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Standard Operating Procedure

Procedure: Fermentation of Biomass

Department: Biorenewable Resources & Technology

Building/ Room Number: Biorenewables Research Laboratory (BRL) 1114

Supervisor: Jacqulyn Baughman

Procedure Overview: Pre-weighed samples of biomass or char (as received condition) are burned completely in a sealed combustion chamber containing 30 atmospheres of pure oxygen. The heating value of the material is determined by the temperature increase in the surrounding water jacket. Heating values are standardized against benzoic acid. Samples are ignited using an electrically charged wire and a cotton string "wick." The sample chamber is flushed and cooled with water after each run.

Health and safety information for materials used: Compressed air and oxygen are under high pressure and will accelerate a fire; tanks should be separated from flammable gases and fires. Ground biomass and char can be fine powders and may cause dust hazards.

Hazard Control Measures:

- Safety glasses
- Proper lab attire
- Nitrile gloves

Waste Disposal Procedures: All contents after fermentation can be dumped into the sink

Decontamination Procedures: Autoclaving

Spill containment and clean up procedures: Yeast, sugars, and fermentation media can be wiped up and thrown in a non-hazardous waste container

Using substances requiring special procedures? Yes

Written By: Zach Bartlett **Date:** 10-7-2011

Approved By:

Detailed procedures, instrument operation and maintenance, emergency contact information and a list of those trained for this procedure are attached.

Equipment Description

The fermenter used in this experiment is a 5L New Brunswick BIOFLO 310 fermenter. It has the options of batch, fed batch, continuous culture. The continuous culture is the option used in the fermentation experiment. The microprocessor controls pH, dissolved oxygen (DO), agitation, temperature, pump feed, antifoam, foam-level, and addition analog/digital inputs and outputs. The systems can be configured as either control stations or utility stations.

The fermentation vessel is designed for 5 liters. The vessel contains a stainless steel head plate, a flanged thick-walled glass tube. The dished head is jacketed for the circulation of temperature-controlled water. Ports in the head plate are provided for: acid-base addition, thermowell for a resistance temperature detector (RTD), a foam probe, a sparger, a harvest tube, a sampling tube, an exhaust condenser, and dissolved oxygen (DO) and pH electrodes. A primary control unit (PCU) is available on the touch screen for monitoring and adjusting fermentation conditions and cables for connecting to probes.

The agitation system is a removable motor located on top of the bearing housing on the head plate. It's connected to the agitation shaft with a multi-jaw coupling for fermentation. The motor speed ranges from 50-1200 RPM. The PCU can control the motor speed. It is possible to cascade the dissolved oxygen to agitation so the agitation speed will vary between the user-specified minimum and maximum set points in order to maintain a set percentage of DO. DO is controlled in the range of 0-200%.

The resistance temperature detector can be controlled through the PCU and may be selected to range from 5°C and 80°C. The RTD is submerged in the thermowell. Aeration is another feature that can be controlled with the PCU. The airflow rate can be regulated by inputting values on the touchscreen. The percentage of oxygen blended with the sparge air can be controlled manually by the user or automatically through the controller by applying the O_2 enrichment function.

The pH is controlled in the range of 2.00-12.00. The pH is sensed by a gel-filled pH probe. Two peristaltic pumps, assigned to acid and base addition parts, connect to the instrument to control pH. The user can select a pH value on the PCU, and acid or base is added to account for change. Foam can be controlled during fermentation by a foam/level probe located in the head plate. The controller operates the antifoam assigned pump that adds defoamer as needed.

The exhaust gases pass into the exhaust condenser where moisture is removed, then returned to the vessel. The remaining air passes through a 0.2µm filter. There is also a sampling system. The sample is attaching to a sampling tube that extends to the lower portion of the vessel. This sampler has a rubber suction bulb to facilitate collection of representative samples without contamination.

Pre-Autoclave Checklist

- Media components, all fermenter parts, glassware, pH meter, stir/hot plates, acid and base solutions, and autoclave supplies are available.
- Autoclave is available and to be used
- **Sample Media Composition (per 1 L working volume)**
    - 20 g of peptone
    - 10 g yeast extract
    - 50 g glucose *Note: glucose is prepared and autoclaved separately to prevent caramelizing; preparation volumes should give a combined volume of 3 L.*

Sample Media Preparation Procedure

1. Place 4L Erlenmeyer flask on stir plate and add stir bar. Add 2000 ml of distilled water to the flask and turn on stirring.
2. Weigh out 60 g of peptone using beaker and bulk balance. Carefully add to flask. Peptone absorbs water and can become sticky on surface. Clean up any spills as soon as possible.

3. Weigh out 30 g of yeast extract and bulk balance. Carefully add to flask.
4. Rinse down side of flask by adding another 500 ml of distilled water. Stir until solution is no longer cloudy (several minutes).
5. Calibrate pH meter and then hang pH probe into flask so that the bulb on the end is just under the solution surface.
6. Adjust the pH of the solution to 4.5 (as read by the pH meter) by adding 10% sulfuric acid drop-wise with a plastic transfer pipette while stirring.
7. Empty flask of medium solution into fermenter. Add two drops of antifoam.
8. Using 1 L flask, prepare OD blank solution by adding 6 g peptone and 3 g yeast extract to 300 ml of distilled water. Stir until dissolved. Label and cover flask with aluminum foil.
9. Using another 1 L flask, slowly add 50 g glucose into 200 ml of DI-water *while stirring*. Heat the solution on a hot plate to dissolve completely.
10. Dilute glucose solution to 450 ml with distilled water and pour into labeled 500 ml addition bottle.
11. Cover a 125ml beaker with aluminum foil (used for yeast later).

Fermenter Preparation and Sterilization

1. Attach fermenter head plate:
    a. Apply thin layer of lubricant around top surface of glass vessel.
    b. Set head plate in place and secure sliding metal ring using four bolts.
    c. Tighten bolts evenly.
2. Calibrate the pH probe:
    a. Remove probe from 4M KCl storage bottle and rinse with DI water.
    b. Connect probe to PCU with cable.
    c. On PCU, select Calibrate button, then pH probe.
    d. Immerse pH probe in 7.00 buffer and allow Raw Input signal to stabilize.
    e. Enter the value 7.00 in box below Set Zero button and press enter.
    f. Press Set Zero button to accept value.
    g. Recap 7.00 buffer solution and rinse probe with DI water.
    h. Enter the value 4.00 in box below Set Span button and press enter.
    i. Press Set Span button to accept value.
    j. Recap 4.00 buffer and rinse probe with DI water.
    k. For best results, repeat calibration.
    l. Insert probe into fermenter carefully, making sure it has a tight seal and the end is covered in solution. (Do leave probe dry or in DI water!)
3. Prepare the fermenter for autoclaving:
    a. Insert dO_2 probe into fermenter.
    b. Place black rubber bearing cover on top of bearing housing to prevent steam damage.
    c. Clamp off the air sparger tube.
    d. Clamp off all tubes that have penetrations which go below the internal liquid level to prevent boiling over during autoclaving.
    e. Remove the RTD from the thermowell.
    f. Remove cables from pH and dO_2 probes; cover probe tops with shorting caps to prevent steam damage.
    g. Disconnect water lines from exhaust condenser and base heat exchanger.
    h. Loosen glass sample bottle on sterile sampler about ½ turn.

i. *Make sure that at least one exhaust point is working* (such as the exhaust condenser) *to prevent pressure build-up*. It is also good to leave a spare port plug loosely set in its hole, which can be re-tightened immediately after autoclaving.
j. Make sure all solutions are covered with aluminum foil, marked with indicator tape and labeled, and that the sugar addition bottle is clamped.

4. Autoclave the fermenter
    a. Get autoclave cart from BRL 1125.
    b. Set fermenter vessel upright on cart and all other solutions in the plastic autoclave pan.
    c. Move fermenter and solutions into autoclave in BRL using cart.
    d. Close autoclave doors by cranking wheel all the way counterclockwise.
    e. On touch screen, turn on jacket power.
    f. Press Select Cycle and enter "444" as the user ID.
    g. Select autoclave cycle for 45 minutes at 121°C.
    h. Autoclave will start automatically and cycle will take about 1 hour.
    i. After autoclaving, carefully remove vessel and solutions from autoclave using heat-resistant gloves and set-up in lab.
    j. Connect water and air lines; attach pH and dO_2 probes to PCU; replace RTD in thermowell; reattach motor drive.
    k. Turn on PCU power so probes can polarize overnight.

Sample Fermentation Description

Fermentation can be done with a wide array of variables. An example of a fermentation procedure is as follows:

1. Parameters
    a. Agitation Rate- 300 rpm
    b. Temperature- 32°C
    c. pH- 4.5-5
    d. Media initial glucose concentration- 50 g/L
    e. Yeast Inoculum- 50 ml brewer's yeast sample

Fermentation is an aerobic process with the given parameters will last for roughly 3-4 hours. After the aerobic process, air will be turned off and fermentation will continue under anaerobic conditions for another 4 hours. Sample will be taken at time 0 and every hour thereafter to measure optical density, glucose concentration and ethanol concentration. Samples are taken in the sample vial. The samples should be prepared for analysis quickly after they are taken so that the fermentation does not continue in the sample vial before analysis. Base (NaOH) will have to be added to the fermenter periodically as fermentation byproducts acidify the media to keep the pH about 4.5.

Pre-Fermentation Checklist

- YSI biochemistry analyzer has fresh membranes, glucose standard, ethanol standard, ethanol buffer. (For instructions on YSI analysis see Appendix A)
- Fermenter and additional solutions are prepared and sterilized.
- Probes have been polarizing for at least 12 hours.
- Yeast culture, all fermenter parts, sample vials, spectrophotometer curettes, micropipette, plastic consumables, volumetric flasks, centrifuge, and NaOH solution are available.

Fermentation Procedure

1. Turn on spectrometer to warm up. Set absorbance dial to 620 nm and pull out filter pull until orange dot is showing. (For instructions on spectrometer analysis see Appendix 2)
2. Make sure water is connected correctly to base heat exchanger and exhaust condenser, then turn on the water by opening the valve on the wall just before the water regulator/filter system. Adjust flow with valve until a slow but steady flow drains into the sink.
3. Heat the fermenter to 32ºC by changing temperature control from Off to Auto, and setting Set Point to 32.
4. While vessel is heating, add glucose solution by threading addition bottle tubing through the lowest peristaltic pump (in the bottom and out the top) and connecting it to one tube on the addition tri-port. Remove the clamps.
5. On the Pumps screen, change the pump power setting to 100% until as much glucose solution as possible has been transferred into the fermenter. Re-clamp the addition tri-port tubing before disconnecting addition bottle.
6. After the glucose has been added and the fermenter is at temperature, set the aeration rate by opening up the air line (*turn the red dial on the wall counter clockwise until gas flows—do not adjust the regulator pressure)* and selecting O2 Enrichment under the Air tab.
7. Set the agitation by changing the agitation control from Off to Auto, and entering the fermentation speed of 300 rpm into Set Point.
8. Calibrate the dO_2 probe:
    a. On PCU, select Calibrate, then dO_2 probe.
    b. Type 0 into the box below the Set Zero, but *do not press Set Zero yet*.
    c. Momentarily disconnect the cable from the dO_2 probe.
    d. When Raw Input stops decreasing, press Set Zero.
    e. Type 100 into the box below the Set Span button (this tells the controller that this is the max amount of oxygen available for the selected aeration rate, medium and agitation speed).
    f. When Raw Input stops increasing, press Set Zero button.
9. Check that pH is between 4.5 and 5. Thread the 4N NaOH addition bottle feed through the middle (base) peristaltic pump and connect to an unused addition tri-port tube.
10. Turn off air flow by pressing Off under the Air tab (this will keep air from bubbling into the NaOH addition bottle while the pump is being primed).
11. Prime the NaOH addition pump by removing the clamps, entering the Pumps screen and repeating pressing and holding the Prime button on the middle (base) pump's control. Continue priming the pump until NaOH is just about to drip into the fermenter.
12. Turn aeration back on.

Inoculation

13. Pour about 50 ml of Old Main yeast sample into sterilized beaker. Keep covered with foil as much as possible.
14. Remove sterile 60cc syringe from packaging and fill with yeast sample from beaker.
15. Remove foil covering inoculation port tubing and connect syringe to luer lock fitting.
16. Holding syringe plunger, unclamp inoculation tubing and slow push down on plunger until sample has been added.
17. Re-clamp inoculation port tubing before removing syringe.
18. Repeat if not all of yeast sample fit into one syringe.

19. Let the fermenter rest (no agitation) for about 10 min while you clean up inoculation glassware, wipe down area with 70% ethanol solution, and fill "blank" spectrophotometer cuvette with blank solution.
20. Start fermentation: reset the agitation to 300 rpm. Under Trends menu, hit Clear button to reset data collection.
21. Immediately take the first sample by opening the red valve on the sterile sampling port, squeezing the red bulb to clear sample tube and create a vacuum, then slowly releasing the red bulb. Sample should flow up into the attached vial. Close red valve and replace full sample vial with an empty one.
22. Sample every hour. After last sample, export trend data with USB drive.
23. Sample analysis (See Appendix 1 & 2)

Clean-up

1. Disconnect fermenter:
    a. Set temperature control to OFF.
    b. Set agitation to OFF and remove drive motor.
    c. Disconnect fermenter from air sparger line, RTD probe, pH and dO_2 probe cables, base and addition lines.
    d. Remove, rinse and store the pH and dO_2 probes.
    e. Carefully remove NaOH addition bottle tubing from peristaltic pump. Disconnect tubing from addition tri-port such that excess NaOH in tubing drains into fermenter or back into addition bottle.
    f. Loosen nut holding sterile sampler in place and lift out assembly for easier cleaning. Wipe down metal tubing and rinse out with distilled water being careful that filter does not get wet.
    g. Disconnect exhaust condenser from head plate and set aside.
2. Clean fermenter:
    a. Rinse water through any used addition tubing to remove residue.
    b. Loosen four mounting bolts below head plate and disconnect metal ring.
    c. Lift off head plate and set rim on edge of sink.
    d. Gently wipe off the bottom of the head plate and penetrations with sponge and warm soapy water, then rinse with DI water.
    e. Set head plate on paper towels on counter to dry.
    f. Empty the contents of the glass vessel into sink.
    g. Set glass vessel in bottom of sink and wash out with warm soapy water.
    h. Rinse glass vessel with DI water several times and set upright on counter to dry.
3. Collect and discard disposables (pipette tips, micro-centrifuge tubes).
4. Wash and rinse remaining glassware (cuvettes, test tubes, addition bottle). Set in racks or hang by sink to dry.
5. Wipe down work area with 70% ethanol solution, then water.

Maintenance

1. pH Probe Maintenance and Storage
    a. The pH probe should be stored standing upright, with the electrode tip immersed in a solution of 3M KCl or a buffer solution between pH of 4.0 and 7.00
2. DO Probe Maintenance and Storage
    a. Use soft facial tissue to clean the DO probe

   b. Check the probe's Teflon membrane to be sure there are no punctures, puckers, or wrinkles. If there are, the DO probe should be replaced.
   c. When it is not in use, the DO probe should be stored standing upright with the shorting cap in place and the membrane isolated from the air environment. At no time should the probe be allowed to rest on its membrane
3. Three month Inspection
   a. Check all controls and accessible items (power switch, connectors, screws, nuts and bolts) to make sure they are properly tightened. Tighten any loose items.
   b. Check all controls and connectors for dust
   c. Check all the O-rings in the head plate and impellers are intact and in good condition. Replace those that are not.

Appendix 1 (YSI Analyzer)

Equipment Description

The YSI analyzer works by using immobilized oxidase enzymes to produce hydrogen peroxide which is converted to a very small electrical current (nA) at a platinum probe. With calibration, these electrical signals are converted into concentration readings. The two biochemical analytes measured in this experiment are glucose and ethanol.

On the next page are two diagrams: one of the main components of the instrument (which can be seen by opening the front cover) and one of the sipper over the sample cell.

The sample cell contains a stir bar and has three probe ports attached to it: on the left, the black probe, the right, the white probe, and in the back, an auxiliary temperature probe. The glucose membrane (red-orange O-ring) should be installed on the black probe and the ethanol membrane (green O-ring) on the white. (The membrane locations can be switched but then the instrument settings must also be changed.) Instructions for how to install/change probe membranes can be found in section 2.4 of the operator's manual (drawer below instrument). Glucose membranes, standard solutions and buffer concentrates are stored in the 2059 fridge; ethanol membranes in the freezer. New membranes should be installed the day before analysis, as they require time to stabilize.

The buffer bottle should be filled with ethanol buffer (YSI 2787); both glucose and ethanol membranes are compatible with this buffer but the ethanol membranes are not compatible with the glucose buffer (YSI 2357), which may have been used for glucose analyses from other experiments. Generally, it is a good idea to make up new ethanol buffer for each fermentation; one concentrate bottle normally makes enough for one fermentation (~450 ml).

For this experiment, the buffer bottle should be filled with ethanol buffer, the calibrator bottle with glucose 2.5 g/L standard solution, and the test tube holder/station #2 with a test tube containing 2.0 g/L ethanol standard. The sample turntable (station #4) is located to the left of the instrument. Spare parts and a preventative maintenance kit are found in the drawers below the analyzer.

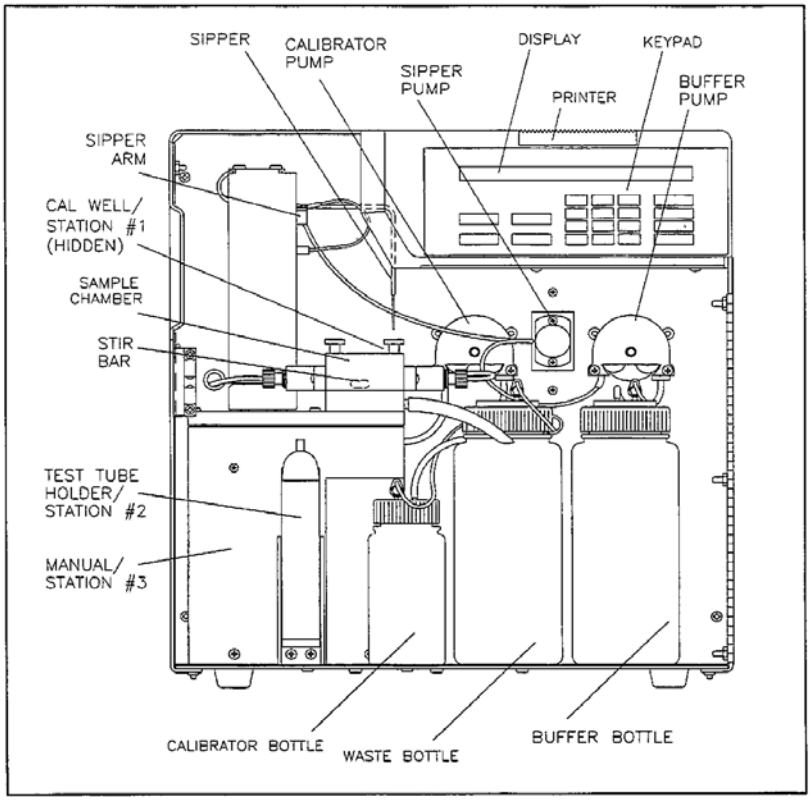
The sipper swings left to right, and raises/lowers. Among it functions are to fill and flush the sample chamber with buffer, and aspirate/dispense standards and samples from the various stations into the sample chamber. It uses conductivity to sense liquid levels when aspirating samples; any prepared standards, therefore, need to have ionic strength. The sipper cannot puncture sempta but can penetrate Parafim film (if needed to cover standards and samples which evaporate easily

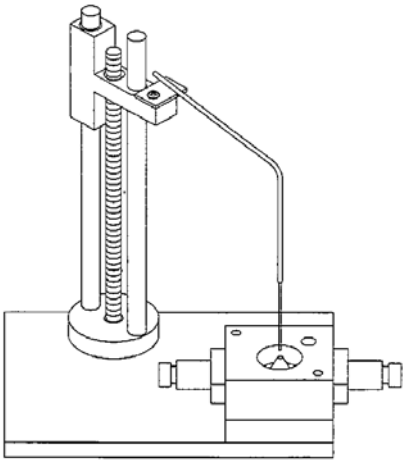
Instrument Operation

1. The instrument should say "Please select instrument mode" or be in the Standby Mode. If in Standby Mode, press the grey STANDBY button→2) Yes to leave Standby.

2. Verify/change analysis settings (manual section 2.9):
    a. MENU→2) Setup→2) MeasParameter→
        i. 1) SampleSize = 10 µL
        ii. 2) CalMethod → Two Stations: Black station # = 1, White station # = 2 (this means that the glucose standard is in the normal calibration bottle (1) and the ethanol standard is in the test tube holder (2))
        iii. 3) Black: Chemistry = Dextrose, unit = g/L, calibration value = 2.50 g/L, end point = 30 sec.
        iv. 4) White: Chemistry = Ethanol, unit = g/L, calibration value = 2.00 g/L, end point = 45 sec.
3. Check to make sure that the appropriate solutions are in the appropriate containers. The ethanol standard in the test tube holder should be covered with Parafilm to decrease ethanol evaporation. See section 4.1 for more details on solutions and settings for glucose and ethanol analysis, keeping in mind that ethanol buffer must be used.
4. To analyze samples, the instrument has to be in Run mode; this can be reached by pressing the grey RUN button. When the machine enters Run mode, it will first verify that the baseline probe currents are stable and below 6nA, then verify the calibrations.
    a. If you get a baseline error, you can check the currents by pressing MENU→ 3) Diagnostics → 3) Probe. Flushing while in diagnostics can often help lower the baselines, especially if there is a new membrane that hasn't had time to stabilize.
    b. If a calibration print-out lists unstable, it means that the calibration currant value shifted too much over time. The machine will automatically try again and normally get it by the second or third try.
    c. See also probe maintenance (section 7.5) and troubleshooting (section 8.2).
5. Once calibration is complete, the screen should read "Ready to Sample at Station #4" for the turntable. Make sure all samples are now uncapped and in position on the turntable.
6. To set up turntable sampling, press MENU → 3) RunMode → 1) SampleProtocol → 5) Turntable: Start in position: (enter position of first sample, normally 1, and press Enter); Number of samples: (enter number of samples you want analyzed); Level Sensing: On. Press 0 several times to return to "Ready to Sample" screen. For more sampling options, see manual section 3.2.
7. Press blue Sample button. When prompted, enter sample number and hit Enter. It is easiest to write a sample number on the side of each test tube, position the test tubes in ascending order, and enter the first test tube number as the sample number. This will mean that the sample number from each test tube prints out on the results.
8. When sample run in complete, remove test tubes from turntable and load the next set. If 2 hours passes without a sample being run, the machine returns to the Standby mode, in which case, the RUN button can be pressed to re-calibration and re-enter Run mode.
9. When you are finished for the day ***and if*** the instrument needs to be ready for ***glucose-only*** analyses, please reset the following settings (see Step 2):
    MENU→2) Setup→2) MeasParameter→
    a. 1) SampleSize = 25 µL
    b. 2) CalMethod → One Station: #1 (this means that the glucose standard is in the normal calibration bottle).
    c. 3) Black: Chemistry = Dextrose, unit = g/L, calibration value = 2.50 g/L, end point = 30 sec.
    d. 4) White: None

Leave the machine on "Please select instrument mode screen" or STANDBY.





Appendix 2 (DU 640 UV/Vis Spectrophotometer)

Equipment Description

Like most spectrophotometers, this instrument measures absorbance by taking light from a UV or visible light bulb, isolating a certain wavelength by passing the light through a monochrometer, then measuring the change in light intensity before and after it passes through a sample cell. This instrument was originally purchased for kinetics work and has several accessories not found on most instructional lab spectrophotometers, including: a sipper for manual and turntable sampling, a flow cell, and a transporter that holds a six-cell compartment with temperature control. For this experiment, only one cell position will be used for manual blanking and sample reading.

Cuvettes and cuvette caps (if needed) are found in the cabinets on the west wall of 2059, to the left of the spectrophotometer. These are disposable and can be thrown away after use. Make sure to touch only the cloudy sides of the cuvette and wipe down the clear sides with a KimWipe before placing it in the light path.

Instrument Operation

1. Turn on instrument using switch on the back (right side) of the spectrophotometer and the button on the front of the monitor. Make sure the sample compartment lid is closed.
2. The spectrophotometer will run some hardware diagnostics as it powers up, including moving the transporter back into position 1. When finished, a screen will appear that lists components and Passed/Failed. All components should say Passed unless they are not installed. (See main manual section 11.1 for Troubleshooting if necessary.)

Note: If Light Path reads Failed, do not worry; occasionally the light path test is run while the transporter is still moving, causing the light signal to be blocked.

3. Press Quit to reach the main screen.
4. On the bottom left of the screen, click on [VIS OFF] to turn on the visible bulb; once on, it will read [VIS ON] in red.
5. To make sure that the transporter is in the correct position (position 1), click on DEVICES on the bottom, then click on the 1 box below TRANSPORT. Click Exit.
6. Fill a cuvette with blank media solution and place it in position 1 in the sample cell (position 1 is farthest from you towards the back of the instrument). Click on BLANK to initially blank the instrument.
7. Click on RediRead on the bottom of the screen; this is the mode you will normally be in to read samples.
8. Set the parameters:
    a. Click on Wavelength, enter 620 with the mouse and click OK.
    b. Make sure Read Mode is set to [Abs] for absorbance.
    c. Read Avg Time is fine at 0.50 sec.
9. It is a good idea to re-blank the instrument before taking sample reading. This can be done by placing a cuvette of media blank in the sample cell and clicking on ReadBlank.
10. To read a sample, place a cuvette with the sample in position 1 of the sample cell and click on ReadSample. The absorbances will generally vary a little if pressed again; one can either go by the first reading or take several and average them. The screen can list the results of up to 8 samples. These can be cleared by clicking on Clear. (The Print function is not hooked up and will not work; students must manually record the data.)

When you are done for the day, remove any used cuvettes from the sample cell, and turn off the spectrophotometer and monitor.

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Training Sign-Off

Trainee	Date	Trainer