LABORATORY EXPERIMENT IN BIOCHEMICAL ENGINEERING

Ethanol Fermentation

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The need for didactic experiments that will prepare our students for their professional futures, together with the importance of the ethanol industry in Brazil, led us to design and construct an experimental bench-scale kit for determining the kinetic parameters related to the ethanol fermentation process.

The experiment's design was based on the principles that guided construction of the Didactic Laboratory of Chemical and Biochemical Reactions at our university, which included a laboratory composed of didactic kits for short experiments prepared by the students that would complement classroom knowledge.

A set of three bench-scale fermentors was designed and constructed that would support groups of five students per fermentor. To standardize the experiments, one of them was used in two types of cultivation: the first at low substrate concentration ($\cong 16.7\ gL^{-1}$ of glucose) and the second at a concentration of 60 gL^{-1} to allow fitting of traditional kinetic models without and with inhibition by the product, respectively.

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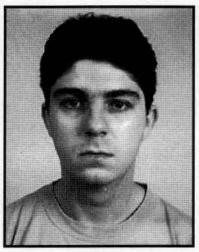

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THEORY

We know that in a favorable environment, simple sugars (monosaccharides) are transformed in ethanol and carbon dioxide (CO_2) by the action of yeasts as follows:

$$\underset{\text{(hexose)}}{C_6H_{12}O_6} \xrightarrow[\text{yeast (30°C)}]{} \underset{\text{(carbon dioxide)}}{2\,CO_2} + \underset{\text{(ethanol)}}{2\,C_2H_5OH}$$

It can be seen that for each mole of hexose, equal molar amounts of ethanol and carbon dioxide are produced.

In fermentation processes that synthesize primary metabolites such as ethanol, cell growth and product generation take place simultaneously. Here the cellular growth and the product synthesis are directly related. Therefore, the ethanol production can be predicted from the cellular growth kinetics. From the hypothesis that the concentration of cells is a good measure of the enzymatic system responsible for the transformation of the substrate into product, it is convenient to define the specific growth rate (μ) as

$$\mu = \frac{1}{X}\frac{dX}{dt} \qquad (1)$$

where dX/dt is the variation of the cellular concentration (X) with time (t).

Several models have been proposed to relate the specific growth rate (μ) to the limiting nutrient and inhibitor concentrations. A classic kinetic model relating the specific growth rate to the limiting substrate was proposed by Monod.[1,2] Working in a continuous process, he obtained the following relationship, also applied to batch processes:

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$$\mu = \mu_{max} \frac{S}{K_S + S} \quad (2)$$

where μ_{max} is the maximum specific growth rate achievable when $S >> K_S$, S is the concentration of growth-limiting nutrient, and K_S is the saturation constant or the value of the limiting nutrient concentration at which the specific growth rate is half of μ_{max}. It is known that this model is valid for cultivations using low initial limiting substrate concentrations (S_0).

In ethanol fermentation, high initial substrate concentrations (S_0) generate high product concentrations (P) that inhibit cellular growth and, consequently, the production of ethanol. Various relationships relating the effect of ethanol concentration (P) to the specific growth rate (μ) of the organism have been reported.[3-5]

One type of relationship that presents similarity to the non-competitive inhibition in enzyme kinetics has been proposed[3] for modeling ethanol inhibition of *Saccharamyces cerevisiae.* A term accounting for ethanol inhibition is added to the simple Monod kinetic model, giving

$$\mu = \mu_{max} \frac{S}{K_S + S} \frac{K_p}{K_p + P} \quad (3)$$

where P is the ethanol concentration and K_p is the product inhibition constant. From Eq. (3), we see that the higher the ethanol concentration, the higher its negative effect will be on the specific growth rate (μ).

Still, in a fermentation process, the concentration of cells (X) and product (P) can be related to the limiting substrate concentration (S) by the yield coefficients

$$Y_{X/S} = \frac{dX}{dS} = \frac{X - X_0}{S_0 - S} \quad (4)$$

$$Y_{P/S} = \frac{dP}{dS} = \frac{P - P_0}{S_0 - S} \quad (5)$$

MATERIAL AND METHODS:

◑ **Microorganism** *Saccharomyces cerevisiae* (commercial Fleischmann's baker's yeast) was grown in two different media for ethanol production.

◑ **Media** Two different media have been used in the experiments: media 1 and 2 in ASSAYS 1 and 2, respectively.

◑ **Medium 1** (low substrate concentration) in gL^{-1}: commercial corn glucose (90% w/w in glucose), 20.0; KH_2PO_4, 5.0; $MgSO_4.7\,H_2O$, 0.4; yeast extract, 3.0; $(NH_4)_2SO_4$, 1.8; commercial antifoam (dilution 1:10), 5 drops; pH=4.6; solvent, distilled water.

◑ **Medium 2** (high substrate concentration) in gL^{-1}: commercial corn glucose, 66.0; KH_2PO_4, 5.0; $MgSO_4.7\,H_2O$, 0.4; yeast extract, 3.0; NH_4Cl, 2.5; commercial antifoan (dilution 1:10), 5 drops; pH=4.6; solvent, distilled water.

◑ **Experimental Equipment** The fermentors were made of glass, adapting 1000-mL "kettle"-type (Pyrex) recipients with flat bottoms. Fermentor lids were made of "technyl" (nylon) and adapted to the opening of the recipients. Agitation was accomplished using magnetic stirrers adapted to the base of the fermentors. The temperature was controlled through solenoid valves activated by bulb and capillary controllers. Wells for controlling and monitoring temperature, and tubes for heat transfer ("U" tubes), sampling, inoculation, and gas exit, were made of stainless steel and connected to the lids of the fermentors.

Devices for determining the volume of CO_2 liberated by the fermentation were constructed of PVC (polyvinylchloride) pipes with internal diameters of 10 cm and heights of 100 cm and connected to the gas exits of the fermentors, based on equipment proposed by Nilsson, et al.[6] A scheme of the experimental apparatus is shown in Figure 1.

◑ **Analytical Methods** Cell concentration was evaluated as dry weight. Broth samples were centrifuged, washed twice, and resuspended with distilled water. Aliquots were diluted and the absorbance of the suspension was measured at 650 nm with a spectrophotometer (Micronal B-395). The concen-

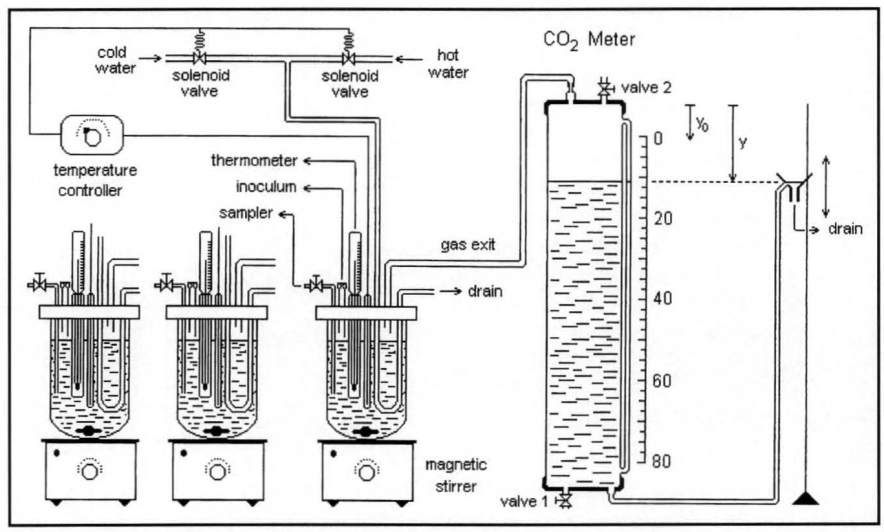

Figure 1. *Schematic of the experimental apparatus.*

tration of the yeast suspension (X) in gL^{-1} was related to the absorbance according to the following calibration curve: X=0.817 Abs, valid for X < 0,45 gL^{-1}. Glucose concentration (S) was determined as total reduction sugars by the colorimetric Somogyi method[7] using the same spectrophotometer. Ethanol concentration (P) was determined by the method of oxidation by potassium dichromate after distillation and indirectly relating to the volume of CO_2 formed.

◑ **Measurement of CO_2 Volume Produced by Fermentation** According to the stoichiometry of the microbial reaction, for each mole of consumed glucose, two moles of ethanol and CO_2 are produced. Therefore, if it is possible to measure the amount of CO_2 produced by the reaction, this can be related to the concentration of ethanol present in the fermentation broth. Such a procedure facilitates the didactic experimental routine as ethanol analysis by the method of oxidation by potassium dichromate or other conventional method is time consuming, requiring the supernatant of the centrifuged sample to be distilled and diluted for titration.

Measurements of the CO_2 volume produced by the fermentation process were accomplished in the following manner. Initially, the pipe was filled with water from the bottom (valve 1) up to the level y_0 measured from the top of the pipe, maintaining valve 2 open. Before cultivation was started, valves 1 and 2 were closed. The CO_2 produced left the fermentor through the gas exit tube and entered from the top of the pipe, pushing the water level down. The water level was monitored by a transparent tube connected vertically to the PVC pipe. According to hydrostatic principles, in order to maintain constant atmospheric pressure in the head of the fermentor, the water level in a thin tube connected to the base of the PVC pipe was controlled manually by draining the water.

The number of moles of CO_2 produced during fermentation can be calculated at any time by the ideal gas law

$$n_i = \frac{P_{atm} V_i}{RT} = \frac{P_{atm} \pi D^2 (y_i - y_0)}{4\,RT} \qquad (6)$$

where

- n_i number of moles of CO_2 evolved at time t=i
- V_i gas volume at time t=i
- R gas constant [R=82.04 atm cm^3/(g-mole K)]
- T gas temperature [K]
- D internal diameter of the PVC pipe [D=10 cm]
- y_i distance from the top of the PVC tube to the water level at time t=i
- y_0 Distance from the top of the PVC tube to the water level at t=0

◑ **Experimental Procedure** Two fermentation assays were carried out at 30°C using the culture media previously presented. First, the 1000-mL fermentors containing 700 mL of culture media were sterilized in an autoclave at 121°C for thirty minutes. After sterilization, the temperature was maintained at 30°C. Then, 100 mL of inoculum activated in a shaker with different cell concentrations (X_0) was added, completing 800 mL of initial working volume. The inoculum is activated prior to its addition to the culture medium in order to eliminate the adaptation stage of the microorganism to the culture medium (the "lag" phase of the process). Samples of 10 mL were withdrawn approximately every half hour, right after inoculation up to complete depletion of the glucose (end of fermentation). At the moment of sample withdrawal, the distance between the top of the PVC pipe and the water level (y_i) was measured. Samples were divided into two aliquots of 5 mL for analysis of the concentrations of substrate (S), ethanol (P), and cellular mass (X). The assays carried out at low and high initial substrate concentrations (S_0) were designated as ASSAY 1 and ASSAY2, respectively. Ethanol concentrations were determined analytically only in ASSAY 2 to be related to the CO_2 volume produced. In ASSAY 1, the ethanol concentrations were determined indirectly by the CO_2 volume produced.

RESULTS AND DISCUSSION

The results obtained in ASSAYS 1 and 2 are shown in Table 1. Usually, fermentation processes are relatively too time-consuming to be used in didactic experiments, but standardization of the assays provided reasonably short experiments of 4.5 and 3.0 hours, respectively.

From the experimental data of ASSAY 2, it was possible to relate the numbers of moles of ethanol and CO_2 generated by the ethanol fermentation. The number of moles of CO_2 evolved (n_{CO_2}) was calculated by Eq. (6) and the number of ethanol moles ($n_{ethanol}$) formed was estimated as

$$n_{ethanol} = PVM_{ethanol} \qquad (7)$$

where P is the ethanol concentration in the broth (in gL^{-1}), V is the broth's volume (in L), and $M_{ethanol}$ is the ethanol molecular weight ($M_{ethanol}$=46).

TABLE 1
Experimental Results Obtained in ASSAYS 1 and 2

ASSAY 1				ASSAY2			
time (h)	S(gL^{-1})	x(gL^{-1})	P(gL^{-1})	time (h)	S(gL^{-1})	x(gL^{-1})	P(gL^{-1})
0.0	16.66	1.08	0.00	0.00	60.00	18.38	0.00
0.5	15.78	1.09	0.46	0.58	48.25	19.80	2.79
1.0	14.94	1.15	1.03	1.17	35.73	22.51	7.18
1.5	12.97	1.37	1.83	1.75	26.60	23.78	10.37
2.0	11.19	1.56	2.40	2.33	15.38	24.43	15.16
2.5	9.68	1.76	3.66	2.92	5.86	25.83	18.35
3.0	6.69	2.06	4.69				
3.5	3.52	2.52	5.72				
4.0	0.88	2.72	7.10				
4.5	0.10	2.84	7.44				

Figure 2 illustrates the good linear relationship between values of $n_{ethanol}$ and n_{CO_2}. The linear regression of the experimental data resulted in a slope of 1.006 and a regression coefficient (R^2) of 0.994, showing that the theoretical-experimental methods, proposed to determine the ethanol concentration indirectly in the broth by the volume of CO_2 produced, generated very good results.

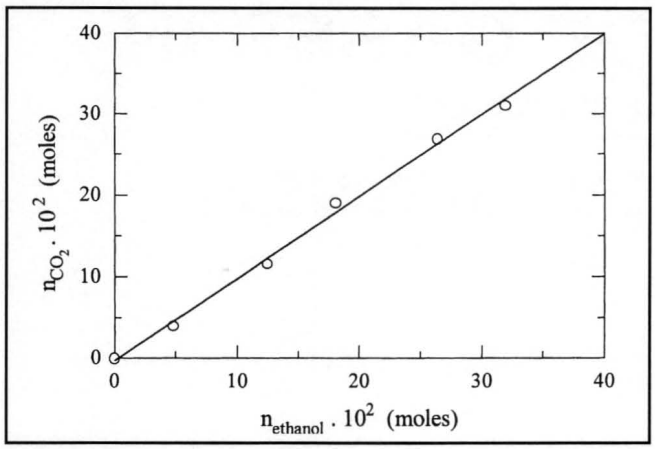

Figure 2. *Relationship between the number of moles of ethanol formed ($n_{ethanol}$) and CO_2 evolved (n_{CO_2}) during ASSAY 2.*

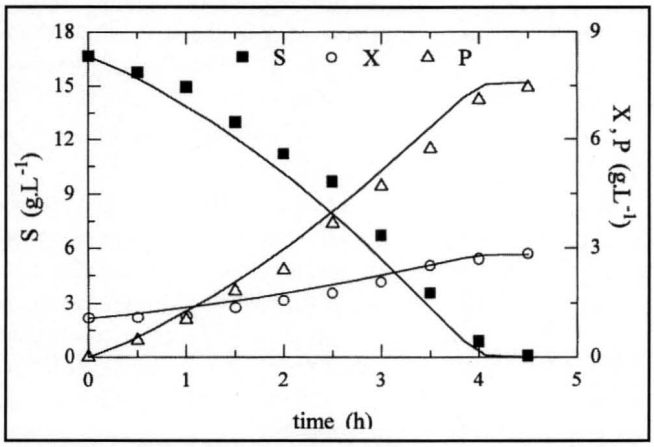

Figure 3. *Fit of Monod's model to the experimental data of ASSAY 1.*

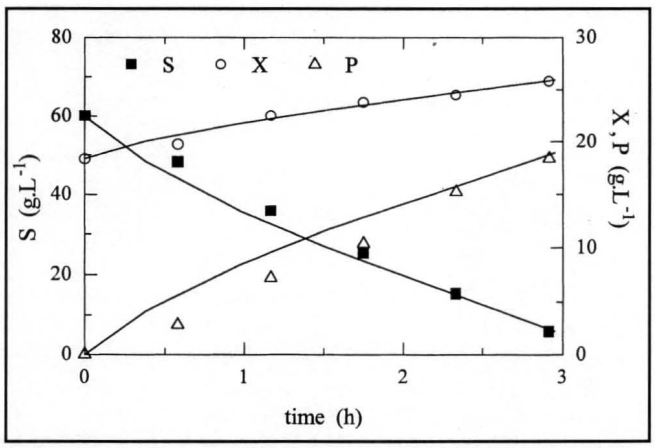

Figure 4. *Fit of the Aiba, et al., model[3] to experimental data of ASSAY 2.*

Yield coefficients $Y_{X/S}$ and $Y_{P/S}$ were determined by experimental data (Table 1) as being: $Y_{X/S}$=0.11—$Y_{P/S}$=0.46 (ASSAY 1) and $Y_{X/S}$=0.14—$Y_{P/S}$=0.35 (ASSAY2), respectively.

Experimental results were analyzed based on two classical kinetic models. Initially, Monod's model (Eq. 2) was fitted to the experimental data of ASSAY 1. The specific growth rate (μ) was estimated by Eq. (1) where dX/dt was calculated from the polynomial equation fitted to the curve X(t). The kinetic parameters, μ_{max}=0.32 h^{-1} and K_S=0.63 gL^{-1}, were obtained by nonlinear regression of μ and S values according to Eq. (2) using Marquardt's algorithm.

In the same way, the kinetic model proposed by Aiba, et al.,[3] was fitted to the experimental values of ASSAY 2, considering the kinetic parameters, μ_{max} and K_S, obtained previously (ASSAY 1). The product inhibition constant (K_p) was estimated as being 6.29.

Figures 3 and 4 illustrate the good fits of the kinetic models of Monod and Aiba, et al., to the experimental values of the assays carried out at low and high initial substrate concentration, ASSAY 1 and ASSAY 2, respectively. A fourth-order Runge-Kutta technique was applied to simulate the predicted curves of S, X, and P as a function of time.

For comparison, Table 2 shows the values of the kinetic parameters evaluated from the data of other workers.[8] It can be seen that the values of the kinetic parameters obtained by the present work are within the range of values encountered in the literature showing that the proposed methodology can be of great value for educational and research purposes.

CONCLUSIONS

The equipment allowed us to perform short-duration didactic experiments (4.5 and 3.0 hours) using low-cost raw materials. The relationship of ethanol/CO_2 produced by the fermentation was very close to unity (1.006), showing the precision of the equipment. Use of the CO_2 meter in didactic

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TABLE 2
Kinetic Parameters

	T (°C)	μ_{max} (h^{-1})	K_S (gL^{-1})	K_p (gL^{-1})	$Y_{P/S}$ (-)
Egamberdiev and Ierusalimskii[8]	28	0.31	-	20.6	0.39
Aiba and Shoda[8]	30	0.43	-	55	0.35
Pironti[8]	30	0.26	15.5	13.7	0.47
Cysewski[8]	35	0.58	4.9	5.0	0.44
Bazua and Wilke[8]	35	0.64	0.24	40	0.52
Hoppe and Hansford[8]	30	0.64	3.3	5.2	0.43
This work	30	0.32	0.63	6.29	0.35-0.46

CONCLUSIONS

This paper presents a simple way to enhance the learning experience in the unit operations laboratory by adding a related design problem to the lab requirements. This idea can be implemented in any classical unit operations experiment such as distillation, absorption, liquid-liquid extraction, or even in a heat-exchanger experiment.

By implementing this approach, the students connect lab work to real-life problems, their creativity is stimulated, and the concepts are learned enduringly. The feedback from the students confirms this. It is apparent that they felt more comfortable the following term when they had to apply these concepts to design absorption, stripping, and even distillation columns as part of the task in the senior design course.

NOMENCLATURE

- a gas-liquid interfacial area per unit total volume [m^2/m^3]
- G_x liquid-phase flow rate per unit cross-sectional surface area [m]
- G_y gas-phase flow rate per unit cross-sectional surface area [m]
- H_{OL} height of an overall transfer unit based on liquid-phase concentrations [m]
- K_x overall mass-transfer coefficient [m/s]
- L liquid-phase flow rate [kg/s]
- m Henry's constant expressed as a ratio of mole fractions
- N_{Ox} number of overall transfer units based on liquid-phase concentrations
- Re_x liquid-phase Reynolds number
- Re_y gas-phase Reynolds number
- S stripping (desorption) factor
- Sc_x liquid-phase Schmidt number
- Sh_{Ox} liquid-phase Sherwood number based on K_L
- V gas-phase flow rate [kg/s]
- x_a mole fraction of solute in the liquid phase at the inlet
- x_b mole fraction of solute in the liquid phase at the outlet

Greek Symbols

- α exponent in Eqs. (1) and (3)
- β exponent in Eqs. (1) and (3)
- γ exponent in Eq. (3)
- φ coefficient in Eq. (3)
- ψ coefficient in Eq. (1)

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experiments of ethanol fermentation dispensed with the distillation of the sample and the chemical analysis of ethanol. The experimental data obtained in the assays allowed fits of a simple kinetic model (Monod) and of one that takes into account the inhibition by the product.[3] Comparison of the kinetic parameters obtained in the present work with others in the literature showed that the experimental device has attained the expected aim.

NOMENCLATURE

- D internal diameter of the PVC pipe [D=10 cm]
- K_p product inhibition constant [gL^{-1}]
- K_S saturation constant [gL^{-1}]
- $M_{ethanol}$ ethanol molecular weight [$M_{ethanol}$=46]
- n_{CO_2} number of moles of ethanol formed
- n_i number of moles of CO_2 evolved at time t=i
- P product concentration [gL^{-1}]
- P_{atm} local atmospheric pressure or barometric pressure [atm]
- R gas constant [R=82.04 atm cm^3/(g-mole K)]
- R^2 regression coefficient [-]
- S limiting substrate concentration [gL^{-1}]
- T gas temperature [K]
- t time [h]
- V broth's volume [L]
- V_i gas volume at time t=i [mL or cm^3]
- X cellular concentration [gL^{-1}]
- y_0 distance from top of PVC tube to water level at t=0 [cm]
- y_i distance from top of PVC tube to water level at t=i [cm]
- $Y_{X/S}$ cell yield coefficient [gg^{-1}]
- $Y_{P/S}$ product yield coefficient [gg^{-1}]

Greek

- μ specific growth rate [h^{-1}]
- μ_{max} maximum specific growth rate [h^{-1}]

Subscripts

- 0 refers to the time t=0
- i refers to the time t=i

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