a low operational cost and a high production per unit volume, and is theoretically the most efficient reactor in terms of enzyme conversion with a particulate biocatalyst.[12] However, at a manufacturing scale, the difficulty in temperature control along the reactor may be an important disadvantage.[13] The performance of an ideal PFR is modeled by eq 3

$$\frac{v_w W}{Q} = S_0 x - K_M \ln(1 - x) \quad (3)$$

For multiphase/heterogeneous reaction systems (e.g., with a particulate biocatalyst in immobilized-enzyme catalysis), a FBR can be used alternatively to a PFR. In a FBR, the fluid is passed upward through the catalytic bed, at high enough velocities to suspend the solid. This process, known as fluidization, imparts good mixing with uniform temperature throughout the reactor.[12−14] The main drawback of a FBR is the high flow rates commonly needed to achieve fluidization and, consequently, the limited contact time between substrate and enzyme in a single-pass operation. The contact time can be increased when operating this reactor in recycle mode, with continuous product removal.[12] Formally, ideal PFR and FBR are modeled by the same equation (eq 3).

The basic kinetics of enzymes is similar whether they are free in solution or immobilized in a matrix. However, in flow reactors with immobilized enzymes, several factors (conformational, steric, partitioning, 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. In this case, continuous enzymatic reactors can be more accurately modeled by substituting the intrinsic K_M and v_w, in eqs 2 and 3, by the kinetic parameters of the immobilized enzyme (designated as apparent kinetic parameters) which take into account the external and internal limitations on mass transfer (real reactors).

TEACHING METHODOLOGY AND LEARNING OUTCOMES

This experiment is designed as an integrated laboratory activity utilizing low-cost, unsophisticated equipment and a simple and safe cell/enzyme immobilization method. It involves 3 h of laboratory time for each student per week over 4 weeks. Students are organized in laboratory sessions (shifts) of three groups with 3 or 4 students/group to perform the experiment autonomously with one instructor's supervision. In each laboratory session, students prepare an Excel data sheet containing the experimental results. All groups in the course share the entire set of data for analysis. After each 2 weeks of experimental activity, the instructor provides a "tutorial" class (of 3 h) to each lab shift to guide students in the treatment of results and mathematical modeling, overall data analysis/discussion and preparation of written reports.

A theoretical background on Protein/Enzyme Engineering (concepts of protein structure, enzyme activity/stability; enzyme kinetics; protein immobilization and reactors for bioprocess application), as well as basic scientific and computational skills, is required for this experiment. At the end of the experiment, students should be able to (i) know and understand the principles of enzyme kinetics and their application to biocatalysis; (ii) deal with equipment and methodologies employed in enzyme-based processes; and (iii) apply basic computational tools to model kinetics and operation of (bio)reactors.

EXPERIMENT

Different methodologies have been reported to study invertase kinetics, including, for example, the colorimetric reduction of 3,5-dinitrosalicilic acid,[15,16] the use of a commercial blood glucose meter[17] or nuclear magnetic resonance spectroscopy.[4] In the present study, invertase from whole yeast cells (a low-cost and accessible enzyme source) is used to introduce students to Michaelis−Menten kinetics, study the effects of enzyme immobilization on the kinetic parameters, illustrate the use of immobilized cells in bioprocesses, and to model the behavior of three types of classical reactors.

Experiment 1: Comparison of Kinetics of Free and Immobilized Enzyme

The goal of the first two laboratory sessions is the determination and comparison of the kinetic parameters of invertase from *Saccharomyces bayanus* cells, free (first session) and immobilized in calcium-alginate (second session). The initial reaction rate (v_0) of sucrose hydrolysis is determined for different initial substrate concentrations (S_0), ranging from 10 to 100 g·L^{-1} and a yeast cell concentration of 0.2 mg·mL^{-1}. The 3,5-dinitrosalicylic acid (DNS) method[15,16] is used to quantify the reaction products (glucose and fructose). The total amount of products is referred as "total reducing sugars" (TRS). In one laboratory session, each group determines the value of v_0 (g TRS·L^{-1}·min^{-1}) for 2−3 out of 8 initial sucrose concentrations (S_0). Results of v_0 vs S_0 are registered in an Excel file, analyzed for "eye-guided" fitting to a Michaelis−Menten profile and enzymatic assays repeated if necessary (outliers). At the end of each session, a whole set of coherent results of v_0 vs S_0 is obtained. Replicates from different laboratory sessions (minimum of three) are used for statistical analysis, and kinetic parameters for both free and immobilized enzyme are estimated from a Lineweaver−Burk (LB) plot and through direct fit of experimental data to the Michaelis−

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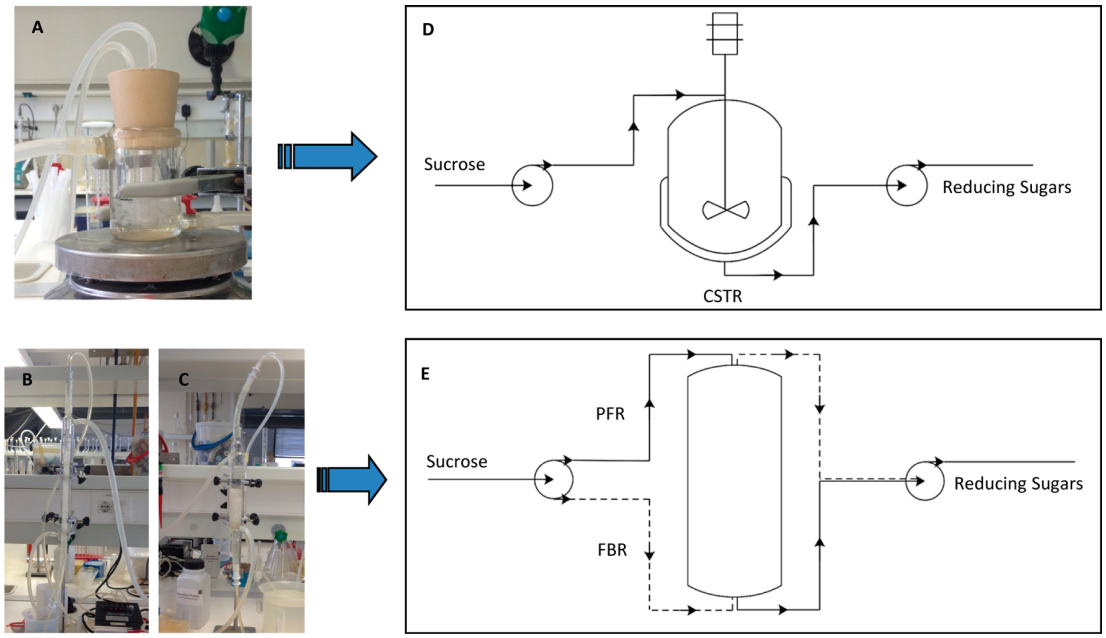

Figure 1. Flow enzymatic reactors used in this laboratory experiment (A) CSTR; (B) PFR; (C) FBR. Schematic representation of experimental setups: (D) CSTR and (E) PFR and FBR.

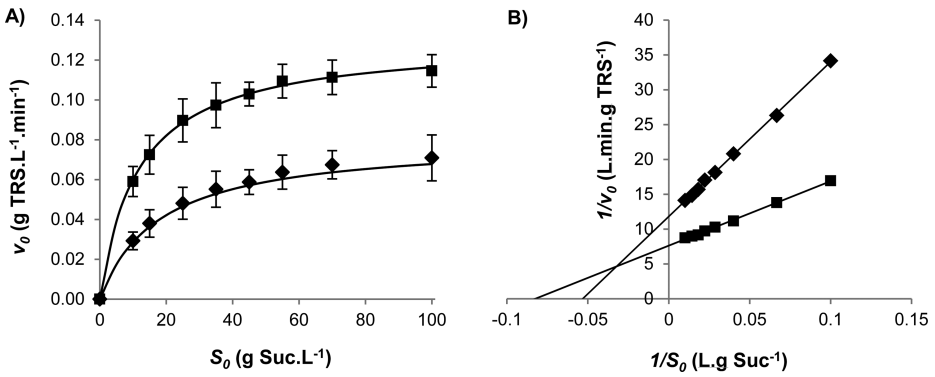

Figure 2. Kinetics of sucrose hydrolysis by invertase from *S. bayanus*. (A) Representation of Michaelis–Menten curves for free (■) and immobilized (♦) cells. (B) Lineweaver-Blurk plots for free (■) and immobilized (♦) cells. All assays were performed at 45 °C and pH 4.5 with 0.2 mg cells·mL^{-1}.

Menten equation. A detailed description of experiment 1 (including the procedure for yeast cell immobilization in calcium alginate) and data treatment is in the Supporting Information.

Experiment 2: Operation of Enzymatic Reactors with Immobilized Enzyme

The main goal of this experiment is to evaluate the performances of flow reactors (CSTR, PFR, and FBR) containing invertase from *S. bayanus* cells immobilized in calcium alginate, operating in continuous at 45 °C and pH 4.5 (Figure 1). This second experimental exercise involves two consecutive laboratory sessions (preparation and operation) with three groups of students. In every session, each group studies one type of reactor (CSTR, PFR or FBR), and at the end, the groups share the results of the three types of reactors for overall analysis/discussion. In the first session, students calibrate the pumps needed to feed the reactors at the desired flow-rates and immobilize *S. bayanus* cells (containing invertase) in calcium alginate beads. The immobilized enzyme is very stable and is kept at 4 °C until use. In the second session, reactors are filled up with the immobilized cells (mass of biocatalyst, W) and studied in continuous operation, using a feed solution with 10 g·L^{-1} of sucrose (S_0) for different flow rates (Q). At each flow rate, samples from the outlet stream are removed in steady state and analyzed by the DNS method to determine the total reducing sugar concentration (TRS) and conversion (x). For each type of reactor, the experimental conversion profile as a function of the feed flow rate is then compared with predicted (theoretical) profiles, which are obtained by solving eqs 2 and 3 in order to obtain the conversion (x), for a given flow-rate, using the kinetic parameters (for free and immobilized enzyme) obtained in experiment 1. A detailed experimental description (including the range of flow rates studied for each type of reactor; how to ensure sampling in steady-state and data treatment/modeling) is in the Supporting Information.

■ HAZARDS

Most reagents used this laboratory exercise are not hazardous substances. Exceptions include acetic acid and calcium chloride

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Table 1. Kinetic Parameters for Free and Immobilized Invertase Enzyme[a]

Enzyme State	Estimation Method	K_M (g Suc·L^{-1})	v_{max} (g Suc·L^{-1}·min^{-1})	v_W[b] (g Suc·g cells^{-1}·min^{-1})
Free	LB	12.1 ± 0.6	0.131 ± 0.003	0.656 ± 0.015
	Direct fit	11.7 ± 0.5	0.130 ± 0.003	0.650 ± 0.013
Immobilized	LB	18.8 ± 0.7	0.085 ± 0.002	0.425 ± 0.010
	Direct fit	19.0 ± 0.6	0.085 ± 0.002	0.425 ± 0.008

[a]Obtained from a LB plot and through direct fit of experimental data to the Michaelis−Menten equation. Average results obtained in 4 lab sessions (quadruplicates). [b]v_w is maximum specific reaction rate (i.e., v_{max} divided by the concentration of cells within the reactor in all enzymatic assays, 0.2 mg cells·mL^{-1}).

(used in the preparation of acetate buffer containing different sucrose concentrations) and 3,5-dinitrosalicilic (DNS) and sodium hydroxide (used for preparation of the DNS reagent). Following recommended safety rules, precautions to prevent skin and eye contact, inhalation and ingestion should be taken; students should wear protective clothes, eyewear and gloves to handle these chemicals or the final working solutions. Links to safety and hazards for all reagents used are included in the Supporting Information.

RESULTS AND DISCUSSION

Effect of Immobilization on Enzyme Kinetics

The kinetics of both free and immobilized enzyme followed the Michaelis−Menten model. As a first approach, v_{max} and K_M were determined using the Lineweaver−Burk plot, a current and convenient way to estimate Michaelis−Menten parameters (Figure 2).

However, LB linearization is based on a double reciprocal plot that favors error propagation.[18] Hence, the kinetic parameters were further tuned by adjusting the experimental values to the Michaelis−Menten model (eq 1), using the Solver tool of Microsoft Excel, through the least-squares method applied to the sum of the square differences between predicted and experimental values. Lineweaver−Burk parameters were used as estimates for iteration in mathematical modeling. Maximum reaction rates (v_{max}) of 0.131 and 0.085 g Sucrose·L^{-1}·min^{-1} and Michaelis constants (K_M) of 11.7 and 19.0 (g Sucrose·L^{-1}) were obtained for free and immobilized invertase, respectively (Table 1). These values fell within the range of values for v_{max} (0.10−0.14 and 0.07−0.10 g Sucrose·L^{-1}·min^{-1}) and K_M (11.0−14.0 and 17.0−20.0 g Sucrose·L^{-1}) for free and immobilized enzyme, respectively, obtained in laboratory classes over several years.

Expected effects of immobilization on the kinetic parameters included (i) biocatalyst denaturation, (ii) hindered activity, and (iii) mass transfer resistance through the alginate matrix. A higher K_M value generally indicates lower substrate specificity. However, in the system under study, the difference observed in the K_M values was apparent and originated from mass transfer resistance effects introduced by the gel-matrix and the generation of a gradient of substrate concentration inside the beads.[13] Occlusion of enzyme-containing cells in calcium alginate is a mild immobilization method that is unlikely to affect intrinsic biological activity.[3,10] Therefore, the reduction observed in v_{max} can also be explained by mass transfer limitations in the substrate transport across alginate beads,[13] which affects its access to the active center of immobilized enzyme molecules (saturation). Mass transfer resistance for each substrate concentration can be quantified by the ratio of v_0 for immobilized and free enzyme (see Supporting Information).

Reactor Operation and Modeling with Immobilized Enzyme

The experimental sucrose conversion by immobilized invertase at different flow rates in CSTR, PFR and FBR is depicted in Figure 3A−C. A flow rate increase resulted in a decrease of contact time between enzyme and substrate (with a negative effect on the conversion) and an increase in fluid turbulence that decreased the stagnant layer of liquid around the beads,[12−14] improving mass transfer (which favors conversion). Data in Figure 3 show that, in all reactors, the first effect is prevalent and substrate conversion decreased when the flow

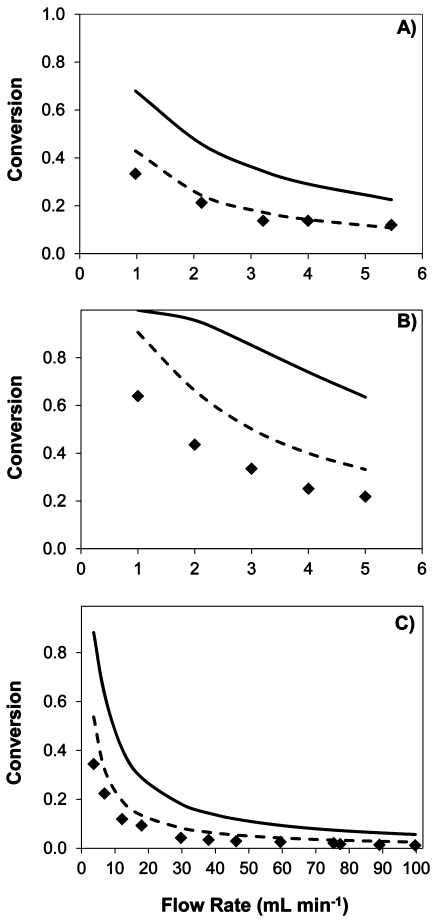

Figure 3. Experimental conversion (♦) obtained for (A) CSTR, (B) PFR, and (C) FBR at different flow rates and respective modeling using the enzyme intrinsic kinetic constants (—) and the apparent kinetic constants (---). Initial concentration of sucrose was 10 g·L^{-1}. Concentration of biocatalyst was 500 mg cells·g Alginate^{-1}. Working volume ≈10 mL for all reactors. Studies were performed at 45 °C and pH 4.5.

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rate increased. The range of experimental results fit to expected values of conversion for each type of reactor under studied conditions (obtained in laboratory classes over several years). For the CSTR, the conversions decreased from 0.345 to 0.125 and for the PFR from 0.640 to 0.218 when increasing the flow rate from 1 to 5 mL·min^{-1}; for the FBR, the conversion decreased from 0.345 to 0.012 when the flow rate was increased from ≈4 to 100 mL·min^{-1}.

With the use of the experimentally determined kinetic parameters (K_M, v_{max}) for the free enzyme (intrinsic parameters) and for the immobilized enzyme (apparent parameters), theoretical conversions were calculated, for each studied flow rate, using eqs 2 and 3 (Figure 3A–C). The calculated theoretical conversions using the intrinsic or inherent enzyme kinetic parameters (i.e., assuming an ideal reactor behavior) are much higher than the experimental values. Using the apparent kinetic parameters in eqs 2 and 3 for mathematical modeling, the calculated theoretical conversions are in better agreement with the experimental data because the kinetic parameters determined for the immobilized enzyme take into account mass transfer limitations due to enzyme immobilization (real reactor behavior). The greater convergence of experimental and "real reactor" profiles in the cases of CSTR and FBR likely resulted from the similarity of hydrodynamic conditions (e.g., fluid agitation) in these two reactors with those of the batch stirred tank reactor (BSTR) used in determination of the kinetic parameters for the immobilized enzyme. By contrast, in the PFR there is no fluid agitation and mass transfer limitations are likely more significant than in a BSTR, which is reflected in the poor agreement of experimental data with the predicted "real reactor" conversion profile.

■ ASSESSMENT

This integrated laboratory experiment has been successfully offered for several years to undergraduate students of B.Sc./M.Sc. in Chemistry, Chemical and Biological Engineering at Instituto Superior Técnico, University of Lisbon, within the scope of Enzyme Science and Technology learning courses. The data presented were gathered from written reports of a laboratory course of the B.Sc. in Biological Engineering (3rd year). Students were evaluated by their learning and execution abilities, with the following grade-weighting criteria: (a) participation in classes (25%); (b) final written report (50%); (c) final oral discussion (25%). Average final classification obtained was 16/20. In students' surveys for assessment of the quality of this laboratory experiment and interest to their formation *curriculum*, over the last four years 86–95% of students inquired found that this experiment was well-structured, promoted cooperative learning while also improving autonomous learning capacity, and provided good knowledge and comprehension of enzyme kinetics/application (average number of answers 57). Negative comments often referred to repetitiveness/moroseness of kinetic studies and "dead-time" in the operation of the reactors (waiting for steady-state sampling). Teachers' commitment, availability to clarify doubts in and out classes, as well as capacity to stimulate participation and promote discussion, were aspects highly scored by the students. Overall, pedagogic quality (including both classes and teachers' evaluation) was classified between 8 and 9 on a 0–9-point scale.

■ CONCLUSION

In the experiment described, students received hands-on experience with immobilization of biocatalysts, determination of enzyme activity and kinetic parameters, operation of enzymatic flow reactors, and fitting of experimental data to theoretical models. Collaborative work promoted students' engagement and active participation within the groups, leading to excellent experimental results.

■ ASSOCIATED CONTENT

Supporting Information

Experimental handout for students, instructors notes containing a list of all required chemicals, necessary instrumentation, advance preparations, detailed analytical methods and relevant information for data treatment. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.
Authors include students that contributed to elaboration of the manuscript.

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