CHEMICAL ENGINEERING SENIOR LABORATORY
CHEG 4139

Bioreactor

Objective:
The laboratory has acquired a bioreactor specifically designed for cultivating cell cultures. In this experiment, *E. coli* serves as the model organism. The growth medium used is Luria-Bertani (LB) broth, which provides essential nutrients for bacterial proliferation. The baseline conditions for the fermentation process are as follows:

Overnight incubation time: 15 hours
Impeller mixing speed: 200 RPM or as determined by students
Incubation/reaction temperature: 37 ℃ or as determined by students
LB Medium

The objective of this experiment is to determine the kinetic parameters of bacterial growth using LB media. You will monitor the optical density (OD) of the bacteria over time and construct a bacterial growth curve. Due to the lengthy nature of a single reactor run, you will only achieve one complete reactor run during the duration of your lab.

Major Topics Covered: Kinetics, Biochemistry, Numerical Methods

Theory:
In this experiment, you will be determining some of the kinetic parameters for the growth of *E. coli.* The relevant equations you may find useful include:

$$\frac{dX}{dt} = \mu X \qquad \mu = \frac{\mu_{MAX} S}{K_s + S}$$

Where X = biomass concentration, μ = specific growth rate, t = culturing time, μ_{MAX} = the maximum specific growth rate, K_S = Monod constant, and S = substrate concentration. You will be measuring the biomass optically using cell density in a UV-vis spectrometer at 600 nm. Determining other parameters will be the focus of this experiment. For relevant information, see the references at the end of this document.

Safety Precautions:
E. coli is a biological agent that requires stringent safety measures during handling. Adhering to these safety precautions is mandatory. Failure to comply with these guidelines will result in immediate removal from the laboratory.

1. Gloves, goggles, and lab coats are required at all times when operating the bioreactor.
2. If you need to leave the reactor bench, first remove and properly dispose of your gloves, then thoroughly wash your hands with antibacterial soap. DO NOT wear your gloves out of lab room or when working in the communal computer space.
3. Always dispose of used gloves in a designated trash bag; do not discard them directly into the general trash bins!
4. Only place contaminated glassware in the wash basin. Assume anything placed in the wash basin is contaminated.

5. Before and after handling the bacteria, sanitize the countertop by spraying it first with a 70% ethanol solution, then with deionized (DI) water, and with the ethanol solution again.
6. When disposing of samples, add some 70% ethanol (or a bleach solution) into a spare plastic bottle. Pour all samples into this bottle, ensuring to rinse cuvettes, centrifuge tubes, etc., with ethanol. This waste solution can be safely poured down the drain once your experiment is completed. Afterward, clean the bottle with soap and water.
7. Always wash your hands with antibacterial soap after removing your gloves.

Procedure: See Bioreactor Operations Method

Due to the lengthy set up of this experiment, the following schedule is recommended:

Lab Session 1

- Clean and autoclave the reactor (with the assistance of the TA and the technician)
- Prepare a minimum of 4 L media
- Clean and autoclave all glassware and pipette tips
- Autoclave media, glassware, pipette tips

Lab Session 2 (Must be completed the afternoon before Lab Period 3)

- Connect the reactor to the control station
- Prepare 4 media flasks and inoculate with bacteria
- Place the inoculated flasks in an incubator-shaker for 15 hours
- Set up pH and DO probes

Lab Section 3 (start between 8 and 9 AM)

- Operate the reactor according to the detailed procedure
- Run the benchtop shaker according to the detailed procedure
- Sample both the reactor and shaker flasks every 30 minutes for 6 to 8 hours until stable conditions are observed for two consecutive points
- Sterilize the reactor and any contaminated glassware
- Complete the cleaning of the reactor and glassware

Since Sessions 2 and 3 will not fall on a regularly scheduled time slot, you must coordinate with your group and the TA to agree on a suitable time to meet.

Analysis:

Your analysis **must** include:

1. A determination of the maximum growth parameter (μ_{MAX}) for the LB media (from your experiment data).
2. Why is the Monod constant not determined for the LB media in this experiment?
3. How do the results vary between the bioreactor and flask? Discuss what these differences indicate about the physical processes occurring in both the bioreactor and the flasks.

Report:

Describe the design of your experiments, including the methodology and the results obtained, accompanied by an error analysis. Provide a thorough and quantitative discussion of your results. Ensure you include your bacterial growth curve (consider using a semi-log plot for clearer visualization). Report all relevant biokinetic parameters. Discuss any discrepancies between the observed and predicted results, quantifying experimental uncertainties or highlighting limitations of the correlations or computational tools used in your analysis.

Pro Tips:

1. The bioreactor is a very expensive piece of equipment. Take the following precautions to avoid damaging or breaking it:
   - When moving the reactor vessel away from the bench, always transport it on a cart or in an autoclave basket.
   - When autoclaving the reactor, always tighten the lid on loosely so the glass has room to expand as it heats.
   - When reassembling the bioreactor, the impeller motor should gently slide onto the impeller. Do NOT try to force it. You may need to readjust the position of the impeller to get a good fit.
   - Do not loosen the support ring attaching the glass reactor cylinder to the reactor base when it is not empty.
2. Never tightly cap anything you autoclave. Either apply the caps loosely or cover open holes with aluminum foil.
3. Only glass or metal materials are autoclavable. The only plastic materials that can be autoclaved are the 100 mL graduated cylinders, the Tygon tubes on the bioreactor, and the pipette tips. Do not put any other plastic laboratory equipment in the autoclave.
4. You may autoclave glassware many days in advance. It will remain sterile as long as the aluminum foil cover is not disturbed.
5. Use thermally reactive autoclave tape on items to ensure the autoclave is functioning as intended.
6. When leaving the lab with anything that may be biologically active, travel in pairs. One partner will carry anything with bacteria, and the other partner will open doors for the first to minimize contamination. Use the basin to carry flasks that contain bacteria.
7. It is wise to prepare an extra inoculation flask. In the event one of your bacteria samples does not grow, you will have a backup.
8. When performing the inoculation, make sure the outside of your flasks and the rubber mat in the incubator/shaker are clean and dry. Do not exceed 300 RPM.
9. Do not turn on the bioreactor unless water is flowing from the faucet line. Do not try to run the reactor unless the reactor is on the base and connected to the water lines.
10. When running the bioreactor, be sure to keep all open ports capped with foil unless taking samples.
11. When preparing diluted samples, always use fresh pipet tips. Be careful not to insert a pipette tip contaminated with bacteria into your fresh media, as this will force you to discard the media solution.
12. When preparing diluted samples do not dilute the flasks. This will just cause continued growth of *E. coli* and prevent stationary phase from being reached.

References:

1. Shuler, M.L., Kargi, F., *Bioprocess Engineering: Basic Concepts.* 2nd Ed., Prentice Hall, New York, (2001).
2. Blanch, H. W., Clark. D. S. (1997), *Biochemical engineering*
3. James E. Bailey, David F. Ollis, *Biochemical Engineering Fundamentals*, 1986
4. James M. Lee, *Biochemical Engineering*, Prentice, 1992 (or copy of ebook, 2003).

Bioreactor & Benchtop Shaker Operations Method

Note: For this full-day experiment, you will run the bioreactor and the benchtop shaker simultaneously.

Step 1: Prepare Media

1. Choose the media type (LB medium)
2. Make 4 liters of media according to the instructions on the reagent container or based on literature.
3. Clean the bench and mass balance if needed

Step 2: Clean and Sterilize Glassware/Media/Pipette Tips

1. Clean the following with alcohol and water, and RINSE THOROUGHLY.
   - Four 125 mL Erlenmeyer flasks
   - Four 250 mL Erlenmeyer flasks
   - Three 100 mL graduated cylinders
   - One glass funnel
2. Cap the glassware with aluminum foil and place a strip of autoclave tape across the foil.
3. Fill the pipette tip box with 1000 μL tips and place a strip of autoclave tape on the box's lid.
4. Place the glassware, pipette tip box, and media in the autoclave. The TA will assist in running the sterilization sequence: S**terilize and Dry**. This sequence will take approximately 2-4 hours.

Step 3: Sterilize Bioreactor

1. If needed, disconnect the impeller motor and place it on the bench.
2. If needed, disconnect the water lines on the reactor and the condenser tube.
3. Carefully move the bioreactor to the sink.
4. Undo the screws in the reactor lid, being careful not to let the support ring fall quickly. Using water and Alconox soap, scrub the inside of the bioreactor and any surfaces on the lid that will be contained within the reactor. Do not forget to unscrew the vent port at the top and clean inside.
5. After rinsing off the soap, reattach the lid. Screw the lid on loosely (the end of the screw should be even with the bottom surface of the support ring).
6. Cover all open holes on the bioreactor with aluminum foil. This includes the holes at the end of the sampling tubes. You do not have to cover the cooling water ports with foil. Place a piece of autoclave tape across the foil.
7. Carefully place the reactor into the autoclave and start the sterilization sequence. This should take 2-4 hours. (To save time, the TA/Technician will autoclave the reactor for you.)

Steps 4-7 are performed the day before the full-day experiment

Step 4: Setup the DO probes

1. Check and refill the DO probe with electrolyte.
2. Cover both ends of the DO probe with the appropriate caps. Spray the probe body with ethanol and bring into the biosafety cabinet.
3. Spray the portable UV lamp with ethanol, then bring it into the biosafety cabinet. (Alternatively, you can use the built-in UV lamp in the biosafety cabinet).
4. Set up the probe on the provided ring stand.
5. Remove the green cap from the end of the DO probe.
6. Sterilize with UV light for approximately 15-20 minutes. When sterilization is finished, leave the probe in the biosafety cabinet until installation time.

Step 5: Assemble Reactor

1. Tighten the four screws on the lid evenly. Move in an X-shape pattern, then go around clockwise to make sure everything is screwed on tightly and evenly.
2. Carefully place the reactor on the base. Make sure the cooling water ports for the reactor and the condenser are aligned. The reactor should sit firmly on the bench. Connect the water lines to their respective ports.
3. Insert the thermocouple into the sheath in the reactor.
4. Insert the DO probe into the bioreactor and reconnect it to the DO cable.

Step 6: Polarize the DO Probe

This step **MUST** be completed at least 6 hours before you begin your reactor run. You may perform this step after sterilizing the probe.

1. Assemble the bioreactor if not done previously. Turn on the bioreactor.
2. Make sure the setting for "Temperature" and "Agitation" control settings are set to "OFF"
3. Connect the DO probe to the appropriate cable if not done previously. The DO probe is now polarizing. Leave the bioreactor on overnight to allow the DO probe to polarize before calibrating.

Step 7: Prepare Inoculation Flasks

This step **MUST** be completed about 15 hours before you begin your reactor run.

1. Bring the sterilized glassware and room-temperature LB media to the designated biosafety cabinet.
2. Properly sterilize the biosafety cabinet and all materials you will place inside it with an ethanol spray.
3. Make sure both your names are written on the flasks; the flasks should also be numbered. Pour 60 mL of LB media into each of the two 125 mL flasks. KEEP THE FOIL COVERS!
4. Thaw the frozen samples of *E. coli* provided to you by gently rolling them between your hands.
5. Once the samples have fully melted, inoculate each of the flasks with 0.5 mL of *E. coli* using the micropipette.
6. Re-cover the flasks with foil.
7. Place the flasks into the incubator/shaker, making sure the flasks are clean and dry on the outside and bottom. Make sure the flasks are in the appropriately sized holders. Set the temperature to 37 °C and the agitation to 200 RPM. Note the time.
8. Sterilize the biosafety cabinet again using the appropriate technique with ethanol and clean all surfaces with ethanol.

Step 8: Prepare the pH Probe

1. Calibrate the pH probe (refer to the Appendix for the procedure).
2. Cover both ends of the pH probe with the appropriate caps. Spray the probe with ethanol, and bring it into the biosafety cabinet.
3. Remove the plastic cap from the end of the probe.
4. Set up the probe on the provided ring stand so that the UV light is directed toward the end of the probe.
5. Sterilize with UV light for approximately 15-20 minutes. When sterilization is complete, leave the pH probe in the biosafety cabinet until it is time for installation.
6. Insert the pH probe into the bioreactor; reconnect the pH probe to the pH cable.

Step 9: Run the Reactor & Benchtop Shaker

To operate the control system for the bioreactor, please use the touch screen to do any change or input exact values.

1. If needed, turn on the water faucet line connected to the reactor, then turn on the bioreactor.
2. Using the autoclaved glass funnel, add 3L of LB Media into the bioreactor.
3. Calibrate the DO probe (see Appendix for procedure).
4. Place the impeller motor onto the reactor. It should sit firmly atop the reactor. You may need to manipulate the impeller manually to get a good fit. You should not try to force the motor onto the reactor.
5. Click the clipboard image in the bottom left of the screen to set the temperature setpoint to 37 °C or as determined by the students.
6. On the same summary screen, set the agitation to 200 RPM or as determined by the students.
7. Fill one 50 mL centrifuge tube with about 25 mL of media. Cap and save for later; this will be used if the samples need to be diluted.
8. Fill the decontamination basin with approximately 3 inches of water and soap
9. Pour some ethanol into the *E. coli* waste bottle.
10. Pipet 1000 μL of fresh media from the centrifuge tube into a clean cuvette. Use this blank to zero the spectrophotometer at 600 nm.
11. Empty the contents of the cuvette into the *E. coli* waste bottle, then place all contaminated cuvettes/glassware into the decontamination basin.
12. Begin the *E. coli* growth in the bioreactor
    a. Once the media in the reactor is at temperature, pour 60 mL of your inoculated bacteria into the reactor carefully.
    b. Immediately extract 1 mL from the reactor using a syringe, place it into a clean cuvette, and measure the absorbance at 600 nm. Empty the contents into the ethanol bottle and place all contaminated equipment into the decontamination basin.
13. Begin the *E. coli* growth in the benchtop shaker
    a. Fill each of the three 250-mL flasks with 100 mL of fresh media
    b. Pipet 2 mL of your inoculated bacteria into each flask
    c. Immediately extract 1 mL from each of the flasks in the shaker using the micropipette, place into a clean cuvette, and measure the absorbance at 600 nm. Empty the contents into the ethanol bottle and place all contaminated equipment into the decontamination basin.
    d. Place the flasks in the shaker to begin the cell growth.
14. Collect optical density readings for the bioreactor and benchtop shaker flasks every 30 minutes. If your readings on the spectrophotometer begin to get high (above 1.000), you will need to dilute the next sample you take with fresh media. Be sure to note the composition of each sample (i.e. how much sample and how much is fresh media).

15. Once your measurements have stabilized for an hour (or you have run out of time), the experiment can be completed. Take your final samples.

Step 10: Clean Up

1. Remove probes from the bioreactor, rinse with DI water into *E. coli* waste, and repeat the sterilization procedure.
2. Pour about 2-3 capfuls of bleach into the reactor for at least 10 min of sterilization. Make sure you turn the temperature setting to "OFF". **DO NOT USE ALCONOX.**
3. Discard the contents of your *E. coli* waste container in the sink, and place the container in the disinfection basin for cleaning.
4. Turn the agitation setting to "OFF" after sterilizing with bleach.
5. Empty the decontamination tub and refill with soap and water. Leave your glassware to soak.
6. Disconnect the cooling water tubes and impeller motor on the bioreactor.
7. Spray down the countertop, control station surface (if contaminated with *E. coli*), and biosafety cabinet with ethanol, then distilled water, then ethanol again.
8. Empty the contents of the bioreactor into a sink, and scrub all surfaces with soap and water. This includes the inside of the reactor, the impeller, and tubes inserted into the reactor, and all exterior surfaces.
9. Return the bioreactor to the control base.
10. Clean the rest of your glassware with soap and water. Leave glassware and bottles on the drying rack to dry. Rinse off the cuvettes with water and dispose of them in the designated waste bag.
11. Clean the countertop and the biosafety cabinet with ethanol, DI water, and ethanol

Appendix

pH Probe Calibration

Note: If autoclaving the probe, then pH probe calibration is done prior to autoclaving

Calibration should be performed prior to inoculation.

1. Connect pH probe to the bioreactor using the appropriate cable.
2. Turn on the water faucet
3. Turn on the main power switch
   • Display calibration screen by pressing the caliper button on the bottom of the screen.
4. Immerse pH electrode into pH 7.00 buffer solution and allow 3-5 minutes for the system to equilibrate. Wait for the RAW mV value to stabilize.
5. Set the pH function to “Zero”
6. Set the display to read 7.00
7. Rinse the pH electrode with distilled water
8. Immerse pH electrode into pH 4.00 buffer solution and allow 3-5 minutes for the system to equilibrate. Wait for the RAW mV value to stabilize.
9. Set the pH function to “Span”
10. Set the display to read 4.00

Dissolved Oxygen (DO) Probe Calibration

Note: If autoclaving the probe, then DO probe calibration is done after autoclaving

When the system is operated for the first time, or when the electrode has been disconnected from the voltage source for longer than 5-10 minutes, the electrode must be connected to the operating O_2 amplifier for polarization prior to calibration. The electrode is polarized and ready for operation after six hours of polarization time. Do not calibrate until the electrode is polarized. Make sure the bioreactor is turned on for the entire duration.

Calibration should be performed prior to inoculation. The DO probe should be immersed in the liquid medium during calibration.

1. Turn on the water faucet if not already on
2. Turn on the main power switch if not already on
3. Display calibration screen by pressing the caliper icon on the bottom of the screen.
4. Disconnect the cable from the DO probe.
5. Set the DO function to “Zero” and wait 3-5 minutes or until reading is stable.
   - Watch the RAW nA value
6. Set the display to read 0% by setting “Zero” to 0.0
7. Reconnect the cable to the DO probe and wait 5 minutes or until reading is stable.
   - Watch the RAW nA value
8. Set the DO function to “Span”
9. Set the display to read 100% by setting “Span” to 100