**CHEMICAL ENGINEERING SENIOR LABORATORY**
**CHEG 4139**

Bioreactor

**Objective:**
The laboratory has acquired a bioreactor for the purpose of growing cell cultures. In the initial attempt to grow *E. coli*, an M9 growth media was used, which used the minimal amount of salt and nutrients to encourage bacterial growth. After a few successful attempts with the M9, groups became unable to grow *E. coli* in the media, prompting a switch to LB media, which is much richer in nutrients. Experimental conditions for the baseline fermentation using M9 media is as follows:

| | |
|---|---|
| Overnight incubation time: | 15 hours |
| Impeller mixing speed: | 200 RPM |
| Incubation/reaction temperature: | 37 °C |
| Glucose loading in M9 growth media: | 0.4% by mass |

Your goal is to compare the kinetic parameters for bacterial growth using the LB media to those of the M9 media. You will measure the bacterial optical density (OD) over time and construct a bacterial growth curve. You will also use a numerical method to estimate the substrate concentration in your reactor as time progresses. Due to the lengthy nature of a single reactor run, you will likely only achieve one complete reactor run during the duration of your lab. As such, it is important to communicate with other groups performing this experiment to prevent tests of repeated conditions. After collection of your own data, you will be given a sample of previously collected data for both the M9 media and the LB media.

Alternatively, you may also attempt to use M9 salts. For additional information, please refer to online resources, supplemental information, and your instructor.

**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*. Some relevant equations include:

$$\frac{dX}{dt} = \mu X \qquad \frac{dS}{dt} = \frac{-\mu X}{Y_{X/S}} \qquad \mu = \frac{\mu_{MAX} S}{K_S + S}$$

Where $X$ = biomass concentration, $S$ = substrate concentration, $Y_{X/S}$ = maintenance coefficient (~0.5 for this experiment), $\mu$ = growth parameter, $K_s$ = Monod constant, and $\mu_{MAX}$ = the maximum growth rate parameter. 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, so the following safety precautions MUST be followed when working with it. Failure to observe these precautions will result in expulsion from the lab.

1. Gloves, goggles, and lab coats must be worn at all times when working with the bioreactor.

2. If you wish to walk away from the reactor bench, first remove and dispose of your gloves, then wash your hands with antibacterial soap. DO NOT wear your gloves around the lab or when working in the communal computer space.
3. **Always throw out used gloves in a separate trash bag**; do not throw out gloves directly in the trash!!!
4. Place only contaminated glassware in the wash basin. Anything that enters the wash basin should immediately be considered contaminated.
5. Before and after working with the bacteria, spray down the countertop with a 70% ethanol solution, then with DI water, then with 70% ethanol solution again.
6. When discarding samples, pour some 70% ethanol solution into a spare plastic bottle. Empty all samples into the bottle, spraying the cuvettes, centrifuge tubes, etc. out with ethanol. This waste solution may be poured down the drain at the completion of your experiment. The bottle must be washed with soap and water after.
7. ALWAYS wash your hands with antibacterial soap after removing your gloves.

**Available Variables:** Temperature, Mixing Speed, Media Type, Media Addendums (M9 only; i.e. glucose concentration)

**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
- Make minimum 1 L media
- Clean and autoclave all glassware and pipet tips
- Autoclave media (sterilize only), glassware, pipet tips

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

- Hook up reactor to control station
- Prepare 4 media flasks and inoculate with bacteria
- Set up pH and DO probes
- Leave in incubator-shaker for 15 hours
- Autoclave remaining media

Lab Period 3 (start between 8 and 9 AM)

- Run the reactor according to detailed procedure
- Run the benchtop shaker according to detailed procedure
- Sample reactor and shaker flasks every 30 minutes for 6 to 8 hours until it reaches a stable condition for two points.
- Sterilize reactor and contaminated glassware
- Finish cleaning reactor and glassware

**Analysis:**
Your analysis **must** include:

1. A determination of the Monod constant, $K_s$, and the max growth parameter, $\mu_{max}$ for your selected conditions. How do the Monod constant and maximum growth parameter vary based on the media you used? What does this observation mean in terms of what is physically

occurring in the bioreactor and in the flask?

**Report:**
Describe the design of your experiments and the results obtained, including an error analysis. Provide thoughtful and quantitative discussion of results. Be sure to include your bacterial growth curve (you may want to consider a semilog plot for this) and glucose concentration curve. Report all appropriate biokinetic parameters. Express any discrepancies between observed and predicted results in terms of quantified experimental uncertainties or limitations of the correlations or computational software used.

**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.
    - 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.
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 pipet 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 the 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 back-up.
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 pipet 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: Sterilize Bioreactor

1. If needed, disconnect the impeller motor and place it on the mount on top of the reactor.
2. If needed, disconnect the water lines on the reactor base 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 in the base and condenser tube 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.

## Step 2: Clean and Sterilize Glassware

1. Clean the following out with alconox and water. RINSE THOROUGHLY.
   - 4-5 125 mL Erlenmeyer flasks
   - 4-5 250 mL Erlenmeyer flasks
   - 3 100 mL graduated cylinders
   - 1 glass funnel
2. Cap the glassware with aluminum foil. Place a strip of autoclave tape across the foil.
3. Fill the pipet tip box with 1000 µL tips. Place a strip of autoclave tape on the box's lid.
4. Place the glassware and pipet tip box in the autoclave and run the sterilization sequence: S**terilize and Dry**. This sequence will take approximately 2-4 hours.

## Step 3: Make Media

1. Choose media type
2. Make media according to instructions on reagent container or based on literature. If using M9 salts, you will need 1X; adjust accordingly.
3. Autoclave media (only use DI water) – Instructor operate the autoclave.
   a. Make sure bottle is open ¼ turn to prevent pressure build-up
   b. Ensure autoclave is set to "**Sterilize**" *not* "Sterilize and Dry"

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

## Step 4: Setup the pH and DO probes

1. Check and refill the DO probe with electrolyte.
2. Calibrate the pH probe (see Appendix for procedure).
3. Cover both ends of the DO probe with the appropriate caps. Spray probe with ethanol and bring into the biosafety cabinet.
4. Spray the UV lamp with ethanol, then bring into the biosafety cabinet. (Alternatively, you can use the built in UV lamp in the biosafety cabinet).
5. Set up the probe on the provided ring stand.
6. Remove the green cap off the end of the DO probe.

7. Set the UV lamp so that the UV light is directed toward the end of the probe. Turn on the UV lamp.
8. Sterilize with UV light for approximately 15-20 minutes. When sterilization is finished, leave the probe in the biosafety cabinet until it is time for installation.
9. Cover both ends of the pH probe with the appropriate caps. Spray probe with ethanol, and bring into the biosafety cabinet.
10. Remove the plastic cap off the end of the probe.
11. Set up the probe on the provided ring stand so that the UV light is directed toward the end of the probe.
12. Sterilize with UV light for approximately 15-20 minutes. When sterilization is finished, leave the pH probe in the biosafety cabinet until it is time for installation.

**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 small pegs on the base. Connect the water lines to their appropriate ports.
3. Place the thermocouple into the sheath in the reactor.
4. Insert the pH probe into the bioreactor; reconnect the pH probe to the pH cable.
5. Insert the DO probe into the bioreactor; reconnect the DO probe 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 water source, then turn on the bioreactor.
2. Make sure the setting for “Temp” and “Agit” 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 flasks. KEEP THE FOIL COVERS!
4. Warm the frozen samples of *E. coli* provided to you by 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. Set the temperature to 37 °C and the mixing to 200 RPM. Note the time.
8. Sterilize the biosafety cabinet again using appropriate technique with ethanol.
9. Return the media to the refrigerator and clean all surfaces with ethanol.

**Step 8: Run the Reactor & Benchtop Shaker**

To operate the control system for the bioreactor:

Move the cursor around the screen with the arrow buttons. Press "Alter" to make selections. Use the number pad to input exact values. Use "Enter" to confirm your selections.

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 2L 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. Set the temperature control setting to "Prime" and press "Enter." Let the reactor run at this setting for roughly 30 seconds, then set the temperature control setting to "P-I-D."
6. Set the desired mixing rate and temperature using the control panel. Make sure that the "Control" setting for both agitation and temperature is set to "P-I-D."
7. Fill one 50 mL centrifuge tube with about 25 mL media. Cap and save for later; this will be used if the samples need to be diluted.
8. Fill the contaminated 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 bottle of ethanol, 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 50 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 2.5 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 tub.
    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. (If using M9 media, you can measure glucose levels with glucose test strips)
16. Once your measurements are stabilized for an hour (or you've run out of time), the experiment has completed. Take your final samples.

**Step 9: Clean Up**

1. Remove probes from bioreactor, rinse with DI water into *E. coli* waste and repeat sterilization procedure.
2. Pour about 1-2 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, and biosafety cabinet with ethanol, then distilled water, then ethanol.
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 “Screen” until “Calibration” is selected, then press “Enter”
4. Immerse pH electrode into pH 7.00 buffer solution, then allow 3-5 minutes for the system to equilibrate
5. Set the pH function to “Zero”
6. Set the display to read 7.00 under the “Zero” column
7. Rinse the pH electrode with distilled water
8. Immerse pH electrode into pH 4.01 buffer solution, then allow 3-5 minutes for the system to equilibrate
9. Set the pH function to “Span”
10. Set the display to read 4.01 under the “Span” column

### 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.

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 “Screen” until “Calibration” is selected, then press “Enter”
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
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.
8. Set the DO function to “Span”
9. Set the display to read 100% by setting “Span” to 100