A BATCH FERMENTATION EXPERIMENT FOR L-LYSINE PRODUCTION

In the Senior Laboratory

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Biochemical processes are finding increasing application in the chemical industry for the production of a wide variety of products from renewable resources. These products include pharmaceuticals, consumer and food products, fuel additives, industrial enzymes, and many others. They are typically created using batch processing, a marked departure from the more traditional continuous processes for commodity chemicals. Recent graduates from chemical engineering programs are finding more opportunities for employment in industries that use biochemical processes and perhaps fewer opportunities on a percentage basis in traditional commodity chemical and petrochemical production.[1]

Biochemical processes are complex, involving multiple steps in converting raw material into products. In addition, preparation steps and downstream separations are not typical of traditional chemical processing. Examples of chemical engineering laboratory experiments using biochemical processes have recently appeared.[2-4] In these experiments, ethanol is typically produced in short-duration experiments that are, by necessity, abbreviated and less complex than most industrial fermentations. In order to prepare undergraduates for opportunities in biochemical processing and to provide a laboratory experience with a complexity similar to a commercial process, we have developed a batch fermentation experiment to produce L-lysine for the senior laboratory.

In this experiment, student groups produce L-lysine, an essential amino acid, from a glucose minimal defined media in batch culture (fermentation). In this article, we will describe the pedagogical approach, the objectives for a semester-long design of experiments, and key results from the fermentation experiment.

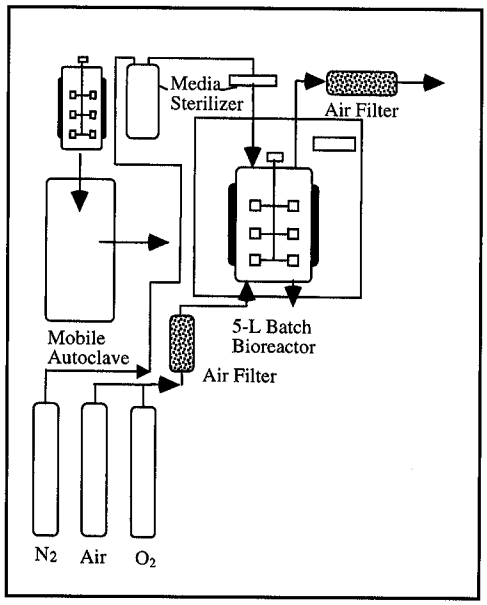

Figure 1. *Batch fermentation experiment for L-lysine product from a defined minimal media containing glucose*

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OVERVIEW OF THE EXPERIMENT

The L-lysine batch fermentation experiment is shown schematically in Figure 1. It is conducted using a 5-liter bioreactor (New Brunswick Scientific BioFlow3000) and a data acquisition and control system (New Brunswick Scientific BioCommand). With this system, students study the kinetics of microbial growth and L-lysine production under controlled conditions of temperature, pH, dissolved oxygen (DO), and agitation. Auxiliary equipment includes a mobile autoclave sterilizer (New Brunswick Scientific) and a media microfiltration unit (Fisher Scientific).

Approximately 60 to 100 senior-year chemical engineering students annually conduct the batch fermentation experiment in the "Chemical Plant Operations Laboratory" course. Due to the complexity of this experiment, students work in teams comprised of two 4-member groups. The fermentation experiment requires two to three days of continuous operation to complete due to the slow kinetics of cell growth and L-lysine production. Table 1 shows the sequence of events for this experiment.

TABLE 1
Schedule for L-lysine Experiment

Orientation	Week 1
Proposal preparation	Weeks 1 and 2
Pre-laboratory check-in	Start of week 3
Laboratory experiment	During week 3
Post-laboratory oral presentation	During week 4
Final report preparation	Weeks 4 and 5

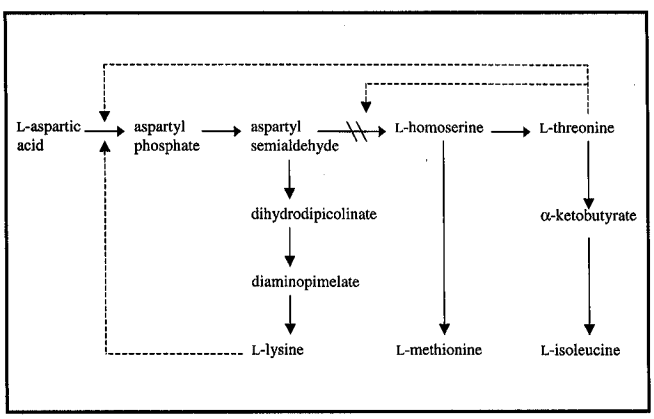

Figure 2. *Feedback inhibition for regulation of L-lysine synthesis within the cell. Dashed lines indicate feedback inhibition of key enzymes in the metabolic pathway (solid lines).*

TABLE 2
Experiment Plan for Cell Growth and L-lysine Production
(Amino acid base case values are L-threonine (150 mg/L), L-methionine (40 mg/L), and L-leucine (100 mg/L)

	Glucose Concentration (g/L)	
Amino Acid Concentration	*20*	*30*
1. Low (50% lower)	Team 2	Team 5
2. Base case	Team 1	Team 4
3. High (50% higher)	Team 3	Team 6

PEDAGOGICAL OBJECTIVES

The first pedagogical objective for the fermentation experiment is to introduce the students to biochemical process equipment and to explain the key steps for production of a biochemical product. Because most of the graduating seniors have little or no biochemistry or biochemical engineering experience, the experiment objectives are geared toward an introductory treatment. Prior to conducting the experiments, we give a 2-hour orientation and provide background information on L-lysine production using *Corynebacterium glutamicum* (American Type Culture Collection, ATCC No. 21253), we conduct a tour of the laboratory, and hold a discussion of experiment objectives.

We give background information in an oral presentation to the 8-member student team and describe cell growth in the context of the major growth stages: lag, exponential, deceleration, stationary, and death. Specific metabolic characteristics of *C. glutamicum* are described as shown in Figure 2.[5] We further explain that due to a mutation in the cellular DNA by chemical treatment, this cell cannot convert aspartyl semialdehyde to L-homoserine. In order to grow the cells on a glucose minimal medium, L-methionine, L-isoleucine, and L-threonine must be added in trace amounts. Once these supplemented amino acids are consumed by growth, any remaining glucose is converted to L-lysine rather than cell mass. We explain that concerted feedback inhibition of the enzymatic conversion of L-aspartic acid to a aspartyl phosphate is relaxed as L-threonine is consumed, thus allowing overproduction of L-lysine. When these concepts are understood, we tell the students that cell growth and product formation are expected to occur separately in the batch culture. One of the objectives for the student teams is to test this hypothesis and also to determine if the amount of supplemented amino acids controls the maximum concentration of cells in the fermentation.

The second part of the orientation is a tour of the laboratory facilities. We describe each piece of equipment and explain its purpose in the production of L-lysine. We emphasize the importance of maintaining sterile conditions and show the students the two methods of sterilization used; steam autoclaving for the bioreactor and microfiltration for the growth media. We discuss scale-up and the need for coordinating processes at smaller scales to support production at a larger scale (*e.g.*, flask-scale cultures for inoculating the fermenter and the associated equipment). Finally, we explain that the safety aspects of the laboratory are consistent with Biosafety Level I requirements (Center for Disease Control, CDC). The

last part of the orientation is a discussion of handout materials (available upon request by e-mail from <drshonna@mtu.edu>) and a schedule for meeting the requirements as outlined in Table 1.

Another pedagogical objective is to test the effects of initial glucose and amino acid concentrations on L-lysine production and cell growth in a *design of experiments*. As shown in Table 2, this design of experiments involves six teams during the semester. The goal is to involve the student teams in a

Safety is integrated into all aspects of the undergraduate chemical engineering laboratory experience. . . In the design phase . . . a thorough safety review of the bioprocessing equipment, procedures, chemicals, and biological organisms was conducted.

continuous improvement exercise and to increase their understanding of how fermentation parameters affect cellular growth and L-lysine production. Each team conducts an experiment at different initial glucose and amino acid concentrations. During the semester, as experiments are completed and results become available, sharing the data with the other student teams is intended to increase the level of understanding about this fermentation process for the entire class. Student teams share their results by attaching reports and presentations to an e-mail to the instructor—the cumulative results (as shown later in Table 6) are then organized and disseminated by the instructor to the student teams (by e-mail attachment) during the final days of the semester.

EXPERIMENTAL METHODS

Following the orientation, each team prepares and submits a proposal in which students demonstrate their familiarity with the process equipment, the objectives, laboratory safety (chemical, physical, and biological hazards), sample calculations, and the market aspects of their product. Because of scheduling limitations, during the 52-hour experiment the teams are split into two groups. One group from the team initiates the fermentation over a 4-hour period. This involves formulating the growth medium, assembling and autoclaving the bioreactor, sterilizing the medium and transferring it to the bioreactor using microfiltration, calibrating O_2 and pH probes, and finally inoculating with flask-grown cells. During the next 48 hours, all students in the team periodically sample for cell growth, glucose consumption, and L-lysine production (no sampling is done between midnight and 8 a.m., however).

Each run in the experiment plan is conducted under identical conditions of temperature (30°C), pH (7.0), dissolved oxygen (50% of saturation with air), and duration (52 hours). The experiment objectives given to each team are shown in Table 3. The maximum specific growth rate is obtained by applying the Monod equation[6] to the definition of the specific growth rate, μ, as

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{1}$$

where X is the concentration of cells in the medium (g/L). The Monod equation is

$$\mu = \mu_{max}\frac{S}{K_S + S}\frac{A}{K_A + A} \tag{2}$$

where μ_{max} is the maximum specific growth rate constant (hr^{-1}), S and A are the concentration of glucose and supplemented amino acids, respectively (g/L), and K_S and K_A are the half saturation constants (g/L). At the start of the fermentation, $S >> K_S$, $A >> K_A$, and therefore $\mu = \mu_{max}$ in Eq. (1). The solution to Eq. (1) for exponential growth is

$$\ell n\frac{X}{X_0} = \mu_{max} t \tag{3}$$

For cell growth, samples from the bioreactor are taken at 2-hour intervals on the first day and at 4-hour intervals on the second and third days. Mass concentrations are obtained by first measuring the absorbance (at 500 nm wavelength, A_{500}, Milton Roy Spectronic 21D) and converting those values using the conversion factor, y (g dry cell wt./L) = 0.5x, where x is A_{500}. Every 8 hours, samples are taken for glucose

TABLE 3
Fermentation Experiment Objectives

1. Determine maximum specific growth rate, μ_{max} (hr^{-1})
2. Measure glucose consumption (g/L)
3. Measure L-lysine production (g/L)
4. Determine cell growth yield, $Y_{X/S}$
5. Determine L-lysine production yield, $Y_{P/S}$

TABLE 4
Major Steps in the Experiment Procedure for L-lysine Production in Batch Culture

1. Assembly of fermenter and microfilter for steam sterilization
2. Steam sterilization of fermenter and microfilter
3. Media preparation
4. Filter sterilization of culture media
5. Calibration of pH and dissolved oxygen probes
6. Initialize data acquisition
7. Fermentation
8. Sampling for cell, glucose, and L-lysine
9. Analysis of glucose and L-lysine samples
10. Shutdown and clean-up of fermenter

and L-lysine analysis by filtering 5 ml of cell culture solution through a 0.2 μm (polycarbonate, 25 mm dia. Millipore GTTP02500) membrane and into a closed capped vial (20 ml) to remove cells. These samples are then stored in a refrigerator (4°C) until the end of the experiment, when they were analyzed together by the second group of the team. Glucose concentration is analyzed using the hexoskinase/glucose-6-phosphate dehydrogenase method (INFINITY Glucose Reagent, Sigma Scientific) and L-lysine concentration by using the saccharopine dehydrogenase assay (Sigma Scientific S-9383). The yield of cell growth on glucose consumed ($Y_{X/S}$) is calculated as $Y_{X/S} = \Delta X/\Delta S$ and the data are taken over the exponential and deceleration growth stages. The yield of L-lysine produced on glucose consumed ($Y_{P/S}$) is calculated as $Y_{P/S} = \Delta P/\Delta S$ and the data are taken over the entire fermentation period, but especially during deceleration and stationary stages of cell growth (when L-lysine production occurs). Although different student groups conducted the initiation and sample analyses portions of the experiment, the group that was not "on duty" was encouraged to drop into the laboratory to observe the activities of the other group, and many students did so when their class schedules permitted.

TABLE 5
Composition of Defined Minimal Media for L-lysine Production using *C. glutamicum.*
(All values are per liter of final solution)

- 20 grams D-glucose
- 5 g $(NH_4)_2SO_4$
- 8 g K_2HPO_4
- 4 g KH_2PO_4
- 0.2 g $MgSO_4 \cdot 7\ H_2O$
- 1.0 g NaCl
- 0.5 g citric acid
- 20 mg $FeSO_4 \cdot 7\ H_2O$
- 50 mg $CaCl_2 \cdot 2\ H_2O$
- 150 mg L-threonine
- 40 mg L-methionine
- 100 mg L-leucine
- 1 mg biotin
- 1 mg thiamine · HCl
- 10 ml 100x trace salts

100x Trace Salts Solution; per liter of distilled water

- 200 mg $MnSO_4$
- 6 mg H_3BO_3
- 4 mg $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$
- 100 mg $FeCl_3 \cdot 6\ H_2O$
- 1 mg $ZnSO_4 \cdot 7\ H_2O$
- 30 mg $CuSO_4 \cdot 5\ H_2O$

(pH of this solution adjusted to 2 to avoid precipitation)

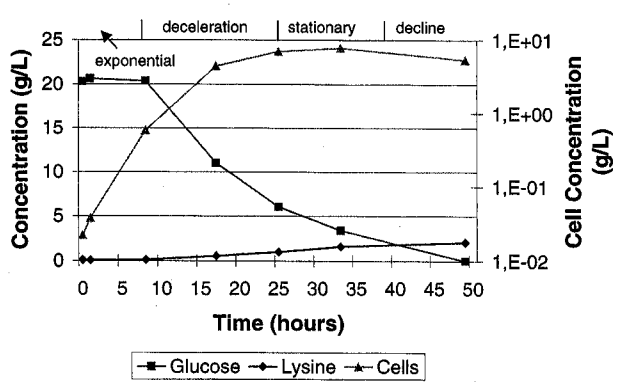

Figure 3. *Results for cell growth, glucose consumption, and L-lysine production for initial concentrations of 20 g/L of glucose and base case amounts of amino acid supplements.*

The major steps in the fermentation procedure are shown in Table 4. Table 5 shows the composition of the defined medium for the fermentation per liter of solution. Handout materials for this experiment can be obtained in electronic format (PDF file) by contacting the author at <drshonna@mtu.edu>. Materials available include an overview of the semester-long experiment plan, an introduction to bioprocess safety issues, and detailed steps in the fermentation preparation, start up, and sample analysis.

RESULTS AND DISCUSSION

Figure 3 shows a set of results for the cell growth, glucose consumption, and product formation for these experiments using *Corynebacterium glutamicum.* Cell data shows four stages of batch growth: exponential, deceleration, stationary, and declining. Glucose is consumed fastest during the exponential and deceleration stages and more slowly during the stationary and declining stages. L-lysine production is most rapid during the deceleration stage and increases to the greatest amount during the decline stage. This observation is consistent with the metabolic pathway shown in Figure 2, with L-lysine production in *Corynebacterium glutamicum* being greatest after the added amino acids are largely consumed and cell growth ceases, and during the period that concerted feedback inhibition of the L-lysine metabolic pathway is released. The students are made aware of the difference between growth-associated versus non-growth-associated product formation. Figure 3 provides an example of mixed growth-associated product formation—that is, intermediate between the two types. Results from the remaining experiments (for the most part) showed similar trends for the batch culture data.

Table 6 shows the results for all six teams from the semester-long experiment plan. For the 20 g/L initial glucose concentration experiments, the maximum cell concentration decreased (from 9.5 to 4.0 g/L) when the initial amino acid concentration was decreased by 50%, but cell concentration did

not increase (it decreased slightly from 9.5 to 8.5 g/L) as expected from the metabolism shown in Figure 2, when the initial amino acid concentration was increased by 50%. The absence of this additional cell growth may be due to the increase in L-lysine production. An increase in the initial amino acid concentration of 50% did increase the ultimate L-lysine concentration (from 2.09 to 7.5 g/L), whereas a decrease in the initial amino acid concentration did not significantly change the L-lysine concentration.

For the 30 g/L initial glucose experiments, again the maximum cell concentration decreased (from 8.0 to 3.9 g/L) when the initial amino acid concentration was decreased by 50%, but (contrary to the 20 g glucose/L results) the ultimate L-lysine concentration increased (from 2.55 to 10.0 g/L). The results for 30 g glucose/L and 150% amino acid concentration were compromised because the dissolved oxygen probe failed during the run, causing the culture to become anaerobic and changing the cell growth and L-lysine production characteristics. This team proceeded in the same manner as the other teams. They measured cell concentration, plotted a cell growth curve, measured glucose consumption and lysine production, and calculated all growth and yield parameters. The purpose for doing this in this case was to measure effects of anaerobic growth conditions on fermentation performance.

The cell growth yield, $Y_{X/S}$, varied from 0.27 to 0.99 for these experiments, with the exception of the last experiment, which became anaerobic, as mentioned previously. These values are in the range typically found for aerobic culture on glucose and similar growth substrates.[6] The highest value violates a carbon mass balance, however, which predicts a maximum biomass yield of

$$Y_{X/S} = \frac{g\,bio.}{0.5\,gC}\,\frac{72\,gC}{180\,gsugar} = 0.8\,gbio./gsugar$$

for typical values for biomass dry weight fraction carbon of 49-51%. Most likely, this erroneous result came from measurement error on glucose, as the cell mass measurement is more accurately obtained. The L-lysine production yield varied over the range of 0.14 to 0.60 for the various experiments.

The results from this experiment plan for cell growth and L-lysine production confirm the student's prior understanding regarding metabolism for this culture, as shown in Figure 2. Maximum cell growth did decline approximately in proportion to the decrease in the initial amino acid concentration, although it did *not* increase with increasing amino acid concentration. Additional experiments are needed to reduce uncertainty in measured results, which may help explain the higher-than-possible biomass yield observed in one of the experiments. Enhanced L-lysine production was observed compared to the basecase conditions for two experiments, 20 g glucose/L, 150% amino acid concentration and 30 g glucose/L, 50% amino acid concentration. From the results thus far, however, the exact mechanism for this enhanced production is not yet understood.

Table 6, along with a summary narrative of the results from the entire set of experiments, was developed by the instructor and disseminated by e-mail attachment at the end of the semester to the students who participated in the fermentation experiments. The narrative contained a summary of key results for these fermentation experiments:

1. *The supplemental amino acids limit the maximum cell concentration that is achieved during fermentation.*
2. *Cell growth and L-lysine production appear to occur in separate stages of the fermentation.*
3. *It is possible to increase L-lysine concentration by the end of the fermentation by altering initial glucose and amino acid concentrations.*

This end-of-semester summary provided the cumulative results needed to address the two most important experiment objectives: testing the hypothesis that maximum cell concentration in the fermentation is affected by the initial concentration of supplemented amino acids and identifying whether initial glucose and amino acid concentrations could be altered to enhance L-lysine production.

The Department of Chemical Engineering at Michigan Tech has an assessment program for the evaluation of student learning outcomes. As required by ABET 2000 Criteria, we use these assessments to monitor student proficiency in mastering chemical engineering fundamentals and for improving faculty teaching effectiveness.

In this assessment program there are eight major efforts,

TABLE 6

Summary of Student Team Results from the Fermentation Experiment Plan

(Base case concentrations of amino acids [L-threonine, L-methionine, and L-leucine) are given in Table 5)

	Team 1	*Team 2*	*Team 3*	*Team 4*	*Team 5*	*Team 6*
Initial Glucose Concentration (g/L)	20	20	20	30	30	30
Initial Amino Acid Concentration	Basecase	1/2 Basecase	150% Basecase	Basecase	1/2 Basecase	150% Basecase
Maximum L-lysine Concentration (g/L)	2.09	2.21	7.51	2.55	10.0	0
Maximum Cell Concentration (g/L)	9.5	4.0	8.5	8.0	3.9	2.1
μ_{max} (1/hr), Max. Specific Growth Rate	0.38	0.50	0.33	0.43	0.42	0.30
τ_d (hr) Doubling Time	1.82	1.39	2.09	1.6	1.64	2.31
$Y_{X/S}$ (g cells/g glucose)	0.47	0.33	0.35	0.27	0.99	0.02
$Y_{P/S}$ (g L-lysine/g glucose)	0.14	0.60	0.32	0.23	0.60	0

one of which is an assessment of student outcomes in the Senior Laboratory. From a critical reading of student team reports by members of the faculty, we evaluate how well students communicate in writing, the thoroughness of data analysis and discussion of results, how well they function in teams, and how proficient they are in understanding the experimental system, in developing an experimental plan, and in conducting that plan. We include this fermentation experiment in the assessment plan for the Senior Laboratory.

It is apparent from reading these reports that the students understand the basic concepts of microbial growth and growth stages during batch fermentation, concerted feedback inhibition of enzymes for amino acid production, and growth-associated and nongrowth-associated product formation. Thus, from the one-hour orientation and out-of-class readings from the handout materials, the students appear to be assimilating and retaining the biochemical concepts needed to interpret the experimental results. Also, the majority of student teams have demonstrated that they are up to the task of carefully executing the detailed experimental procedures provided to them, although admittedly a good deal of faculty and teaching assistant supervision is required to achieve good results.

SAFETY CONSIDERATIONS

Safety is integrated into all aspects of the undergraduate chemical engineering laboratory experience at MTU. In the design phase, before any equipment was purchased, a thorough safety review of the bioprocessing equipment, procedures, chemicals, and biological organisms was conducted. The physical and chemical hazards in this laboratory are common to other chemistry or chemical engineering laboratories: contact or ingestion of concentrated HCl and NaOH; flammability hazards; hazards of high-pressure bottled air, O_2, and N_2; and hazards of poor housekeeping.

In addition to the chemistry laboratory safety concerns, *Corynebacteria glutamicum* is classified as a Level 1 biohazard (the lowest biohazard classification). To mitigate the additional hazards of biological agents, the Center for Disease Control (<www.cdc.gov>—search for biosafety) recommends specific standard practices including

- *Restricting access to the laboratory*
- *Washing hands with antimicrobial soap prior to leaving the laboratory*
- *Disinfecting all work surfaces with 75% ethanol after any spill*
- *Decontamination (by autoclaving or use of 3% bleach) of all cultures, growth media, equipment, and disposables after use*

Proper preparation of growth media, sterilization of equipment before use, sterile transfer of growth media into the reactor, and proper inoculation techniques are critical to the success of fermentation, and all these aspects expand the students' awareness beyond the traditional chemical engineering experience. Couple this with the biosafety program, and students are well prepared to enter this exciting area of the chemical engineering profession.

CONCLUSIONS

A batch fermentation experiment to produce L-lysine was developed for the Chemical Engineering Senior Laboratory at MTU. The experiment objectives and procedures are appropriate for an introductory treatment of batch fermentation processes, microbial growth, and metabolism. A semester-long experiment plan has been implemented to test for the effects of initial amino acid and glucose concentrations on cell growth and L-lysine production in long-term experiments (52 hours).

The distribution of tasks between the two student groups in each team appears to result in a reasonable level of student effort in this long-term experiment. Judging from the oral and written reports, the students appear to understand the fundamental biochemical principles (provided during a pre-laboratory one-hour orientation and from handout materials) at a level sufficient to interpret experimental results. Considering that most students had little or no prior biochemistry education, this outcome is viewed as positive.

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