MEMORANDUM

Department of Chemical Engineering
Michigan Technological University

TO: BioUO Lab Team Cycle 2, Week 1 L-lysine Production in a Bioreactor
    Experiment starts Tue. Feb. 9, 2004 in room 205 (CSEB)

FROM: David R. Shonnard
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DATE: 22 March, 2004

SUBJECT: L-Lysine Fermentation Objectives

Introduction
Fermentation involves the growth of microorganisms in various bioreactor configurations for the purpose of obtaining some product. The product may be a biomolecule (protein, enzyme, amino acid, etc.) or the cells themselves. The growth characteristics of the microorganisms as they metabolize the carbon source is a very important aspect of the process. In this experiment, you will grow an auxotrophic (the cell lacks the ability to biosynthesize one or more key amino acids required for protein / enzyme production and growth) mutant strain of the bacterium, Corynebacterium glutamicum, on a six carbon sugar (glucose), using an automated bioreactor operated in batch mode over a 2-3-day period. You will monitor for the change in concentration of the cells, glucose, and L-lysine in the culture over time. The task of the Tuesday group will be to set up and run the experiment. Both groups will take samples periodically over the run. The Thursday group tasks will analyze the samples for glucose and lysine and shut down the experiment and clean up. One report will be submitted by the Team.

Objectives
You are to;
1. Conduct a batch growth experiment of this bacterium utilizing the carbon substrate glucose in a defined growth medium over a 2-3-day period. Culture the bacteria under the following set of conditions; 30 °C, pH = 7.0, dissolved oxygen at a setpoint of 50% of saturation value (in contact
with air), **20 g/L glucose**, and **1/2x base case concentrations of amino acids**. The media preparation instructions are provided as part of this assignment.

a) Periodically measure the absorbance of the samples (about 5 ml) taken from the bioreactor at 500 nm wavelength ($A_{500}$ for cells) using a visible spectrophotometer (Milton Roy 21D), and the absorbance at 340 nm ($A_{340}$) for both glucose and L-lysine. The conversion between absorbance and cell numbers is $y = 1.034 \times 10^9 x$, where $y$ is the cell numbers per milliliter of solution and $x$ is $A_{500}$. If you wish to express cell concentration in units of mg dry cell weight, you can convert from cell numbers ($y$) to mg by knowing that there are 0.5 mg dry cell wt. for each $10^9$ cells. From the data over this time period, identify

i) the main growth stages and then

ii) calculate the maximum specific growth rate ($\mu_{\text{max}}$) for this bacterium growing on glucose and

iii) its doubling time at maximum growth rate (hrs). Your group will develop the calibration curves to convert from $A_{340}$ to concentrations of glucose and L-lysine using the provided assay procedures.

iv) From your absorbance data for glucose and L-lysine, calculate the yield coefficients for cell growth on glucose ($Y_{X/S}$) and for L-lysine production on glucose ($Y_{P/S}$). For this yield calculation, only consider the period of time when cells are actively growing for ($Y_{X/S}$) and when lysine is being produced for ($Y_{P/S}$).

2. Using the first draft procedures provided to you, conduct the laboratory experiment (in the presence of the faculty advisor and TA) and note improvements to be made. Integrate these improvements into the existing procedures (this statement of objectives and procedures will be attached to email) paying particular attention to the safety and operational aspects. For example, are there ways to speed the preparation for the experiment safely? You should submit these improved procedures as appendices to your final report and mark them in bold print. Also, you should thoroughly discuss the procedure improvements in the body of the final report and address why they are needed.

3. Discuss your cell growth and L-lysine production results in the context of the known metabolism of *Corynebacterium glutamicum*. For example, does final cell concentration in culture increase or decrease with increase or decrease in initial amino acid concentration? To answer this you will need to have access to the results from previous cycles. I will provide you with those results, but please remind me. Is L-lysine production growth associated or non-growth associated and which of these would you expect from your knowledge of
Corynebacterium glutamicum metabolism and pathway for L-Lysine production? Are measured yields in the range expected from the literature? Compare to data from your handouts.

You may wish to contact me well in advance of preparing your laboratory proposal in order to acquaint yourself with the equipment, the location of any chemicals used in this experiment, and the use of the computer data acquisition equipment. In addition, read over the handout material prior to writing your proposal in order to gain a better understanding of microbial growth kinetics.
Procedures (Sequential Order)
To start the experiment, the Bioreactor unit and the Media Filtration unit have to be sterilized. The first step will then be autoclaving these two pieces of equipment. It is recommended to sterilize the Media Filtration prior to the scheduled experiment day, to save time. The procedure follows a sequential order.

Before starting, a couple of tips on using pipetters is needed. The TA and/or Instructor will demonstrate the use of the micropipetter and the pipette gun. For the micropipetter, depress the plunger only half-way down to draw liquid into the pipette. Press the plunger all the way down to expel liquid. For the pipette gun, never draw liquid into the gun through the end of the pipette.

I. PREPARATION OF THE MICROFILTER FOR STERILIZATION
The microfilter has to be assembled before the sterilization (do this step on Monday night)
1. Secure the three legs of the assembly with the hex head wrench.
2. Place a new 0.2 \( \mu \text{m} \) pore size filter centered onto the filter screen on the bottom half of the microfilter assembly.
3. Place the top on and hand tighten the three wingnuts.
4. Attach the tygon tubing onto filter outlet, making sure the hose clamps are loose.
5. Plug the open ends of the outlet tygon tubing with glass wool and wrap them with foil. Both ends of the inlet tubing should be covered with foil. The unit is now ready for sterilization.
6. Autoclave by following the autoclaving instructions

II. AUTOCLAVING INSTRUCTIONS

Material that can be sterilized:
(a) Glass: Type 1 borosilicate containers only
(b) Plastic: Heat resistant plastic containers only
(c) Liquids: Do not sterilize cultures or chemical solution containing salt water, strong acids or alkalis. Also, do not cover any liquid with a closed screw cap or other stopper.

The specific item that will need to be sterilized for the bio process experiment are:
(a) Media-filtration unit: This includes the filter stand with the filter screen, the adapter that goes to the inoculation port of the reactor, and the tygon tubing associated with the media filtration step, i.e., the hose from the pressure vessel to the filter and the one from the filter to inoculation port of the reactor.
(b) The Bioreactor unit: this includes the reactor vessel, the condenser, the sampling device, sparger tube, gases inlet hose and filter, and the condenser hose.

Getting Started:
1. Make sure that the power switch is turned off and check to see that all of the water is drained from the reservoir. Once drained make sure that the drain valve is closed. Drain valve is located bottom left front behind the small door.
2. Turn the door handle counterclockwise until it stops. Open the door by turning the arm counterclockwise.
3. Take the baskets out of the autoclave
4. Locate the “V” shaped water level indicator through the center of the heater cover and pour in distilled or deionized water until the wedge of the V is submerged. Three liters of water are required.

5. Remove the exhaust tank from the unit located on the left bottom corner of the autoclave. Fill the tank with distilled or deionized water up to the LOW level mark. Insert the exhaust hose back into the tank, making sure that the end of the hose is completely submerged in the water. Secure the tank pushing it in, alternately turning the gasket at the end of the hose left and right. Load the exhaust tank back into the unit after making sure that the hose is not bent or twisted.

6. Gently place the items to be sterilized in the chamber.

7. Close the EXHAUST KNOB.

8. Close the door by turning the arm clockwise. Turn the handle clockwise until it is tight. Feed the MAGNETIC LATCH under the handle and connect it to the SAFTEY SWITCH on the lower panel.

9. Turn the MAIN POWER SWITCH on. The flashing of the DOOR Lamp will indicate that the door is securely shut. The initial setting temperature and pressure will flash alternately and the “C” and “MIN” indicator lights will flash in sequence, confirming the standby status of the unit.

The unit is now ready for operation.

**Sterilization procedure:**

1. The unit has a default temperature of 121 C. The allowable range of sterilization temperatures is 105-126 C. To set the desired temperature, press the TEMP button and use the arrow keys to either increase to decrease the setting in one-degree increments.

2. The allowable time for sterilization has a range from 1 –180 minutes. Use the default setting of 15 minutes for sterilization. In order to change the default settings, press the TIME button and use the arrow keys to adjust the setting in one-minute increments.

3. Press the START button. A short beep will sound and the C indicator lamp will light to indicate the start of the process. When the temperature reaches 80 C, the digital display will register the temperature.

4. The high-pressure lamp will flash when the temperature reaches 98 C.

5. The unit will maintain the temperature at 100-101 C for about 4 minutes while it evacuates the chamber and builds pressure.

6. When the temperature inside the chamber reaches the set temperature, the timer will start will start to operate and the timer operation segment will begin to flash.

**Completion of the cycle:**

1. When the process is finished, a beep will sound and the C indicator will flash indicating the end of the sterilization cycle. The digital display will show the temperature of the chamber.

2. The depressurizing step will now start. The EXHAUST KNOB may be opened very slightly to expedite the process after the temperature reaches 90 C.

3. A short beep followed by a longer one will be emitted when the pressure inside the chamber equals the atmospheric pressure.

4. When the temperature inside the chamber has reached 80 C five beeps will sound in succession, signifying the end of the entire process. Turn the MAIN POWER SWITCH off.

5. Disconnect the MAGNETIC LATCH of the handle from the SAFTEY SWITCH and attach it to the MAGNET PLATE on the handle.
6. Turn the handle counter clockwise and open the door. The door lam will cease to flash once the door is opened.
7. Remove the sterilized items from the chamber. Wear insulated glove when removing the items, as they will be hot.

III. FERMENTER ASSEMBLY
Assemble the fermenter with the assistance of the TA or the faculty advisor, have extra care when handling the fermenter.
1. If needed, insert and secure the sparger tube, harvest tube thermowell, and sampling tube into their respective ports.
2. Attach the condenser onto the top of the reactor.
3. Plug the remaining openings in the top plate with glass wool and cover them with aluminum foil.
4. Fill the openings of the sample probe with glass wool and cover with foil.
5. Wrap the end of the condenser in foil and glass wool.
6. Add 100 ml of de-ionized water to the bottom of the reactor before autoclaving.
7. Make sure the lid of the bioreactor is connected and centered.
8. Autoclave the fermenter (follow the autoclaving instructions).

IV. MEDIA PREPARATION (Base Case Formulation-Modify as Needed)
While the fermenter is being autoclaved, the media preparation can be done. Check with the faculty advisor on how to adjust for high or low amino acid concentration prior to starting.
A. Defined Media
Make a total of 4.5 liters of distilled-water based media from the following stock solutions.
The ingredients for the media on a basis of 1 liter of distilled water are:
20 grams D-glucose (Add Glucose last!!)
5 g (NH4)2SO4
8 g K2HPO4
4 g KH2PO4
0.2 g MgSO4• 7H2O
1.0 g NaCl
0.5 g Citric Acid
20 mg FeSO4• 7H2O
50 mg CaCl2• 2H2O
150 mg L-threonine
40 mg L-methionine
100 mg L-leucine
1 mg biotin
1 mg thiamine•HCl
10 ml 100x Trace Salts
Set 20 ml aside prior to adding Glucose for the Glucose Monitoring measurement (Part XII).
Label this sample "defined media -glucose". After adding Glucose, set 20 ml aside.
Refrigerate these samples after using.
100x Trace Salts Solution: per liter of distilled water (provided)
200 mg MnSO₄
6 mg H₃BO₃
4 mg (NH₄)₆Mo₇O₂₄•4H₂O
100 mg FeCl₃•6H₂O
1 mg ZnSO₄•7H₂O
30 mg CuSO₄•5H₂O (next page please)
(pH of this solution adjusted to 2 to avoid precipitation)

Base and Acid Solution Preparation
300 ml of 6 N NaOH
500 ml of .1 N HCl

B. Complex Media (not for this run!!)
Make a total of 4 liters of distilled-water based media from the following stock solutions.
The ingredients for the media on a basis of 1 liter distilled water are:
10 g tryptone
5 g yeast extract
5 g D-glucose
3 g K₂HPO₄
1 g KH₂PO₄

First make the stock solution from the ingredients, making sure that they are at the right amount
for 4 liters (for instance, 5 grams of D-glucose * 4 liters = 20 grams D-glucose). Use clean
conical flasks or beakers of the appropriate size. Employ cleaned magnetic stirrers to help in
dissolving the reagents in the water.

When you have a well-mixed solution, add some hydrochloric or sulfuric acid to it and bring the
pH to around 7.0 (use the pH meter to monitor the pH). Use the magnetic stirrer to get a
homogeneous solution.

V. MEDIA FILTRATION
1. Remove the magnetic stirrer bar from the media flask with a magnetic wand. Pour the
solution into the high-pressure vessel. Close the lid of the high-pressure vessel securely.
2. Make sure that the nitrogen cylinder valve is closed, then connect the high pressure hose to
the inlet port of the high-pressure vessel. Make sure that the inlet valve and the pressure
relief valve are closed.
3. Check the tygon tubing for breaks or tears. Connect the outlet port of the vessel to the
upstream (top) port of the media-filtration unit using a length of unsterilized tygon tubing.
Make sure that the outlet valve is closed and the hose clamps tightened to prevent leaks.
4. Securely screw in the adapter to the inoculation port of the bioreactor vessel. Connect this
adapter to the downstream (bottom) port of the media filtration unit using sterile tygon tubing
and secure all hose clamps. Inspect this tygon tubing for tears or breaks.
5. Make sure the valve on the N₂ regulator leading to the hose is shut. Open the main N₂ valve
on the tank. Adjust the regulator to 10 psi. Start the nitrogen flow to the vessel by opening
the valve on the N₂ regulator leading to the hose. Open the inlet valve to the high-pressure vessel.

6. Open the outlet valve on the outlet port of the high-pressure vessel. The pressure exerted by the nitrogen should drive the media through the filter and into the bioreactor vessel.

7. When the flow of the media into the bioreactor has slowed to a trickle, shut the outlet valve of the high-pressure vessel. Open pressure relief valve on microfiltration unit. Remove tubing and disconnect from the bioreactor.

8. Unscrew the adapter to the inoculation port of the bioreactor vessel and close the port.

9. Shut off the nitrogen flow to the high-pressure vessel at the nitrogen cylinder main valve, maintaining a pressure of 10 psi.

10. Carefully open the pressure-relief valve on the high pressure vessel to vent out the nitrogen in the vessel by standing it on end, keeping the valve leading from the regulator to the hose open, and the N₂ inlet valve on the high pressure vessel open. Keep the pressure-relief valve open until all pressure is released. Close valve on N₂ regulator outlet.

11. Disconnect the hoses. Rinse the vessel with distilled water. Also clean the filter out by removing filter then running distilled water through it.

12. Fill all open ports on filter with glass wool and cover with tin foil.

13. Close all valves.

VI. FERMENTER OPERATION

These steps must be performed with the close supervision of the TA or the faculty advisor.

1. Put thermocouple into its port.

2. After the media is in the bioreactor, the inlet air hose must be connected to the console port.

3. Turn the oxygen and air cylinders on and regulate the pressure to 10 psig. Turn on outlet valves on regulators.

4. Connect reactor jacket water lines.

5. Connect the condenser water lines to the condenser. Make sure to have the supply lines connected correctly.

6. Seal all air vents from the top of the reactor to vent gases through condenser. Affix flexible tube to top of condenser gas output and lead up to the vent system trunk.

7. Turn on the water valve and the power to the fermenter console.

8. Place agitator motor on top of the fermenter impeller and plug the power cord into the console port.

9. On the fermenter console keypad, the computer should already enter FERMENTER MODE.

10. Enter MASTER SCREEN.

11. Enter TEMP LOOP and set to MODE to PRIME. Keep it here for about a minute.

12. Enter TEMP LOOP and set to PID mode, then set the desired temperature set point.

13. Change AGIT 1 mode to PID. (check for water flow out) Set AGIT to 200.


15. Calibrate the pH probe outside of the fermenter. First, connect the pH cable to the probe and the console. Set up the pH buffers.

16. Enter CALIBRATION screen using the console keypad.

(a) Place probe into 7.0 pH buffer solution

(b) Set pH FUNCTION to READ.

(c) Wait until the value is stable.

(d) Set FUNCTION to ZERO
(e) Enter the value of 7.0 into the ZERO column
17. Setting up the SPAN
   (a) Set pH FUNCTION to READ
   (b) RINSE PROBE with distilled water
   (c) Place probe into 4.0 pH buffer solution
   (d) Wait until the value is stable
   (e) Set FUNCTION to SPAN
   (f) Enter the value of 4.0 into the SPAN column
18. Return to READ mode and return to MASTER SCREEN.
19. Turn off impeller by turning AGIT 1 mode to off.
20. Remove the motor. This will allow for less obstruction when inserting the probe.
21. Sterilize the pH probe with ethanol and insert into the fermenter. Place motor on the top of reactor.
22. Turn on impeller by turning AGIT 1 mode to PID.
23. Calibrate the dissolved oxygen probe by doing the following. Set AIRFLOW to the fermenter to 1.0 (L/min) by entering CALIBRATION screen and making sure the DO is set to READ.
24. Enter Gases screen and set to MANUAL mode. Air = 100, N₂ = 0.
25. Return to MASTER screen and change right control loop to AIR 1 mode, adjust the air flow using the valve knob located on the right side of the fermenter until the flow rate is reading about 1.0 – 1.5 L/min. Note: on Wednesday during the morning or afternoon, you will need to up the flow rate of gas to 2.0 - 2.5 L/min in order to maintain the DO = 50.
26. Return to CALIBRATION screen and set DO to ZERO MODE, and set ZERO column to 0.0 on key-pad. Fill DO probe with polarographic O₂ filling solution and assemble probe. Sterilize exterior with 70% ethanol and insert into reactor carefully. Attach DO lead to DO probe.
27. Wait until the DO reading achieves a steady value. This may take about 15 minutes. Set DO to SPAN mode. Set the SPAN to read 100 (indicating that the media is saturated with air, and reads 100 on the console).
28. Make sure both the pH and DO are in READ mode.
29. Return to MASTER SCREEN.
30. Enter AGIT LOOP and set the agitation speed to 50 rpm and change control to PID.
31. Set the DO2 loop to 02 mode and AGIT 1 LOOP to DO mode.
32. Set DO at 50% or desired Set Point. Go to AGIT LOOP again, and set the maximum agitation rate to 200 rpm.
33. Set up the feed for the NaOH solution by placing the feed tube in feed pump 1. Place the lower end of this tube in the NaOH solution (= 6 N) and connect the upper end of this tube to the appropriate port on the bioreactor. IMPORTANT: Place the NaOH flask in a plastic bucket to prevent it from being knocked over and spilling. Also, verify that the pump rotation (clockwise) will deliver the solution to the bioreactor.
34. Change pH control to PID. Verify that Right Control Loop, Feed 1, is designated as the base pump. Change Set to 10.
35. Attach the sampling valve to the sampling port.

VII. COMPUTER PROGRAM SETUP
1. Once the computer has loaded, go to START, PROGRAMS, NBS, BIOCOMMAND. This will start up the program that will be recording the readings.
2. Next in the window go to CONFIGURE menu, PROCESS SETUP. This should now put you in the log file.
3. Under PROCESS DESCRIPTION place the following information: experiment, group number, group members, date, temperature, pH, and DO values. Keep log interval at 1 minute.
4. Next click DETAILS, PROCESS LOOPS. In this menu click on AGIT, DO2, TEMP, AND PH (with set point = 7). Click OK.
5. At this point you should save your program and give it a name.
6. Click FILE menu, VIEW PROCESS.
7. Now click TOOLS, TOOL BOX. This will show the toolbox. Click on the graph button, to generate a graph. Now use the hand tool to click on the graph. A menu will pop up. Select:

<table>
<thead>
<tr>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation</td>
<td>50</td>
</tr>
<tr>
<td>DO2</td>
<td>0</td>
</tr>
</tbody>
</table>

Click OK.
8. Click back on the pointer tool and create another graph. This time in the pop-up menu select:

<table>
<thead>
<tr>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>20</td>
</tr>
<tr>
<td>pH</td>
<td>0</td>
</tr>
</tbody>
</table>

Click OK.
9. Now the process is ready to read data.

VIII. INOCULATING THE BACTERIA AND TAKING SAMPLES
Dr. Shonnard will provide the bacteria that will be inoculated into the fermenter.
1. Add 0.25 ml of polypropylene glycol into the fermenter to prevent foaming.
2. Through the inoculation port add 40 ml of the bacteria solution.
3. To get a sample from the bioreactor, unscrew the sampling vial slightly, squeeze the rubber bulb, retighten the sampling vial, and open the sampling valve slightly until about 5 ml has been taken. Unscrew the vial to remove the sample. Before each sample is taken, a sample should always be taken and discarded to ensure that the sample vial is not ‘contaminated’ with the previous sample remaining in the downtube. After a sample is taken and analyzed, the sample should be discarded in a beaker or plastic container to be disinfected with bleach at the end of the experiment.
4. Take samples every 2 hours for the measurement of cell absorbance.
5. To take absorbance readings of the samples, follow procedure number IX.
6. Take samples every 8 hours starting at time = 0 for glucose and L-lysine. Withdraw about 6 ml into the sample vial. Filter about 3-4 ml of this sample using the syringe and filter cassette (0.22 or 0.45 µm pores size filters) into a disposable 20 ml capped vial. Label with time and date and place in the refrigerator. These will be analyzed at the end of the experiment for glucose and l-lysine concentration.

IX. READING THE ABSORBANCE (SPECTROMETER OPERATION)
1. Turn the power switch on.
2. Use the mode button to change to absorbance mode.
3. Change the desired wavelength by using the knob next to the wavelength screen.
4. To calibrate the spectrophotometer, use sterile media as your calibration media. Insert the clean cuvette in the sample reader filled with sterile media up to the fill line. Use the “INCREASE KNOB” to calibrate to .000.
5. Take the sample in a clean cuvette, once more filling the cuvette up to the fill line. Discard the first sample to prevent contamination from previous samples. Put the cuvette in the sample reader and wait a few seconds until it is stable, take the reading from the screen.
6. After finishing taking the samples, turn the power switch off.
7. Unplug the machine.

X. SHUT DOWN
1. Change the agitation control mode to OFF. Remove the motor. Turn the pH, DO, and Temperature control modes to OFF.
2. Close gas control valve on reactor and tanks, note final tank pressure, close regulator exit valve, and end data acquisition (See step 3). Switch off the main power.
3. Stop the program – go to Configure menu, choose Process Setup, then Details, and click End Process. Exit BioCommand. Go to XI.
4. Turn the water off and disconnect H2O links entering and leaving the bioreactor and the condenser.
5. Drain NaOH from tubing into the flask. Take NaOH flask, cover it with parafilm and return to the storage cabinet for acids and bases.
6. Remove condensor and drain water from hoses. Remove sampling port and wash.
7. Go to XV for Clean Up.

XI. Data Retrieval Procedure
1. Go to START. This pulls a pull-up menu from the bottom left corner of the screen.
2. Select Dbviewer. Select File, then Open. Double click on your database file.
3. Go to View. Select Loop Data.
4. Double click on each loop under “Available Loops” that you wish to display. The loop name will then move from “Available Loop” to “Loops to Display”.
5. Select your preferred time mode: calendar or decimal hours.
6. Select your preferred step size (usually 1). Click box for show all.
7. Click OK. It is now retrieving data…
8. Now click and drag mouse to select all data.
9. Click Export.  
10. If saving to a disk, switch drive to a: Give file a file name. Hit Okay. Data was sent to disk. Data retrieval complete.

XII. Procedure for Using Glucose Monitor for Measuring Glucose in the Fermenter Experiment

A. MEDIA PREPARATION (See part IV)

B. GENERATE CALIBRATION CURVE
Because the glucose monitor (ReliOn Testing System) is designed and calibrated for use with fresh capillary whole blood samples. When it is used for measuring the glucose concentration in the fermenter, a calibration curve must be developed beforehand whenever a new box of ReliOn Blood Glucose Test Strips is opened. This is because different box of glucose test strip has different lot number and calibration code. And for the proper measurement, (1) Make sure that the lot numbers on the calibrator, the test strip packet, and the test strip insert sheet all match. (2) Check that the calibration code stored in your monitor matches the calibration code printed on the calibrator and on the test strip insert sheet. (pg23, ReliOn, User’s Manual)

For generating calibration curve, we first dilute the defined media (A) by the defined media without glucose (B) into seven different glucose concentrations: 0g/l, 1g/l, 2g/l, 3g/l, 4g/l, 5g/l, 6g/l. For example, you can get the above concentrations by mixing different amount of A and B in seven small vials, in Table 1.

Table 1 Sample dilution scale for generating calibration curve

<table>
<thead>
<tr>
<th>Glucose Concentration (g/l)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (ml)</td>
<td>0</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>B (ml)</td>
<td>10</td>
<td>9.5</td>
<td>9</td>
<td>8.5</td>
<td>8</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

We can then read the glucose concentration results from the glucose monitor for prepared concentration from 0~6g/l using the glucose monitor. A sample test result is shown in Table 2 for Lot number: 25916, Calibration Code: 40b.

Table 2. Sample glucose monitor reading

<table>
<thead>
<tr>
<th>Glucose concentration (g/l)</th>
<th>Glucose Monitor Reading (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>LO</td>
</tr>
<tr>
<td>1</td>
<td>104</td>
</tr>
<tr>
<td>2</td>
<td>276</td>
</tr>
<tr>
<td>3</td>
<td>402</td>
</tr>
<tr>
<td>4</td>
<td>484</td>
</tr>
<tr>
<td>5</td>
<td>589</td>
</tr>
<tr>
<td>6</td>
<td>CK/KE/HI</td>
</tr>
</tbody>
</table>
LO means “Low, glucose level less than 1.1 mmol/L, or 20 mg/dL). CK/KE means check ketone, because the monitor is designed to monitor the diabetes and this value is considered high to human health. HI means “High, glucose level higher than 600 mg/dL, 33.3 mmol/dL).

A calibration curve developed from the above data is shown in Figure 1

![Calibration Curve](image.jpg)

Figure 1. A sample calibration curve (Lot number: 25916, Calibration Code: 40b)

From the calibration curve, we know when we measure the glucose concentration in the fermenter, the actual reading should be:

\[
\frac{\text{glucose monitor reading}}{\text{slope of the calibration curve}}
\]

The slope of the calibration curve is 1.2031 in this case. Also, it should be noted that the glucose monitor reading’s unit is in mg/dl.

As mentioned above, this calibration curve can’t be used generally. It is for a specific lot number and calibration code. When a new box of ReliOn Blood Glucose Test Strips is opened, a new calibration curve should be created following the above procedures.

C. TEST SAMPLE’S GLUCOSE CONCENTRATION

First, use the glucose monitor to test the sample from fermenter, if reading is CK/KE/HI, it means its glucose concentration is higher than the upper assay range limit (600 mg/dL) of the
glucose monitor’s specification. Then we need to dilute the sample from the fermenter using the defined media without glucose (B) to make its glucose concentration lower than 600 mg/dL.

When we can get the actual reading from the glucose monitor, we can use the calibration curve and dilution factor used when diluting to calculate the sample’s glucose concentration as:

\[
\text{Glucose monitor reading}/(\text{slope of the calibration curve} \times \text{dilution factor})
\]

D. How to Use the ReliOn Blood Glucose Monitor

1. Calibrating the monitor
   (pg 16~23, User’s Manual)
   Calibration is the process of programming your monitor for each new box of test strips. The calibrator stores important information about the test strips that your monitor needs to know to give you accurate blood glucose reading.

2. Test the glucose level
   (pg 24~32, User’s Manual)
   Note: Display messages
   (Pg 43~52, User’s Manual)

XIII. LYSINE ENZYME ASSAY PROCEDURE

Intro

As this is a lengthy procedure that involves many chemicals and steps, it is suggested that you gather and clean hardware and locate chemicals at the beginning of the procedure. If you are unsure how to use the Mettler H80 balance to measure the minute quantities required by this procedure, need assistance in calibrating the pH meter, or have any other questions ask your TA or Dr. Shonnard. When performing this procedure plan ahead and be meticulous, but most of all be patient. Your results will be as accurate as you make them.

Hardware needed:

- 2 125mL Erlenmeyer flask
- 8 test tubes
- 2mL volumetric pipet
- 5mL volumetric pipet
- disposable pipet w/ bulb
- pH meter w/ calibration samples
- 100mL graduated cylinder
- 4-5 specs (spectrophotometer tubes)
- 40-200μL Oxford BenchMate
- 100-1000μL Oxford BenchMate
- Tips for both Oxford Benchmates
- spectrophotometer

Chemicals needed:

- KH₂PO₄ (Potassium Phosphate Monobasic)
- EDTA (Disodium Ethylenediamine tetraacetate)
- L-lysine
- HCl or NaOH(concentrated)
NADH (β-Nicotinamide Adenine Dinucleotide, Reduced Form)*
α-Ketoglutaric Acid*
Saccharopine Dehydrogenase*

* chemical stored in freezer, remove only as needed

Procedure

A. Preparation of Solutions for 20 Assays (10 Calibration Standards and 10 Experimental Samples)

1. Reagent A (KH$_2$PO$_4$ (FW=136.09 g/mole) and EDTA (FW = 372.24 g/mole))
   a. Measure 1.3609 g KH$_2$PO$_4$ (100 mM) and .0372 g EDTA (1 mM) on the analytical balance and transfer to a 125-mL flask.
   b. Add 100 mL of distilled water to these reagents to make a solution of 100 mM of KH$_2$PO$_4$ and 1 mM of EDTA using a 100-ml graduated cylinder.
   c. Mix thoroughly by using the Fisher Vortex Genie 2.
   d. Calibrate the pH meter using calibration standards. Carefully add concentrated HCL or NaOH (about 6 N), using a disposable pipet while swirling, until the pH of the resulting solution is 6.8. Cap this solution with parafilm.

2. Reagent B (NADH, FW = 709.4 g/mole)
   a. Measure .0098 g β-NADH (.23 mM) on the analytical balance.
   b. Add 60 mL of Reagent A to the β-NADH using 100 mL graduated cylinder
   c. Mix thoroughly by using the Fisher Vortex Genie 2.

3. Reagent C (α-Ketoglutaric Acid, FW = 146.1 g/mole)
   a. Measure .0233 g α-Ketoglutaric Acid (79.8 mM) on the analytical balance.
   b. In test tube add 2 mL of Reagent A to the α-Ketoglutarate using a 2 mL pipet.
   c. Mix thoroughly by using the Fisher Vortex Genie 2.

4. Reagent D (L-Lysine, FW = 182.65 g/mole)
   a. Measure .0.050 g L-Lysine (54.75 mM) on the analytical balance.
   b. In test tube add 5 mL Reagent A to the L-Lysine using a 5 mL pipet to make a 10 g Lysine / L solution.
   c. Mix thoroughly by using the Fisher Vortex Genie 2.

5. Reagent E (Saccharopine Dehydrogenase, mix right before using)**
   a. Measure .0004 g of Saccharopine Dehydrogenase (2 units of enzyme) on the analytical balance and add 2 mL of Reagent A using a 1 mL pipet in a test tube.
   b. Mix thoroughly by using the Fisher Vortex Genie 2.
** 1.0 unit of enzyme per ml of Reagent E.

B. Dilutions of the Calibration Standards for Lysine

1. Starting with the 10 g/L (54.75 mM) lysine solution already prepared, dilute in order to get several lysine concentrations distributed fairly even from 0 to 10 g/L.
2. Approximately 5 different concentrations will be sufficient to achieve an effective calibration curve.
3. Dilute from the 10 g/L lysine solution every time.
4. For example, if you wanted to prepare a 5 g/L solution from the 10 g/L solution, you could add 1 ml of Reagent A to 1 ml of the 10 g/L solution in a new labeled vial, making sure to use separate 2 ml pipets.

C. Preparation of the Spectrophotometer

1. Clean 4 or 5 spectrophotometer tubes thoroughly.
2. Set the spectrophotometer to a wavelength of 340 nm.
3. Add distilled water to the white line of a clean spec and use this throughout the experiment as an equipment blank spec.
4. Use KimWipes to clean this spec, place it in the spectrophotometer, and zero out the absorbance.

D. Preparation of the Enzyme Spectrophotometer Tubes for Lysine Calibration and Sample Analysis

1. Add 2.75 mL of Reagent B to a spec using a 2 mL pipet.
2. Add .1 mL of Reagent C to the same spec using a 40-200 µL Oxford BenchMate.
3. Add .1 mL of Lysine Calibration Standard or experimental sample to the same spec using a 40-200 µL Oxford BenchMate.
5. Clean the equipment blank spec with a KimWipe, place it in the spectrophotometer, and re-zero.
6. Remove the equipment blank spec, clean the enzyme test spec with a KimWipe, place it in the spectrophotometer, and record the absorbance (wait until constant).
7. Remove the enzyme test spec and add .1 mL of Reagent E using a 40-200 µL Oxford BenchMate.
8. Immediately, mix thoroughly by using the Fisher Vortex Genie 2.
9. Clean the enzyme test spec with a KimWipe, place it in the spectrophotometer, and record the decrease in absorbance every 20 seconds for 5 minutes. Consistency in time between mixing and beginning of recording absorbance is crucial.

XIV. HARVESTING AND MICROFILTER OPERATION (not for this run!! Skip to XV)
This procedure is carried out after the fermentation is completed. The object is to separate the bacteria from the extracellular solution. The recycle mode of filtration will be used for this
The reason for this is that a tangential filtering technique is employed to achieve the separation. In this technique, the flow is parallel to the filter instead of being perpendicular to it. An advantage of this technique is that the filter does not get clogged. However, multiple passes may be required before the separation is completed, and hence the recycle mode. The steps for doing this are as follows:

1. Turn off the gas tanks to the process.
2. Remove the pH and D. O. probes, and the thermocouple. Clean them thoroughly with soap and distilled water before putting them away.
3. Place the peristaltic pump and the micro filter next to bioreactor and connect the pump to the electrical outlet. Make sure that the pump speed is set at zero.
4. Connect the harvest port of the reactor to the feed end of the microfilter. Also, connect the inoculation port to the retentate end of the microfilter.
5. Run the permeate end of the microfilter to a large collection vessel.
6. Clamp the feed hose in the peristaltic pump. Adjust the clamp setting to 3.
7. Start the pump. If the hose from the reactor comes in from the left, turn the switch to the clockwise mode of operation; otherwise use the counterclockwise mode.
8. Slowly increase the pump speed until a steady flow is achieved. A speed of 3 is suggested.
9. Run the pump until the retentate going back to the vessel consists mostly of cells. The retentate flow should be very cloudy.
10. Switch the pump off. Disconnect the feed hose from the harvest port of the reactor.
11. Immerse the feed hose in a vessel containing distilled water. Start the pump and run it until the permeate and retentate streams become clear. Then switch the pump off.

XV. CLEAN UP
1. Remove air filter from the top of the reactor and the console and place it in drawer. Remove probes carefully (Instructor or TAs only).
2. Remove the vessel from the reactor console. In the fume hood, fill it with bleach solution and let it stand for a few minutes.
3. Scrub the vessel and its parts with detergent solution to remove any residue. Pay special attention to the baffles and the agitator blades.
4. Thoroughly rinse all the parts with distilled water.
5. Place the reactor back on the console.

XVI. Preparation of the Proposal
You will need to review this information for a pre-lab check-in to be scheduled at least one day before you operate the equipment. The check-in will also include a short quiz to determine individual group member understanding of the process, including safety procedures. At the pre-lab check-in, each group member should have a clear understanding of the group objectives. Each group member should demonstrate mastery of the procedures and safety features of the experiment. The expected results for the experiment should be discussed. This
should include some indication of how you will treat the data, including how you will plot and graphically display the data.

The format of the pre-lab proposal and of the final report should follow the format in the course syllabus. Attention to good writing principles and effective use of figures and tables is expected. Please make arrangements to schedule the check-in with me no later than one day before operation of the pilot plant.
SAFETY GUIDELINES

CM4120 CHEMICAL PLANT OPERATIONS LABORATORY
BIOPROCESS EXPERIMENTS: FERMENTATION

Biological Safety Guidelines

The microorganism being used in this course is a pure culture composed of the bacterium *Corynebacterium glutamicum*. Although this strain of bacteria is not pathogenic (harmful to human health), in this laboratory we will treat them as if they may be and therefore, *aseptic techniques* will be used in handling the microorganism.

The Centers for Disease Control/National Institutes of Health (CDC/NIH) have developed guidelines for proper handling and disposal practices of potentially disease causing microorganisms in the laboratory. They were developed to reduce exposure of laboratory personnel to hazardous agents and prevent their release to the atmosphere. The CDC/NIH guidelines describe four levels of biosafety practice, or levels of containment, which govern standard laboratory practice and facility features and operation. Primary containment concerns control of exposure to personnel within the laboratory and is accomplished through the use of proper laboratory techniques and safety equipment. Secondary containment has the objective of controlling the release of biohazardous agents to the environment and involves the design of the facility itself and it's operation.

The biosafety levels are designated in ascending order by the degree of protection provided. Biosafety level 1 is appropriate for use with microorganisms not known to cause harm to adult human health and therefore has the lowest level of protection. Biosafety level 2 is suitable for agents with moderate impacts to human health and the environment. Biosafety level 3 is used for infections agents which cause serious disease or are potentially lethal through exposure by inhalation or ingestion. Biosafety level 4 is required for work with agents which have a high individual risk of life-threatening disease. In this laboratory, standard laboratory practice consistent with biosafety level 1 will be used with additional precautions to limit environmental exposure.

Laboratory Practice and Techniques

There are standard laboratory practice which apply to all biosafety levels.

- limit access to the laboratory
• decontamination of work surfaces daily or after a spill of viable material
• prohibit mouth pipetting
• prohibit eating, drinking, smoking, and applying cosmetics in the laboratory.
• ban the storage of food in cabinets and refrigerators used to store laboratory agents
• wash hands after handling viable materials before leaving the laboratory
• minimize the creation of aerosols
• if possible, all handling of viable microorganisms will occur in the laminar flow hood.

In addition to these standard practices of laboratory operation, there are several important steps which will be taken to limit exposure to the outside environment. These precautions affect the proper disposal of viable materials.

• all disposable pipettes which contact viable microbes will be disposed in biohazard bags.
• all solutions containing viable microorganisms will be steam autoclaved or chlorinated.
• all biohazard bags will be steam autoclaved before disposal in the trash.

**Safety Equipment**

Except for the steam autoclave, there is no additional safety equipment required for this laboratory other than strict adherence to proper aseptic techniques as described above.
Specific Report Requirements

The PreLaboratory Proposal should follow the same format as in CM4110. Since you are using a SOP, the JSA should not contain a detailed step by step procedure. What it should contain is a brief summary of each section of the run, i.e. autoclaving, media preparation, microfiltration of media, etc., together with the safety implications of that section, and the special precautions that must be taken to prevent against any potential hazards.

The PreLaboratory Proposal should also contain a detailed schedule as to who will be doing what and when throughout the run. The Experimental Strategy should identify key concerns during the run and also should indicate what variables you will monitor during the run and why.

The Oral Presentation should present an overview of the run, some indication of past performance, a comparison to your measured performance, quantitative conclusions, and finally any specific recommendations.

The Final Report for this run should be an expanded Executive Summary containing all pertinent data and drawing appropriate conclusions and recommendations. Careful reference to past run performance is essential. In this expanded Executive Summary it might be useful to use tables and graphs to effectively convey important information. Error limits should accompany all data used in the Executive Summary.

All sections of the portfolio should be prepared by the entire team under the direction of the Team Leader. The Safety Officer should ensure that laboratory operations are carried out safely and that safety concerns are documented in the PreLaboratory Proposal, in the Oral Presentation, and in the Final Report. The Team Leader and the Safety Officer should be made known to me and Dr. Fisher by the end of the first week of the cycle.

Generally, I grade following the instructions provided in the course syllabus. The grading is based primarily on completeness and technical quality. However, having stated that, I do look for clear presentation of the material, brevity and conciseness, and ease in reading. Ease in reading means that there are no mis-spelled words, no grammatical errors, no errors in punctuation, no run-on sentences, no shifting verb tense within paragraphs, no shifting from active to passive or the other way around within paragraphs, and neatness in presentation of graphs and tables. I prefer past tense because you are describing what your group did during the experiment and subsequently in calculations. I also prefer a passive voice in the report, though this is not a requirement and I will not deduct points if an active voice is used correctly. For instance, if you want to use the active voice in the letter of transmittal and a passive voice in the body of the report, that would be fine. However, using an active voice in certain sections of the report may be a mistake. For instance, in the background sections and the introduction of the report, an active voice doesn't seem to be the best.

I hope these comments will help you in preparing your report. If you have additional questions, please contact me either in person or through e-mail.

David R. Shonnard, Associate Professor, Department of Chemical Engineering, MTU