Biofuels Laboratory Manual

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by Northeast Biomanufacturing Center & Collaborative (NBC^2)

Global Biomanufacturing Curriculum

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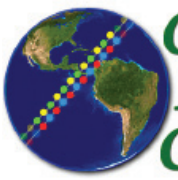


GLOBAL BIOMANUFACTURING CURRICULUM

Lab #1 - Microbial Growth & Analysis

Objectives

In this lab you will learn how to perform simple calculations and prepare different growth media based on the knowledge of the molarity of the different components. You will learn how to use a pH meter to adjust the pH of different solution and growth media. You will further monitor the growth of a culture of a known hydrogen producing bacterium, called *Enterobacter aerogenes*, over time. You will use spectrophotometry to determine the turbidity (optical density) change of the bacterial culture over time and apply the serial dilution and plating method to determine the concentration of viable cells (colony forming units per ml; cfu/ml). Optical density (OD_{550nm}) will then be correlated with colony forming units of *E. aerogenes* growing under the lab conditions. You will also determine the cell count of a given algae culture using hemocytometry and correlate with the optical density of the algae culture under investigation. You will finally learn how to prepare a bacterial culture for long-term cryo-storage by preparing glycerol stocks.

Introduction

Microorganisms, such as bacteria, yeast and algae, are important life forms commonly used in the biofuels field and in research laboratories. Bacteria such as Enterobacter species have great potential for future biohydrogen production and bioremediation. Yeast play an important role in industrial scale production of bioethanol from sucrose and starch. Microalgae, such as Chlorella, have great potential for future production of algae oil-derived biodiesel and food supplements. All of these microorganisms can be easily grown in bioreactor environments, such as fermenters and photobioreactors, in an aqueous environment called growth medium. Growth media can be either a defined medium where all components are known, or a complex medium which exact composition is not known. Routine and successful use of microbial life forms under controlled biotechnological settings requires that the cells are vigorously growing, i.e. in their exponential stage of growth, or that an exact known quantity of cells be available for use in biological research. For these reasons, it is valuable not only to understand the growth conditions, but also the growth patterns of these microbial life forms and to be able to accurately quantify the amount of cells in a given bacterial or algae culture. Once a successful culture with a given isolated microbe has been established it is important to be able to prepare stocks with aliquots of an established culture for long term storage and preservation. In this lab exercise, you will learn, 1) to prepare a growth medium for later inoculation of a given algae species, 2) to measure the concentration of viable bacterial cells (in colony forming units per ml) using the standard plate count (SPC) method, 3) measure the optical density (turbidity) of a bacterial culture over time using spectrophotometry, 4) count the number of cells of a given algae culture with the help of a hemocytometer (Neubauer chamber), and 5) prepare glycerol stocks of a given bacterial culture.

1. The Spectrophotometer

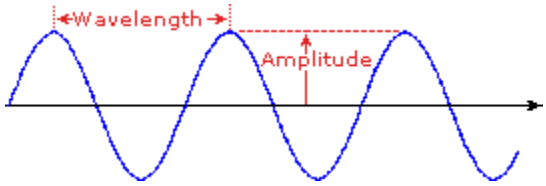
In the early 1940s, the CalTech chemist **Arnold Beckman** developed the first **spectrophotometer**, which - ever since - advanced to one of the most important lab equipment in modern biochemistry and bio-analytical labs worldwide. His famous Model A which was equipped with a tungsten light source and a glass prism, however, did not have enough power in the UV range and the prism was not suitable for spectrophotometry in the UV wavelength range. Today, modern, high performance spectrophotometer are equipped with **deuterium AND tungsten lamps** and (in comparison to the "classical instruments" which could only read one sample at a time), can handle multiple samples

at the same time cutting the amount of analysis time. The latest models of spectrophotometers offer endpoint, kinetic, and spectrum reading modes. Modern spectrophotometer come with bandwidths of < 1.8 nm and a maximum scan speed of 6200 nm/minute.
Before being able to professionally use and work with a spectrometer, we one should have a good understanding of the working principle of a modern spectrophotometer.
A **spectrophotometer is a high precision machine**, comprised of fine-mechanical, optical and electronic components (see Graphic below), which is able to measure and analyze the intensity of light after passing through an analytical cell or cuvet.
Today a spectrophotometer is employed in analytical labs to **measure the amount of light (= intensity)** that is absorbed by an inserted sample containing a molecule (or cells) to be identified or to be determined regarding its amount. With the help of a spectrophotometer:

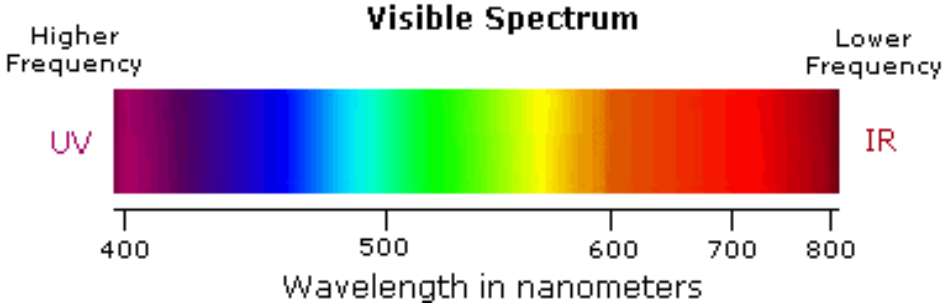
a. **the concentration of a known molecule can be determined (see Lambert-Beer Law)**
b. **the optical density (turbidity) of a cell culture can be determined**
c. **a unknown molecule can be identified (see Absorption spectrum)**
d. **the activity and catalytic properties of an enzyme can be determined (see kinetic and endpoint enzyme assays** (see BTEC293-Lab#2)

Working Principle:
Most molecules are able to interact with electromagnetic energy, most importantly with electromagnetic energy in form of **light**. As a matter of fact, all organic compounds interact ultraviolet (UV) or visible (VIS) light. If molecules interact with light they absorb parts of the total wavelengths of visible light (= **spectrum**) in a process called **absorption**.

Light as electromagnetic waves with defined wavelengths λ



The color qualities and corresponding wavelengths of the light spectrum



Other wavelengths pass the molecule freely without interaction and energy loss in a process called **transmittance**. Different molecules interact with different wavelengths of light; i.e. have a unique so-called **absorption spectrum**, which can be used for their molecular identification.

Important Parts of a modern spectrophotometer are:

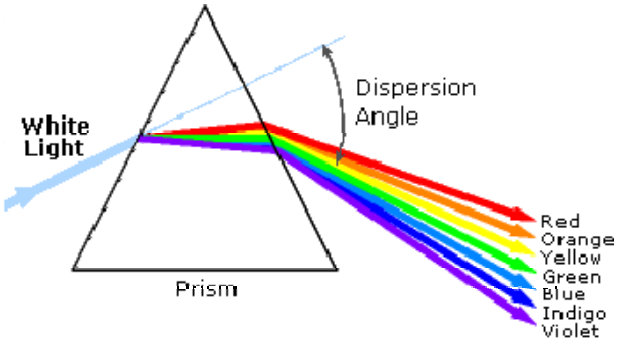
a. Light source:

- A spectrophotometer is equipped with a strong light source which can be either a:
    1. **Tungsten** light bulb
        → creates light with wavelengths from 350-1000nm
        → used for visible light or VIS spectrophotometry
    2. **Hydrogen** bulb
        → creates UV light with wavelengths between 200 and 400 nm
        → used for UV light spectrophotometry

b. Monochromator:

- important component that creates **monochromatic light** which is send through a cuvette containing the light absorbing substance
- Monochromatic light is light in which all photons have the same wavelength
- Monochromatic light is achieved with the help of a:
    1. **Quartz prism**
        → resolves wavelengths of the visible light spectrum between 200 nm and 800 nm (see **Figure** below)

The spectral dispersion of visible light in a quartz prism



OR alternatively a

    2. **Grate**
    - resolves wavelengths from 185 nm to 1000 nm both are the central, high precision part of a spectrophotometer

c. Slits & Lenses

- the selected wavelengths of light emanating from the prism or grate of the monochromator are directed by a series of lenses, mirrors and slits to the measurement (or sample) cell

d. **Measurement Cell**

- the measurement cell holds the **cuvette** pass through containing the dissolved substance to be analyzed
    - the cuvet can be made up from different materials:
        1. Clear polystyrene plastic cuvets → used for VIS spectrophotometry
        2. Clear quartz cuvets → used for UV spectrophotometry

e. **Photodiode or Photomultiplier**

- the light transmitted through the sample falls on the photocathode of a phototube or photomultiplier

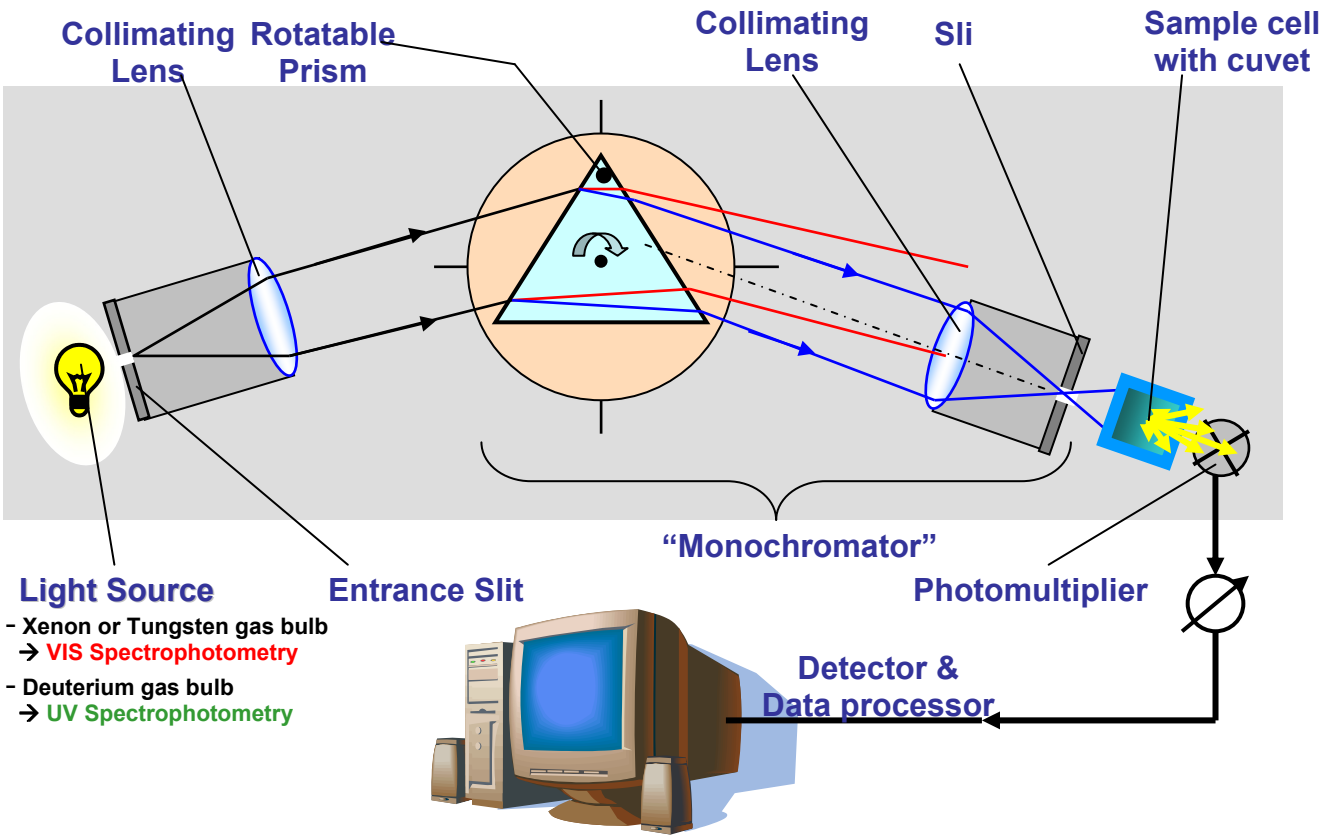
- an electrical current generated in the photo-detector is amplified and send to an intrinsic data processor which calculates the absorbance

f. Gauge or Monitor

- shows the calculated absorbance reading of the sample in the light path of the measurement cell

The **Figure** below gives you an overview on the important components and the path of light in a typical spectrophotometer.

Components & Detection Principle of a Spectrophotometer



As with any technical devise, a spectrophotometer has limitations. Molecules which do not interact with light, i.e. do not absorb light photons, cannot be analyzed with a spectrophotometer; accurate analysis of molecules and especially proteins in the UV range is difficult because of the high (blank) absorbance of many solvents.

2. The Lambert-Beer equation

In spectrophotometry one observes that the amount of light of a defined **wavelength (λ)** which is absorbed by a substance (= **absorbance A**) is directly proportional to, 1) the number of molecules per volume (or **concentration c**) of that absorbing substance in the light path and 2) directly proportional to the length of the **light path (d)** through the solution. The length of the light path in the measuring cells of all modern spectrophotometer is identical to the length of the measurement cuvet and is standardized to 1 cm.

This relationship of these parameters is mathematically expressed as the **Lambert-Beer Equation** as described below:

$$\mathbf{A_\lambda = E_m\ c\ d}$$

A_λ = absorbance at defined wavelength λ
c = concentration of substance (mol/l = M)
d = length of light path/cuvet (1cm)
E_m = absorbance constant or molar extinction coefficient ($cm^2\ M^{-1}$)

It is important to know, that the proportionally or absorbance constant E_m depends on the wavelength λ of light and the nature of the absorbing substance. Values for E_m are found tabulated for various substances in various solvents and for different wavelengths. Knowing the identity of a substance and its absorbance constant at a given wavelength, one can accurately determine the concentration of that substance in a given sample. The photon detector (photomultiplier) of the photometer actually measures (counts) the amount of photons freely passing through the measurement cuvet, means the light transmission (T), but calculates the absorbance (A) based on following mathematical connection.
Light transmission or **Transmittance T** (usually given in %) is defined as light intensity I of substance path divided by light intensity I_o (of control or blank path):

$$\mathbf{T = I / I_o}$$

The intensity of light (I_0) passing through a so-called **blank cuvet** and the intensity of light (I) passing through the **sample cuvet** containing the substance are measured by the photometer (see **Figure** below). The blank cuvet only contains a solution X without the substance to be determined, while the sample cuvet contains the substance to be analyzed dissolved in its appropriate solution X. The choice of solution X depends on the physico-chemical characteristics of the substance, e.g. solubility, pK-value, functional groups, etc. Solutions can be water, buffered salt solution, hexane, ethanol, or methanol.
The photomultiplier detects and amplifies the two intensities I and I_o (detected in photons/sec) and the experimental data are used by the spectrophotometer to calculate the two quantities: Transmittance (T) and Absorbance (A).
Absorbance A which usually ranges from 0 (no absorption) to 2 (99% absorption) is defined as:

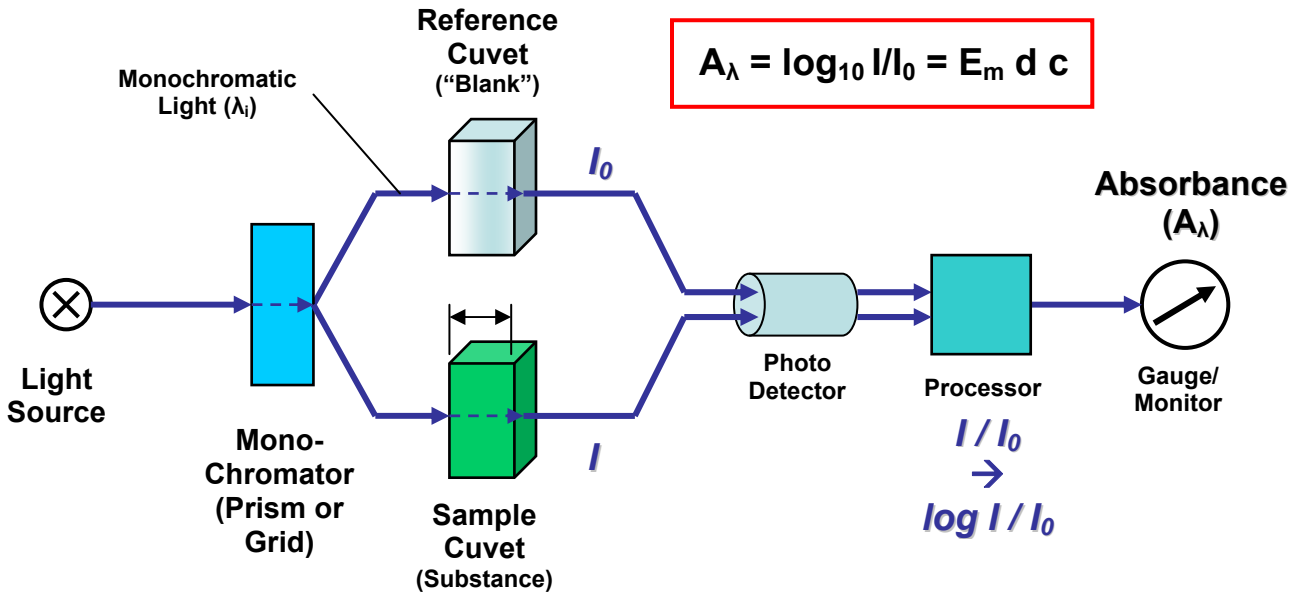
$$\mathbf{A = \ln I / I_o}$$

Sometimes values are given as Extinction E instead of Absorbance A.
Extinction E is defined as:

$$\mathbf{E = \log_{10} I / I_o}$$

The difference between A and E is a scaling factor of *ln10*.

Absorbance & The Lambert-Beer Equation



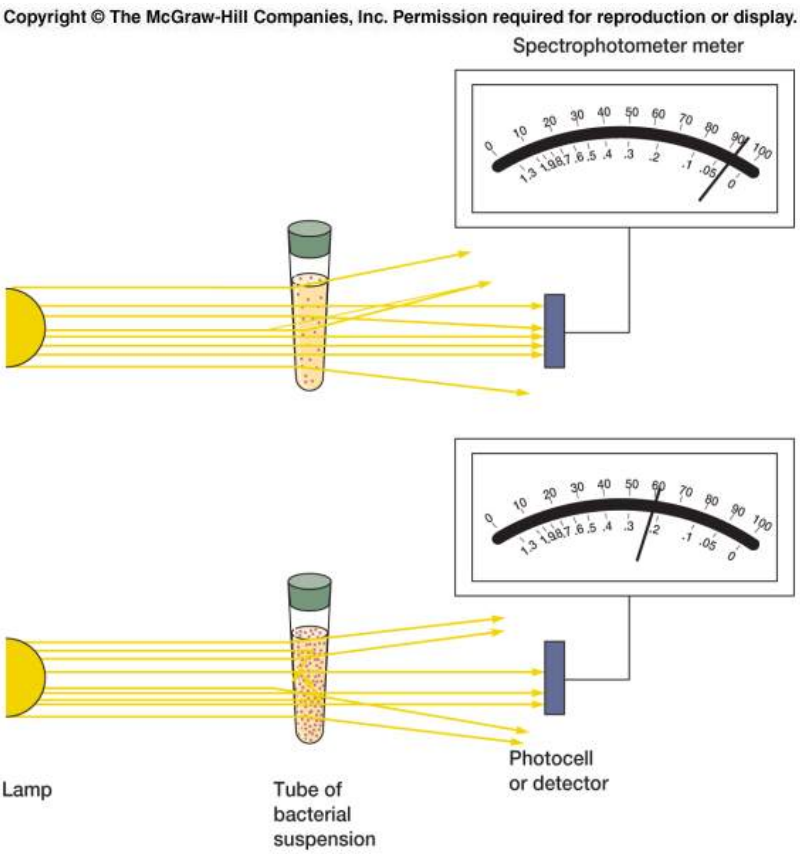
3. Optical Density (OD) or Turbidity/Microbial Mass Measurement Method

This method which is based on the measurement of the increase in biological cell mass over time. The biological cell mass increases over time due to continuous cell divisions and the resulting growth of a given microbial population in a growth medium-filled vessel. A rapid, sensitive and reliable technique to measure the microbial growth over time is with the help of **spectrophotometry**. Spectrophotometric monitoring of microbial growth is based on the fact that microorganisms scatter certain wavelengths of light which strikes them in a special reaction tube called a **cuvette**. The degree of light scattering or **optical density (OD)** of a microbial suspension is proportional to the cell biomass in the vessel (see **Figure** below). Generally, at a cell density of 10^7 bacteria/ml the medium appears turbid and cloudy leading to an increased light scattering. Scattering is usually measured in form of light absorption of the cell broth at a wavelength of between 550 – 660nm. Plotting of the measured OD at a constant wavelength (usually 600-660nm) over time allows the establishment of a typical **growth curve.**

Besides spectrophotometry, microbiologists rely on many different methods to analyze bacterial growth in a laboratory setting for final calculation of biomass, growth rates and generation times. However, no single method introduced below is always best or ideal. The final choice of the bacterial growth technique depends on the available experimental tools, the experimental question and situation.

Measurement of optical density of a bacterial culture

--> allows growth curve establishment



Spectrophotometric determination of the optical density (OD) of a microbial culture has the advantage that it is relatively quick in determining microbial growth. But this method has the disadvantage that it does not yield the actual bacterial count nor is it able to discriminate between viable (living) and dead microbes within a given sample. A method of choice to get this information is the following one.

4. Serial dilution method & Determination of colony forming units (cfu)

This is a good method to determine the number of viable (living) cells, e.g. bacteria, in a given sample. This method, which often referred to as the **standard plate count (SPC)** or viable count method, is widely used in routine microbiology and biotechnology laboratories.

A sample is diluted in a series of dilution blanks as shown in the Figure below. Defined aliquots (e.g. o.1 ml or 1.0 ml) of the prepared dilutions are then plated onto the surface of a solid media, i.e. an agar plate, and the number of bacterial colonies growing on the plates are counted after incubation for 24 – 48 hours. It is assumed that the bacterial cells in the starting sample are diluted to an end point where a single cell divides and gives rise to one visible colony on the plate. The number of bacteria in the original sample is determined by multiplying the number of colonies on a plate by the corresponding **dilution factor (DF)**. However, the assumption that a colony represents the descendants of a single cell is not always correct. Bacteria which grow in cell clusters or cell chains, such as Streptococci species, will also give rise to only one colony on a plate. Because of

the uncertainty in how many actual cells form a colony, counts by the SPC method are documented in **colony forming units (cfu)**.
Only bacterial colony numbers between 30 and 300 cfus are considered to be statistically valid. If the cfus are greater than 300, there is a probability that overcrowding and competition on the plate may have inhibited some cells from growing and successfully forming a colony. Less than 30 cfus could involve a sample error or pipetting mistake and lead to an underestimate of the bacterial cell count.
Despite the fact that the SPC method is a good method which allows an accurate determination of viable bacterial cells it has its limitations. The SPC method can be biased especially when one tries to apply this method for determining viable bacterial counts in environmental samples, e.g. soil. Since specific media and conditions are used, the SPC method would severely underestimate the numbers of bacteria in a soil sample where many of the bacteria will not grow favorably in the media used. Under these conditions the SPC method would most likely favor the growth of aerobic heterotrophic bacteria which grow at neutral pH values. The standard conditions of the SPC method does most likely not support the growth of strict anaerobic soil bacteria, chemolithotrophs, or bacteria that prefer to grow at extreme temperature or pH values.
However, in determining the number of bacteria present in bioreactor samples, water, milk or food, the SPC method is the procedure universally used. It is relatively easy to perform and gives excellent results with these samples.
In this lab we will use the SPC method to determine the number of bacteria in a given bacterial culture and later correlate this cell number with the corresponding optical density of this culture. Generally, the procedure consists of prepare several serial dilutions of the original bacteria sample in growth medium (LB) as illustrated in the **Figure** below. Aliquots of the diluted bacterial samples are then spread onto the surface of LB agar plates and incubated for 24 hours at 37^{o}C and then examined. A plate that shows between 30 and 300 colonies is selected for counting and determination of the cfu.

Following **microbial counting techniques** are widely applied in biotechnology and biological research.

5. Petroff-Hauser Counting Chamber

This microscopic determination method determines the **bacterial number** in a given sample through direct counting with the help of a glass-made counting chamber. It is a relatively easy, inexpensive and quick method which only requires a compound microscope and a glass slip covered, special glass chamber, the Petroff-Hauser Counting chamber. The surface of the counting chamber has an etched in counting grid which holds a small volume of bacterial suspension. The average number of bacteria in the chamber's 25 squares section of the grid are counted and the cell density is calculated by knowing the dimensions of the chamber as well as the dilution factor, applying the following procedure:

25 squares cover an area of:	1 mm^2
the chamber's depth is:	0.02 mm
counting **chamber volume**:	0.02 mm^3 = 0.02 µl = 2 x 10^{-5} ml

Example:

- you pipette a 1:100 diluted bacterial cell suspension into the chamber
- you count 69 bacteria (= $\mathbf{n_c}$) within the 25 square section of the chamber

The cell count given in number $\mathbf{n_B}$ of bacteria per ml is calculated as following:

General Formula:

$$n_B = n_c \times 100 \times 2 \times 10^5 = \_\_\_\_ \text{ cells / ml}$$

For our example that calculates into:

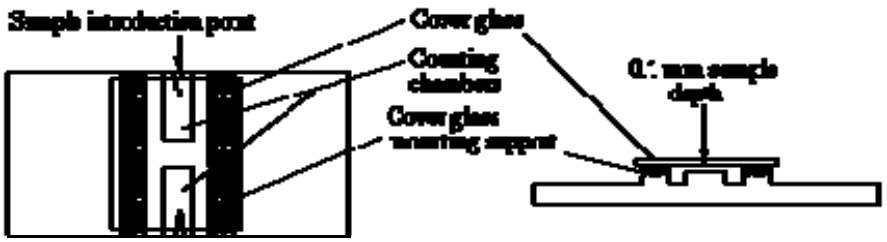
$$n_B = 68 \times 100 \times 2 \times 10^5 = 1.36 \times 10^9 \text{ cells / ml}$$

The disadvantage of this method is that it is difficult to discriminate between live and dead bacteria. It does not allow to get an exact number for the viable cells in a given sample.

6. Cell Counting using a Hemocytometer (Neubauer Chamber)

For microbiology, cell culture, microalgae cultivation and many other applications that require use of suspensions of cells it is necessary to determine the count of cell per volume or cell concentration. In case of larger cells than bacteria, for example red blood cells or microalgae, scientists measure the cell count with the help of a type of counting chamber called a hemocytometer. A hemocytometer is a counting devise originally designed for performing blood cell counts. In this course you will use a hemocytometer to count the number of microalgae in a given algae culture.

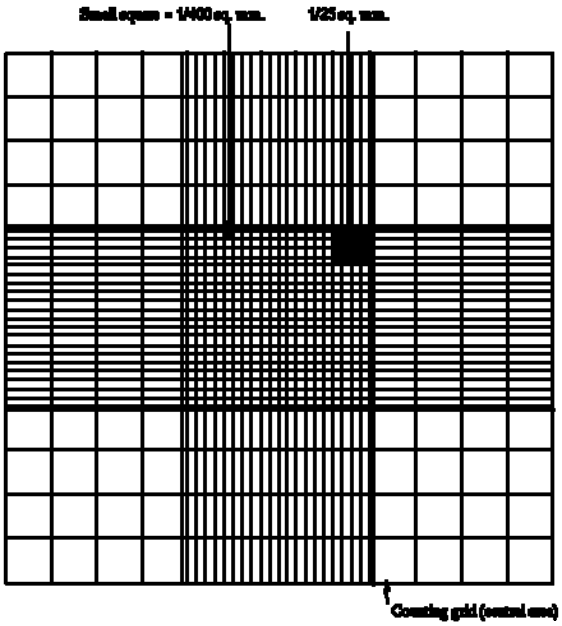
A hemocytometer, which is used in combination with a compound microscope, is a thick glass slide with two counting chambers (see **Figure** below). The polished surface of each counting chamber has an etched in counting grid which will hold the introduced sample of cell suspension. holds a small volume of bacterial suspension. The number of cells in the central section of the chamber's 25 squares section of the grid are counted and the cell density is calculated by knowing the dimensions of the chamber as well as the dilution factor.



To prepare the counting chamber the mirror-like polished central surface is carefully cleaned with lens paper. A glass cover slip is also cleaned. Coverslips for counting chambers are specially made and are thicker than those for conventional microscopy, since they must be heavy enough to overcome the surface tension of a drop of liquid. The coverslip is placed over the counting surface by sliding it over the previously wetted surface of the cover glass mounting support. The cell suspension under investigation is slowly introduced into one of the V-shaped wells with a Pasteur pipette or other type of micropipettor. The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered. The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low (TM: 40x) magnification power. Final counting of the algae cells in the chamber will be performed at a total magnification of 400 times (400 x).

It is essential to be extremely careful with higher power objectives, since the counting chamber is much thicker than a conventional slide. The chamber or an objective lens may be damaged if the

user is not careful. One entire grid of a standard hemacytometer with Neubauer rulings can be seen at TM 40x (4x objective lens) as shown in the **Figure** below. The main divisions separate the grid into 9 large squares (like a tic-tac-toe grid). Each of the nine basic squares has a surface area of one square mm, and the depth of the chamber is 0.1 mm. One basic square of the counting grid has a volume of: 1 mm^2 x 0.1 mm = 0.1 mm^3 or 0.1 µl or 1/10,000 ml.



Suspensions should be dilute enough so that the cells or other particles do not overlap each other on the grid, and should be uniformly distributed. To perform the count, determine the magnification needed to recognize the desired cell type. For the microalgae you will be using in this course, total magnification (TM) of 400 times (400x) is sufficient to do the cell counting. Now systematically count the cells in selected squares so that the total count is 100 cells or so (number of cells needed for a statistically significant count). For large cells this may mean counting the four large corner squares and the middle one. For a dense suspension of small cells you may wish to count the cells in the four 1/25 sq. mm corners plus the middle square in the central square. Always decide on a specific counting patter to avoid bias. For cells that overlap a ruling, count a cell as "in" if it overlaps the top or right ruling, and "out" if it overlaps the bottom or left ruling.

Suppose 187 algae cells were counted in the 25 small (1/25 sq mm) squares in the center of the grid after introducing a sample of a 1/5 diluted algae culture into the chamber.
Since each square has an area of 1/25 mm-squared (that is, 0.04 mm-squared) and a depth of 0.1 mm, the total volume in each square is (0.04) x (0.1) = 0.004 mm^3. Since 25 squares with a combined volume of 25 x (0.004 mm^3) = 0.1 mm^3-cubed were counted with 187 cells in it, the cell count (in cells per milli-liter) is counted as following:

Cell density (CD) = dilution factor (DF) x cell count (CC) x 10^4 (cells/ml)

CD = 5 x 187 x 10^4
= 9.35 x 10^7 cells / ml

LABORATORY ACTIVITY

Note: You will be working in pairs for this activity, however, each student should be able to perform all tasks associated with this lab activity if asked by the instructor to do so. Also, each student is individually responsible for all documentation and data analysis.

Day 1:

Activity 1: Bacterial Culture Growth and Sample Collection

1. Using the aseptic technique to maintain sterility during this step, transfer 100 ml of sterile TYP medium to a sterile 500ml Erlenmeyer flask. Label the flask with your names (initials) and the table number.
2. Aseptically remove 2 ml TYP medium and pipette this volume into a sterile 15 ml conical tube. Label this tube "TYP blank" and set aside (to be used in Activity 3).
3. Aseptically inoculate the 100 ml TYP flask with 5.0 ml of a fresh overnight (stationary) *Enterobacter aerogenes* (Ea) culture. Mix by gently swirling the flask.
4. Immediately (*while maintaining sterility*) remove 2 ml of the inoculated culture from the flask and transfer to a sterile tube which you previously labeled as "T_0". Immediately place the tube on ice (*this will be your first time point sample*).
5. Without delay, place your inoculated *E. aerogenes* culture in a shaking water bath incubator and incubate at 37^{o}C under continuous shaking.
6. After 30 minutes, remove 2 ml of the bacterial culture in the flask (*stop the shaking mode but leave the flask in the water bath*) and transfer to a sterile tube previously labeled "T_{30}". Immediately place the tube on ice for later measurements (see Activity 3). Turn the shaking mechanism back on and continue incubating the bacterial culture under constant shaking.
7. Repeat step 6, removing a 2 ml sample every 30 min. The samples in the tubes will be accordingly labeled "T_{60}", "T_{90}", etc.
8. Prepare a chart similar to the one below and record the actual times the samples were removed from the flask.

Sample Time Point (min)	Time Sample taken	Actual time from start (min)	Cell Count (cfu/ml)
t = 0	e.g. 7:15 PM	0	
t = 30	e.g. 7:47 PM	32	
t = 60			
t = 90			
t = 120			
t = 150			
t = 180			

Activity 2: Preparations of samples to determine the number of colony forming units per milliliter (cfus/ml).

Note: You will be handling a lot of samples in this portion of the lab activities. Make sure that you apply the aseptic technique to assure sterility of your prepared samples. Also, carefully label all materials (e.g. tubes and plates) ahead of the activities to avoid later stress and confusion of samples. Since the growth of the bacteria in the collected samples is halted once the samples are place on ice, you do not need to rush when performing the tasks to plate the bacteria!

Generally, prepare a dilution series of each of the previously collected bacterial culture samples, T_0, T_{30}, T_{60}, etc., as follows:

1. For each time point (T_0, T_{30}, T_{60}, etc.), label 4 sterile microfuge tubes with the time point and the dilution factors 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}.
2. Starting with the microfuge tubes corresponding to the time point of the first collected bacterial sample (= T_0), pipette the following volumes of TYP medium under sterile conditions into the microfuge tubes:
   - sterile pipette 990ul TYP to the tubes labeled "10^{-2}" and "10^{-4}"
   - sterile pipette 900ul TYP to the tubes labeled "10^{-5}" and "10^{-6}"
3. Working sterilely throughout this procedure, pipette 10ul of the 2 ml T_0 culture sample (from Activity 1) to the microfuge tube labeled "10^{-2}".
4. Vortex briefly
5. Using a new sterile pipette tip, pipette 10ul from the 10^{-2} tube to the 10^{-4} tube
6. Vortex briefly
7. Using a new sterile pipette tip, pipette 100ul from the 10^{-4} tube to the 10^{-5} tube
8. Vortex briefly
9. Using a new sterile pipette tip, pipette 100ul from the 10^{-5} tube to the 10^{-6} tube
10. Vortex briefly
11. Label the bottom of 3 sterile TYP agar plates with your name (initials & table number), the time point of the samples (e.g. T_0) and the dilution factors 10^{-4}, 10^{-5}, and 10^{-6}.

Note: Only the 10^{-4}, 10^{-5} and 10^{-6} dilutions will be plated to determine the cfus per ml. However, do NOT discard the 2ml culture samples (stored on ice!) you previously collected from the bacterial culture at different time points. These samples will be used to determine the optical density (OD) of the bacterial cultures by spectrophotometry in Activity 3 below.

12. Plate, by using the spreading technique, 100ul of each dilution (10^{-4}, 10^{-5}, 10^{-6}) on the surface of the correspondingly labeled plate
    - to spread the bacterial cells uniformly on the plate surface, sterile pipette 100ul to the center of the plate
    - sterilize a glass spreader ("hockey stick") by dipping it in ethanol, then igniting the ethanol on the spreader by moving the spreader into the flame of a Bunsen burner (this will allow the ethanol to burn off)

    DO NOT HOLD THE SPREADER DIRECTLY INTO THE FLAME AS THIS WILL HEAT UP THE GLASS, POSSIBLY RESULTING IN THE DEATH OF THE BACTERIA TO BE SPREAD.
    - spread the bacterial cells by pushing the 100ul liquid over the surface of the plate,

rotating the plate as you evenly spread the cells.
- flame sterilize the spreader again as described above when you are finished with the cell spreading

13. Repeat steps 2-12 for the remaining sets of microfuge tubes corresponding to each of the other time points (i.e. T_{30}, T_{60}, T_{90}, etc.).
14. Incubate all plates (bottom up) at 37^{o}C in an incubator overnight (< 24 hours).
15. Count the bacterial colonies on the plates in the next lab session, calculate the cfus and record your results in your lab journal.

Activity 3: Determination of Bacteria Culture Density (Turbidity) by Spectrophotometry.
Note: Once the collected bacterial samples have been serially diluted and plated (part II above), sterile handling of the samples is no longer required!

In this portion of the lab activity, the optical density (OD_{550}), or absorbance, of the collected samples of the growing bacterial culture is measured with the help of a spectrophotometer at a wavelength of 550 nm.

1. Obtain a spectrophotometer and turn it on at least 10-15 minutes before performing the measurements.
   - your instructor will give you a brief demo regarding its proper use
2. Set the wavelength of the spectrophotometer to 550nm and set the proper filter setting.
3. "Blank" the spectrophotometer to "zero" absorbance using TYP medium only.
   - pipette 1ml TYP medium from the tube labeled "TYP Blank" to a clean cuvette
   - use this cuvette only the "blank" to calibrate your spectrophotometer to "zero OD"
4. Measure the optical density (OD_{550}) of each previously collected culture sample, starting with the 2ml sample from the T_0 time point.
   - fill a new, clean cuvette with 1 ml of the T_0 sample
   - read and record the OD_{550} of the sample in the Data Table in the Data Collection Table below;
   - empty the cuvette content into a biohazard container (for liquids), rinse with distilled water, and fill the cuvette with 1ml of the next sample (i.e. T30);
   - continue to collect the OD_{550} readings until all samples have been read

Sample Time Point (min)	**Optical Density/Absorbance** at 550nm
0	
60	
90	
120	
150	
180	

5. After collection of the OD readings for all collected bacterial culture samples, dispose all cultures waste in a biohazard container (for liquids) and discard the used cuvettes in a biohazard container (for solids waste). Turn off the spectrophotometer.
6. Disinfect the work area with 70% ethanol (or other suitable disinfectant).

Activity 4: Determination of the Cell Count of an Algae Culture using a Neubauer Chamber.
Note: Make sure that you apply the aseptic technique while taking samples from the supplied algae culture to avoid cross contamination. Once the collected algae samples have been drawn from the culture flask (bioreactor) sterile working conditions during cell counting are no longer required.

In this portion of the lab activity, you will inoculate a new, scaled up algae culture using an existing algae stock culture ("St") and determine the cell count of the stock culture and of the newly inoculated algae culture at different time points using a Neubauer counting chamber (hemocytometer).

1. Locate the sterile 1 liter Erlenmeyer flask equipped with foam stopper and sealed off aeration tube inlet on your bench. Use a tape and label this flask with your names (initials) and the table number.
2. Disinfect the aluminum foil-capped top section of the flask by spraying it with 70% ethanol and wait 10 minutes for proceeding.
3. Put on gloves, remove the aluminum cap on top of the silicone aeration tube inlet of the flask and attach a 0.22 µm Acrodisc sterile filter to the aeration tube inlet.
4. Using the aseptic technique to maintain sterility during the following steps! Transfer 225 ml of sterile Bristol medium (for Monodus subterraneus or Chlorella protothecoides culture) or 225 ml of sterile Modified Bold 3N medium (for Chlorella minutissima culture) into the sterile 1 liter Erlenmeyer flask.
   YOUR INSTRUCTOR WILL TELL YOU AHEAD OF THIS LAB ACTIVITY WITH WHICH MICROALGAE SPECIES (*MONODUS* OR *CHLORELLA*) YOU WILL BE WORKING DURING THIS LAB
5. Aseptically remove 2 ml Bristol medium or Modified Bold 3N medium and pipette this volume into an empty clean reaction tube. Label this tube "Blank" and set aside (to be used in Activity 5).
6. Aseptically pipette 25 ml of a 7-10 day old phototrophic culture of the microalgae *Monodus subterraneus* (Group A teams) or of the microalgae *Chlorella minutissima* (Group B teams) to the 225 ml algae medium in the 1 liter flask.
   - use a sterile 25 ml plastic pipette to perform this step!
7. Gently mix the flask content by swirling the flask.
8. Aseptically remove 2 ml from the corresponding algae stock culture (Monodus or Chlorella) and pipette this volume into a clean tube. Label this tube "St"" and set aside (to be used in Part V).
9. Remove (*while maintaining sterility*) 2 ml from the freshly inoculated algae culture from the flask and transfer to a tube. Label this tube as "AC_0".
10. Hook up the algae culture to the aeration pump as shown in the **Figure** below) and place the flask with your inoculated algae culture onto the rocker platform positioned in front of a illumination station. Incubate the algae in your flask at 26^{o}C under continuous shaking with

90 rpm. Make sure that the illumination timer is set to a light-dark cycle of 12 hours light and 12 hours dark.

11. Remove a 2 ml sample from this flask at different time points, e.g. day 7, day 14, etc, and follow the steps 8 → 15 below
12. Now, prepare a Neubauer chamber by attaching the cover slip to the chamber.
    - your instructor will give you a demo and show you how to execute this task!
13. Pipette a small aliquot of the previously collected algae culture from the "St" tube into one of the two counting chambers of the assembled Neubauer chamber.
    - use a micropipettor with clean pipette tip to achieve this!
    - make sure that you do NOT overload the first chamber with cells
14. Pipette a small aliquot of the previously collected algae culture from the "AC_0" tube into the second (empty) counting chambers of the assembled Neubauer chamber.
    - again, use a micropipettor with clean pipette tip to achieve this!
    - make sure that you do NOT overload this chamber with cells which will lead to a mixing of cell sample with the first sample!
15. Place the Neubauer chamber under a compound microscope and count the number of algae in both chambers at a total magnification of 400 times (TM = 400x).
    - use the 40x objective lens; make sure that you are very careful while focusing during this step to avoid moving the objective lens into the (much thicker) Neubauer chamber assembly!
16. Count the number of algae cells in the central section of the chamber's 25 squares section of the grid are counted; if the cell count (CC) is >100 make a 1 to 5 dilution of the sample (in distilled water) and then repeat step 9 again.
    - if you dilute one of the samples make sure that you consider the dilution factor (DF) in the later cell density calculation!
17. Calculate the <u>cell density (CD)</u> for each of the collected algae samples (in cells/ml) using the following equation below, record it in the Results Table below as well as in your lab journal.

$$\mathbf{CD = CC \times DF \times 10^4} \text{ (cells/ml)}$$

18. Remove the Neubauer chamber from the microscope after completion of cell counting and clean the chamber with distilled water and wipe dry
19. Return the microscope back to the storage cabinet.

<u>Activity 5: Determination of Algae Culture Density (OD) by Spectrophotometry.</u>

Note: Make sure that you apply the aseptic technique while taking samples from the supplied algae culture to avoid cross contamination. Once the collected algae samples have been drawn from the culture flask (bioreactor) sterile working conditions during the following steps are no longer required!

In this portion of the lab activity, the optical density (OD_{540}), or absorbance, of collected samples of an algae culture are measured at different time points with the help of a spectrophotometer at a wavelength of 540 nm.

1. Obtain a spectrophotometer and turn it on at least 10-15 minutes before performing the measurements.
   - your instructor will give you a brief demo regarding its proper use
2. Set the wavelength of the spectrophotometer to 540nm and set the proper filter setting.
3. "Blank" the spectrophotometer to "zero" absorbance using Bristol medium (Monodus culture) or Modified Bold 3N medium (Chlorella culture) you collected during step 2 of Activity 4.
   - pipette 1ml from the tube labeled "Blank" to a clean cuvette;
   - use this cuvette only as the "blank" to calibrate your spectrophotometer to "zero OD"
4. Measure the optical density (OD_{540}) of a collected sample of your algae stock culture ("St"), i.e. Monodus or Chlorella, and of an algae culture which you collected at different time points, i.e. 0 days ("AC_0"), 7 days ("AC_{7d}"), 14 days ("AC_{14d}").
   - fill a new, clean cuvette with 1 ml of the stock culture sample "St";
   - read and record the OD_{540} of the sample in the Data Table in the Data Collection section below; if the OD is > than 2.0, dilute this sample 1 to 5 and measure the OD again;
   - empty the cuvette content into a waste container, rinse with distilled water, and then fill the cuvette with 1ml of the algae sample (e.g. "AC_0", "AC_{7d}", "AC_{14d}")
   - place cuvette into spectrophotometer and collect the OD_{540} readings for this sample;
5. After collection of the OD readings for the algae culture samples, dispose all cultures in a waste container, collect the cuvettes for cleaning and turn off the spectrophotometer.

Results Table: Algae Culture

Microalgae _____			
Time Point (days)	**Label**	**Cell Count**[&] (cell/ml)	**Optical Density** (OD_{540})
Stock culture	St		
0	AC_0		
7	AC_{7d}		
14	AC_{14d}		
21	AC_{21d}		

Day 2:
Activity 6: Data Collection, Graphing & Analysis

A. Bacterial Culture Density (Turbidity) & Colony Forming Units Data

1. Receive the agar plates from the Part II experimental section above and count the number of bacterial colonies (N_c) on each of the plates with the different dilution factor (DF). Write the colony count in the corresponding column of the Data Table below.

Note: If the plate shows dense growth but colonies are still distinguishable from one another, estimate the number of colonies by sectoring off one forth of the plate, counting the number of

colonies in that quarter, and multiplying by 4. If the number of colonies on the plate is too high to count, simple record for this plate "TMTC" (too many to count).

2. Calculate the number of colony forming units (cfus) per ml, for each time point using the following formula:

$$\mathbf{cfu = N_c \times DF \times 10}$$

- in the formula above, the factor 10 accounts for the fact that only 0.1ml was plated;
- for example, 35 colonies were counted on the 10-4 plate; the cfu then calculates as:

$$\text{cfu} = 35 \times 10^4 \times 10 = 3.5 \times 10^6 \text{ cfu/ml}$$

If more than one plate per time point gave quantifiable results, calculate the cfu per ml for each data point and then calculate the average cfu per ml for that given time point of your growth curve. If multiple data points within a time point give widely divergent cfu per ml numbers, the evaluate the data points for

a. possible known or recognizable errors in procedures for dilution plating
b. statistical significance of the numbers. Colony counts of less than 30, or over 300, per plate are less statistically significant than values which fall between 30-300 colonies per plate.

3. Construct a final summary table showing your experimental results that include each time point, the corresponding OD_{550} readings, raw data of plate counts, and the calculated (average) cfu per ml of culture.
Note: Use the actual times (e.g. 32 min vs. 30 min) in your table.

Results Table: Bacterial Culture

Time* (min)	**OD_{550}**	**# cfu on 10^{-4} plate**	**# cfu on 10^{-5} plate**	**# cfu on 10^{-6} plate**	**Calculated (Average) cfu/ml**
0					
30					
60					
90					
120					
150					

* = use actual elapsed time

4. Using the data compiled in the above table and construct the following two graphs (to be included in your lab journal).
Note: See S&M Chapt. 15 for assistance with graphing and the use of semi-log graph paper.

a. on semi-log graph paper, graph the:
"Time" (x-axis) versus "OD_{550}" (y-axis), and

"Time" (x-axis) versus "cfu" (y-axis)
- use a different symbol and/or color for the two different graphs, and explain in a graph associated legend

THIS GRAPH IS DESIGNED TO GIVE YOU A VISUAL REPRESENTATION OF HOW "cfu" AND "OD" OF A GIVEN CULTURE ALIGN WITH EACH OTHER OVER TIME.

b. on standard graph paper, graph:
"cfu" (x-axis) versus "OD_{550}" (y-axis)

THIS GRAPH IS DESIGNED TO ALLOW YOU TO EASILY CORRELATE A GIVEN "OD550" TO THE NUMBER OF "cfu" PER ML OF A GIVEN CULTURE.

5. Include a brief summary statement of the activities performed, including conclusions to be drawn from the individual activities and discussion of any problems and deviations in the protocol that you encountered.

B. Optical Density of Algae Cultures & Algae Cell Count

1. Use the collected algae data compiled in your "Results Table: Algae Culture" above, including the corresponding OD_{540} readings, and the calculated cell count of your algae culture into the Results Table below and construct the following two graphs (to be included in your lab journal).
Note: See S&M Chapt. 15 for assistance with graphing and the use of semi-log graph paper.

a. on standard graph paper, graph the:
"Time" (x-axis) versus "OD_{540}" (y-axis), and
"Time" (x-axis) versus "Cell Count" (y-axis)
- use a different symbol and/or color for the two different graphs, and explain in a graph associated legend

THIS GRAPH IS DESIGNED TO GIVE YOU A VISUAL REPRESENTATION OF HOW "Cell Count" AND "OD" OF A GIVEN CULTURE ALIGN WITH EACH OTHER OVER
TIME.

b. on standard graph paper, graph:
"Cell Count" (x-axis) versus "OD_{550}" (y-axis)

THIS GRAPH IS DESIGNED TO ALLOW YOU TO EASILY CORRELATE A GIVEN "OD550" TO THE NUMBER OF ALGAE "Cell Count" OF A GIVEN ALGAE CULTURE.

6. Include a brief summary statement of the activities performed, including conclusions to be drawn from the individual activities and discussion of any problems and deviations in the protocol that you encountered.

Activity 7: Cell Storage

In a routine biotechnology and biofuels laboratory environment, where one works with different microorganisms, it is crucial to maintain pure stocks of important, isolated and characterized microbial strains, e.g. bacteria or microalgae, for long-term storage and back-up. In this portion of the biofuels laboratory you will be introduced to common procedures to achieve long-term storage of bacterial or microalgae stocks.

A. Preparing Glycerol Stocks for Cryo-storage of Bacterial Cultures

1) Label a sterile PP cryo-vial with a permanent fine point marker; place the close capped vial on ice
2) Pipette 0.5 ml of sterile 60% (v/v) glycerol (in water) into the cryo-vial
3) Pipette 0.5 ml of a 24 hour culture of your bacterium to the glycerol solution and mix by inverting the close capped cryo-vial several times
4) Freeze the sample slowly by dropping the cryo-vial into a dry ice/ethanol bath or by placing it into the -20^{o}C freezer section of a refrigerator
5) Once samples are completely frozen, long term store the bacterial glycerol stocks in a -80^{o}C freezer or in a liquid nitrogen tank.

Note: Cell viability of the glycerol stock can be easily checked over time by scraping bacteria from the surface of the frozen stock culture using a heat sterilized platinum loop, streaking on a suitable agar plate, e.g. LB agar for E.coli, and evaluating colony growth after overnight incubation at the optimum temperature, e.g. 37^{o}C for E.coli.

B. Preparing Agar Slants for Storage of Microalgae Cultures

1) Sterile pipette 5ml of corresponding molten algae growth medium containing 1.5% agar into a 15ml glass tube (with screw cap), recap and set at a 20-24 degree angle on a special agar slants rack.
   - Team A students use Agar-Bristol medium for *Monodus subterraneus*
   - Team B students use Modified Bold 3N medium for *Chlorella minutissima*
2) When the agar in the tube has solidified (after about 30 min), label the tube (e.g. algae strain, batch number, date, lab ID#, etc.) and place in vertical position in a suitable rack.
3) Use a sterile loop, collect two or three loopful of the desired microalgae from the stock culture and streak the algae in a zig zag pattern up the agar slant in your tube.
4) Incubate for 2-3 days at the suitable temperature (e.g. 25^{o}C for Chlorella) in light with a slightly unscrewed cap on the tube; make sure that no contamination occurs during this growth period.
5) Once cells have grown, seal the tube and store at 4^{o}C, e.g. in a fridge, until further use in future inoculations.

Note: The same procedure can be used to prepare agar slants with bacterial cultures in their corresponding growth media. Bacterial slants are only incubated for 24 hours at (usually) 37^{o}C before stored at 4^{o}C for later use.

Background Reading & Reference

I. See instructor's website or Blackboard site for assigned further readings regarding growth curves, bacteria and microalgae
II. S&M Ch15 for further info regarding graphing and the use of semi-log graph paper
III. Prescott, Harley, Klein. Microbiology (McGraw Hill; 6th edition).
- Ch 6.1, Ch. 6.2. & Ch. 26

Lab #2- Biofuels & Enzymes

Objectives

In this lab you will test the ability of a type of enzymes called cellobiase to increase the conversion rate of a clear substrate called p-nitrophenyl glucopyranoside to glucose and a colored product called p-nitrophenol under different experimental conditions. Cellobiase is an enzyme which breaks down the cellulose breakdown product and disaccharide cellobiose into two glucose molecules. This catalytic reaction of cellobiase is important in the industrial process of making cellulosics ethanol, which is an efficient, sustainable biofuel with the potential to replace petroleum-derive fuels in the future. The substrate p-nitrophenyl glucopyranoside which you will be using in this lab is an artificial molecule resembling the natural cellulose degradation product cellobiose. The experimental parameters studied in this enzyme lab are pH, enzyme concentration, substrate concentration and temperature. You will finally test prepared bacterial and/or fungal extracts as potential natural sources of cellobiase activity.

Introduction

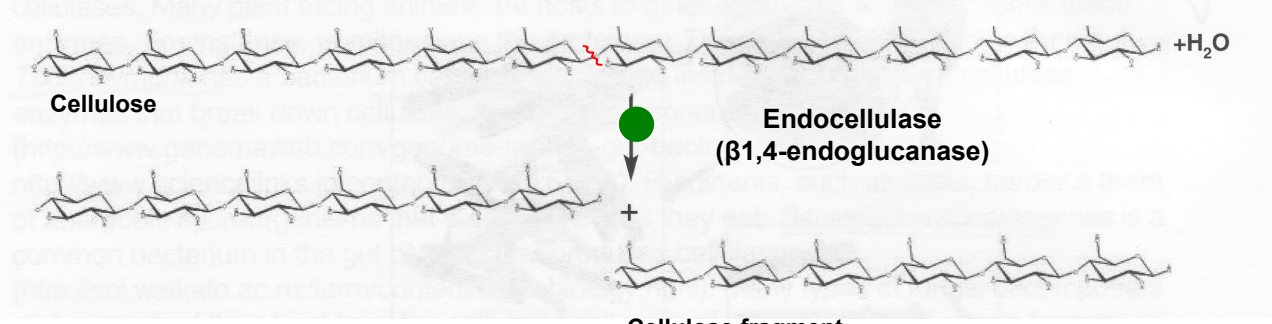
Biofuels, such as bioethanol, biogas or biodiesel, are fuels produced from renewable biomass. Most biofuels, most namely bioethanol and biogas, are generated with the help of microbial life forms such as bacteria and yeast. In order for microorganisms to convert biomass into biofuels through fermentation, certain biomass components, most prominently cellulose and hemicellulose, have to be broken down into smaller, fermentable constituents, most importantly glucose. They achieve this with a unique class of enzymes called cellulases. Enzymes are biological, protein-made catalysts with which help living systems speed up chemical reactions.

In order to understand why cellulosics biomass can serve as a source of sugars, i.e. glucose, which can then converted into biofuels, such as bioethanol, with the help of fermenting microbes, one need to understand the biochemical make up of cellulose.

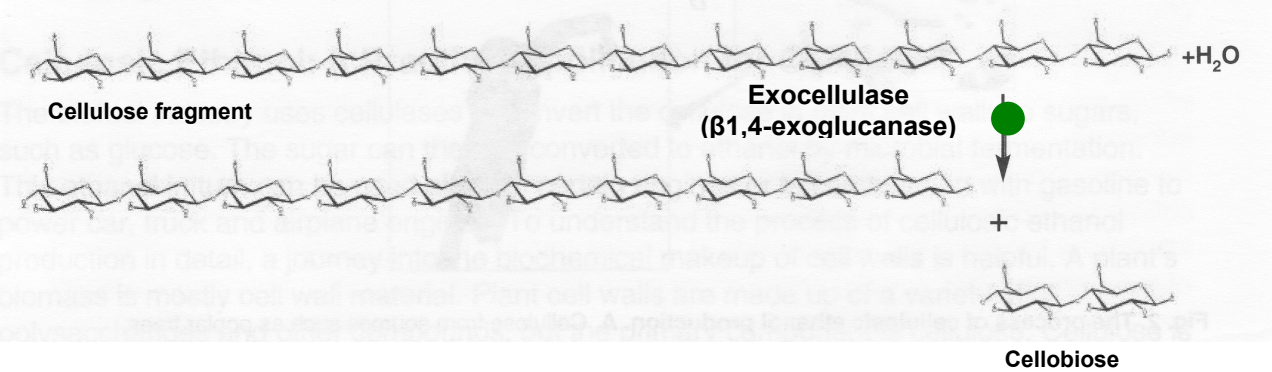
Cellulose, together with hemicellulose, is the major structural component of the cell wall of algae and green plants. It is a linear polysaccharide made up from thousands of glucose molecules linked together via repeated beta (β)-1,4 glycosidic bonds. Each cellulose molecule is attracted to other cellulose molecules via hydrogen bonds that form between their respective glucose molecules. These attractions form cellulose fibrils which are made up from 60-80 individual cellulose strands threaded together. Cellulose fibrils are found together with other polysaccharides and proteins in the primary cell wall of algae and living plant cells which makes this thick cell wall type stretchy and to allow for elongation and plant growth. Other plant cells which are dead at maturity develop a second type of cell wall called the secondary cell wall. Secondary cell walls besides cellulose fibrils contain high quantities of hemicellulose and of a hard phenolics derivative called lignin. Due to the presence of lignin secondary cell walls are more rigid than primary cell walls. They are therefore found in wood and other plant parts, where plant tissues with secondary cell walls have water and nutrient transport function. For cellulosics biofuels production from woody plant biomass, the lignins and hemicellulose must be removed before enzymatic degradation because they interfere with enzymatic break down of the cellulose.

In natural environments cellulose is degraded by a series of enzymes collectively called **cellulases** which are produced by a variety of microorganisms, most namely cellulolytic bacteria und fungi. Together, cellulases catalyze the complete enzymatic digestion of cellulose to fermentable glucose. While animals and humans do not produce cellulases themselves, certain plant biomass eating animals, e.g. ruminants and termites, are able to break down cellulase to glucose because their guts host symbiotic microorganisms which produce these cellulose-degrading enzymes. For example, the guts of "wood eating" termites is home to several protozoans, such as *Trichonympha sp.*, which themselves harbor many symbiotic, cellulose degrading (cellulolytic) bacteria, e.g. bacterium Rs-D17. The rumen, a unique digestive organ of certain grazing, herbivorous animals called ruminants, e.g. cows, buffalos, harbor a

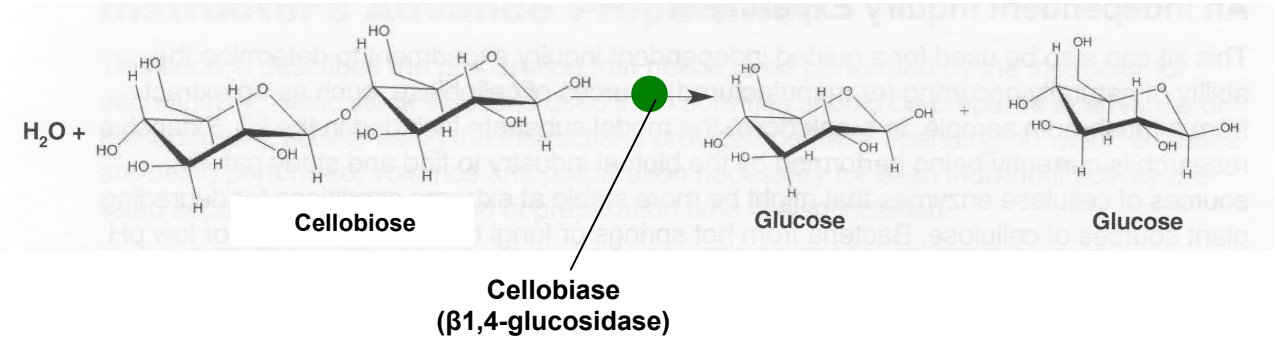
consortium of anaerobic bacteria that help these animals to completely break down the cellulose and other components of their plant food. *Bacteroides succinogenes* is a common bacterium found in the gut of cows that produces cellulases. Not only bacteria can break down cellulose into glucose, but many biomass decomposing fungi derive much of their food energy from the break down of the cellulosic cell walls into glucose with the help of cellulase enzymes. For example, the filamentous fungus *Aspergillus niger* produces cellulases, including a powerful beta (β)-glucosidase (cellobiase), that it secretes from its hyphae into the surroundings where it helps to break down cellulosics materials.
In nature, cellulolytic microorganisms rely on three different types of cellulases, called endocellulases, exocellulases and cellobiases, to achieve the complete degradation of cellulose into fermentable sugars, most importantly glucose.
Endocellulases (or Endo-β1,4-glucanases) are enzymes which cleave the beta-1,4-glycosidic bonds of cellulose at random sites within the cellulose strands (see **Figure** below). As a consequence they create many short cellulose fragments, which themselves can then serves as substrate molecules of the exocellulases.



Exocellulases (or Exo-β1,4-glucanases) are enzymes which cleave the beta-1,4-glycosidic bonds of cellulose from the (usually) reducing ends of this polysaccharide (see **Figure** below). As a consequence exocellulase release the disaccharide cellobiose which then serves as substrate molecule of a class of enzymes called cellobiases.



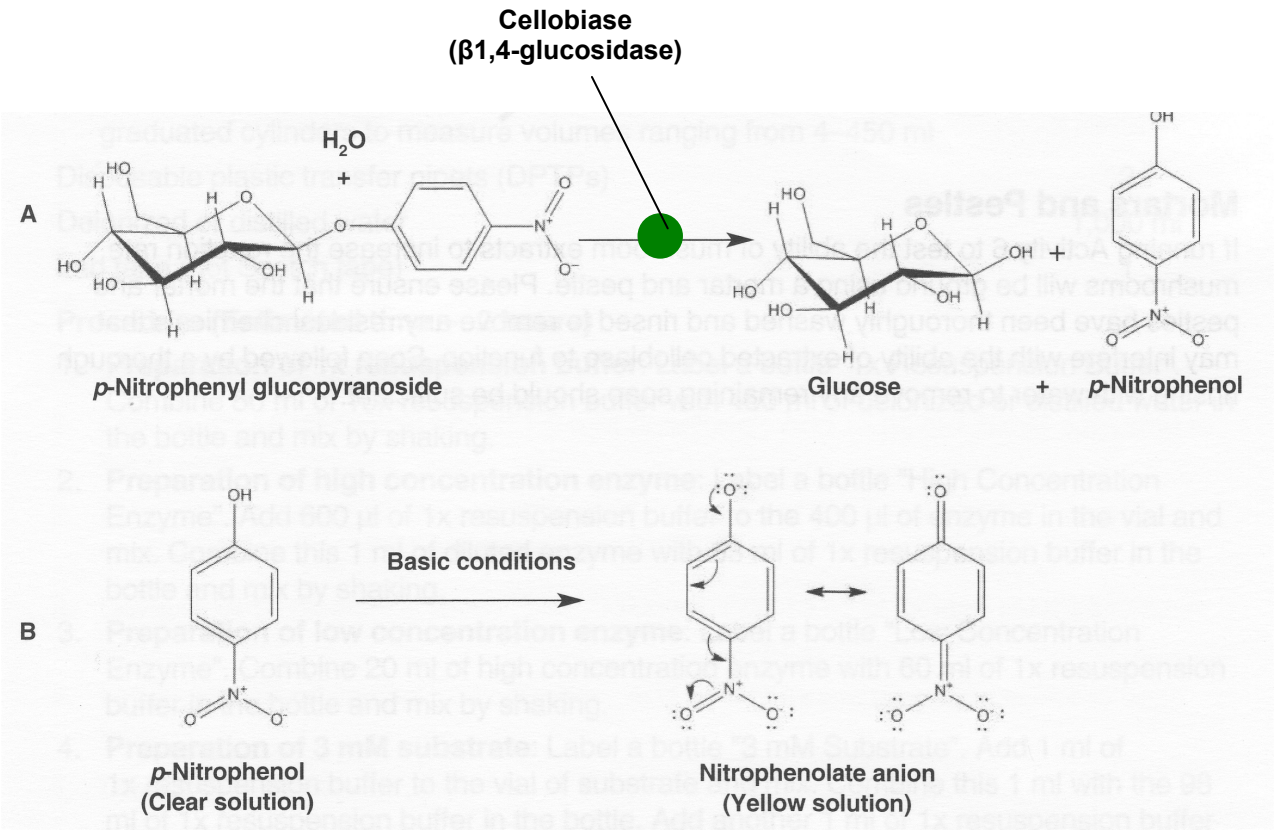
Cellobiases (or β–glucosidases) are enzymes which assure the final break down of cellulose into the fermentable glucose molecules. They catalyze the cleavage of the –glycosidc bond of cellobiose into two molecules of glucose (see **Figure** below). The breakdown of cellobiose by cellobiase enzymes is the final step in producing glucose, the preferred source of sugar of many fermenting microorganisms, from cellulose fibers.



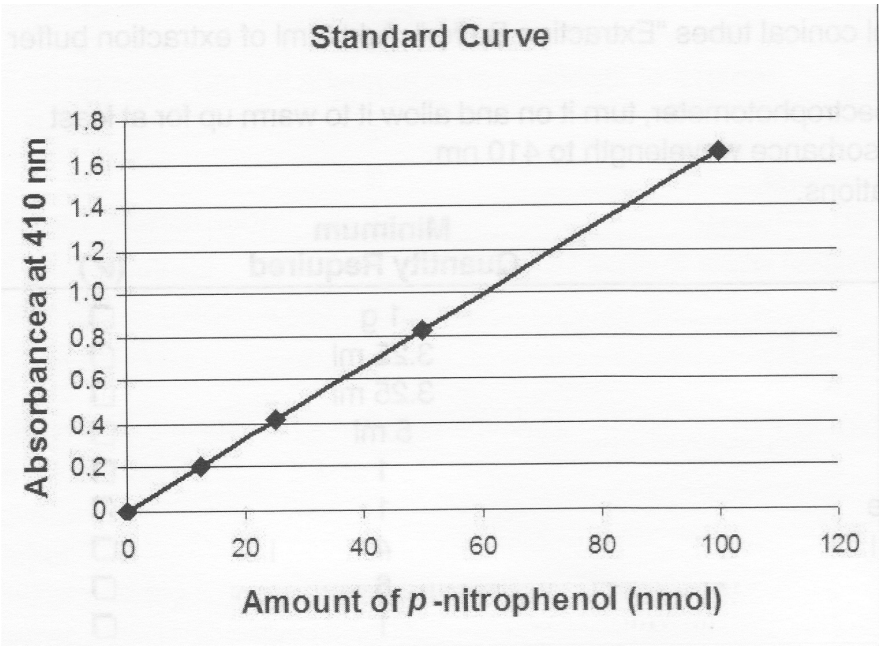
All three types of cellulose degrading enzymes, including cellobiases, are actively being studied and produced on an industrial scale for commercial use in the biofuels industry. Cellobiases are naturally produced by cellulolytic bacteria and fungi which are found in diverse biomass-rich environments, from soils to intestines of herbivorous, biomass-ingesting animals. As mentioned earlier, cellulolytic bacteria have been isolated from very diverse environment, including ruminants, termite guts, and some plants or plant products. Currently, cellulolytic enzymes are intensively investigated for commercial use in the break down of the cellulose in non-food plants, such as switch grass, miscanthus, of crop waste products, such as corn stover, bagasse, or of waste wood products, along with fast growing trees, such as poplar, for production of biofuels from the resulting sugar end product, most namely glucose.

Since cellulose is a very recalcitrant material, the production of biofuels, such as bioethanol, from cellulosics biomass, e.g. wood, corn stover or sugar cane bagasse, is a technological challenge. To retrieve glucose from the cellulose in biomass requires a very complex technological procedure which is comprised of several steps. The collected or harvested plant material first has to be mechanically processed, e.g. with the help of a grinder or shredder, to create smaller more accessible biomass particles. In a second step treatment with weak acids and heat or enzymes (hemicellulases) breaks down the less recalcitrant hemicellulose component of biomass into smaller sugars, most namely arabinose and xylose. This step also helps to remove the wood hardener lignin from the processed biomass. The resulting pulp-like, cellulose rich material is then treated with cellulases to yield the glucose monomers. In a final step, the yielded glucose molecules are metabolized into the biofuel, e.g. ethanol or hydrogen gas, via fermentation in bioreactors.

In this lab you will be working with the enzyme cellobiase and measure the catalytic activity of this enzyme under different experimental conditions. Remember: Cellobiases are the last step in the enzymatic degradation of the polysaccharide cellulose into the fermentable monosugar glucose. Although cellobiose is the natural substrate of cellobiases it can not be easily detected. Since there is also no simple method to quantitatively detect the product, i.e. glucose, of cellobiases, you will be using an artificial substrate molecule for the cellobiase enzyme, called p-nitrophenyl glucopyranoside. Cleavage of this molecule by the cellobiase enzyme yields glucose and a yellow colored co-product called nitrophenolate anion (see **Figure** below) which absorbs visible light with a wavelength of 410nm.



Generation of this yellow colored enzyme product, which reflects the enzyme activity, can easily monitored with the help of a spectrophotometer. To stop the catalytic activity of the cellobiase enzyme and to create the colored product, you will stop the set up enzyme reaction mixtures at different time points by transferring to an alkaline stop solution. When the p-nitrophenol is placed in a basic (alkaline) solution, the hydroxyl group on the nitrophenol loses a proton (H^+) to the hydroxyl anion (OH^-) of the base. This proton dissociation changes the bonding within the phenolic ring, resulting in a change of the absorptive behavior of the nitrophenolate anion molecule. The resulting nitrophenolate anion will absorb violet light (see **Figure** above), reflect yellow light and as a consequence turn the basic nitrophenolate solution yellow. The resulting yellow color can now be accurately detected and quantified (against a set of standards of known concentrations of p-nitrophenol) using a spectrophotometer. Measurement of the absorption of p-nitrophenol standards at a wavelength of 410nm and at different concentrations allows the establishment of a standard curve (see **Figure** below), which is then used to determine the amount of p-nitrophenol product produced by the cellobiase enzyme under different experimental conditions.



The objective of this lab is to find the optimal working conditions, i.e. amount of enzyme, substrate concentration, pH and temperature, of a commercial cellobiase enzyme preparation to assure efficient and cost effective break down of the enzyme substrate cellobiose. Instead of using cellobiose as enzyme substrate you will be using the artificial sugar substrate p-nitrophenyl glucopyranoside which is cleaved by the cellobiase enzyme into glucose and the yellow substance p-nitrophenol. You will indirectly measure the enzyme activity under different conditions by monitoring the production of the yellow p-nitrophenol product with the help of a spectrophotometer. In the final part of this lab you will compare the activity of cellobiase enzymes isolated from different natural sources of cellulolytic enzymes, i.e. fungi and bacteria, and compare these with the specific activity of the commercial cellobiase enzyme.

Concepts: enzymes, enzyme activity, enzyme specificity, enzyme substrate, enzyme product, Michaelis-Menton constant, cellulolytic enzymes (cellulases), cellobiases, cellulolytic microorganisms, including fungi and bacteria

Lab Activity 1: Determine the reaction rate of the cellobiase enzyme at two different enzyme concentrations

In this lab activity you will study enzymatic activity dependent on the enzyme concentration. You will compare the rate of break down of the substrate p-nitrophenyl glucopyranoside into glucose and p-nitrophenol in the absence and in the presence of different amounts of the enzyme cellobiase. Because enzymes have a catalytic turnover, i.e. they can keep catalyzing a given type of chemical reaction over and over again, therefore requiring only small amounts of enzymes to convert large amounts of substrate molecules into the corresponding product.

Because it is difficult to add really small volumes, your instructor has diluted the enzyme with a buffer solution. This will allow you to easily add the required volume that still contains a very small number of molecules of enzyme. To ensure that the buffer used to dilute the enzyme does not affect the rate of formation of the product, you will set up a control reaction containing just buffer and substrate without enzyme. In this exercise you will compare the cellobiase catalyzed breakdown of p-nitrophenyl glucopyranoside to glucose and p-nitrophenol in the presence of two different concentrations of enzyme and in the absence of enzyme in a parallel control reaction.

<u>Procedure</u>

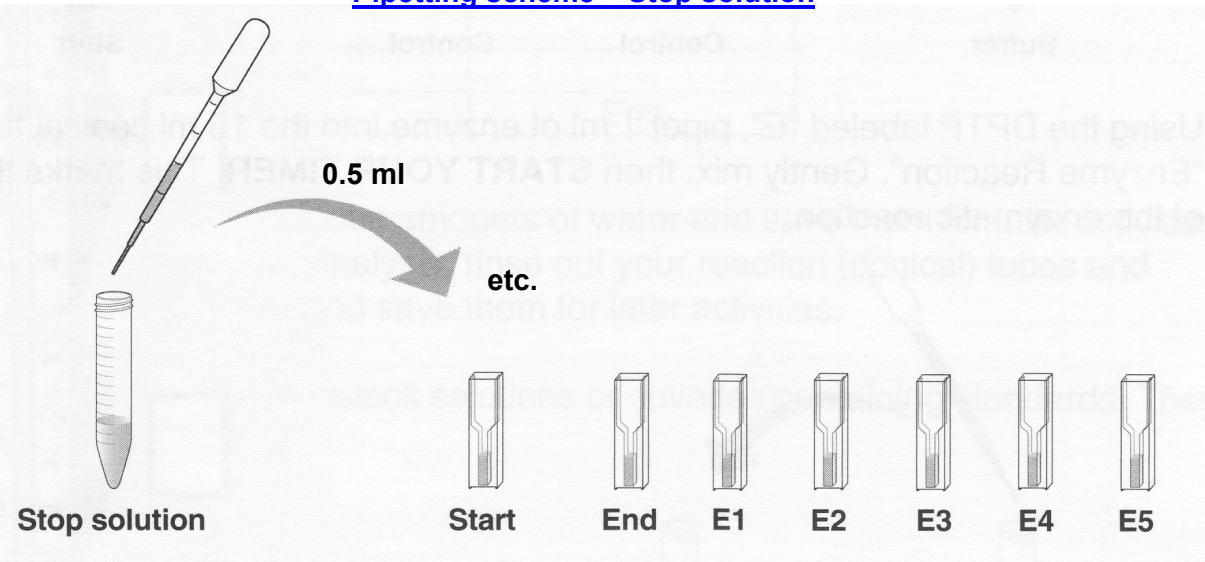
1. **Locate the 15 ml conical tubes labeled "Stop Solution", "1.5mM Substrate", "Enzyme Stock", and "Buffer" on the lab bench. Label each of the tubes with your initials.**

2. **Label five 1.0 ml plastic cuvettes E1-1, E1-2, E1-4, E1-6 and E1-8.**
   - label only the upper most part of the cuvette, so that your label does not interfere with the later absorbance reading

3. **Label the two plastic cuvettes "Start" and "End" on the upper part of the cuvette.**
   - this two cuvettes will serve as control time points (blanks) at the start and end of the reaction and neither cuvette will contain enzyme

4. **Use a clean Pasteur transfer pipette and pipet 0.5ml of stop solution into each of the seven labeled plastic cuvettes (see Pipetting Scheme below).**
   - the stop solution is a strong base, so avoid getting this solution onto your skin or your clothes!
   - after the pipetting work, rinse the transfer pipette well with distilled water and save it for future transfer activities

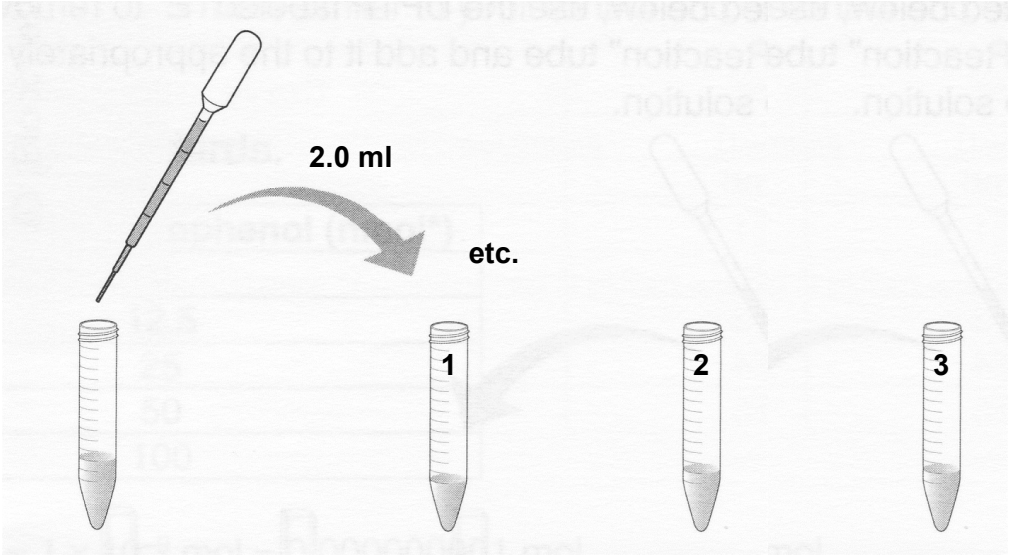
Pipetting scheme – Stop solution



5. **Locate t**
   Low", "Enzyme 2-High", and "Control".

6. **Use a clean Pasteur transfer pipette and pipet 2ml from the "1.5mM Substrate" tube into each of the three previously labeled 15ml conical tubes (see Transfer Scheme below).**
   - after the pipetting work, rinse the transfer pipette well with distilled water and save it for future transfer activities

Transfer scheme – 1.5 mM Substrate solution



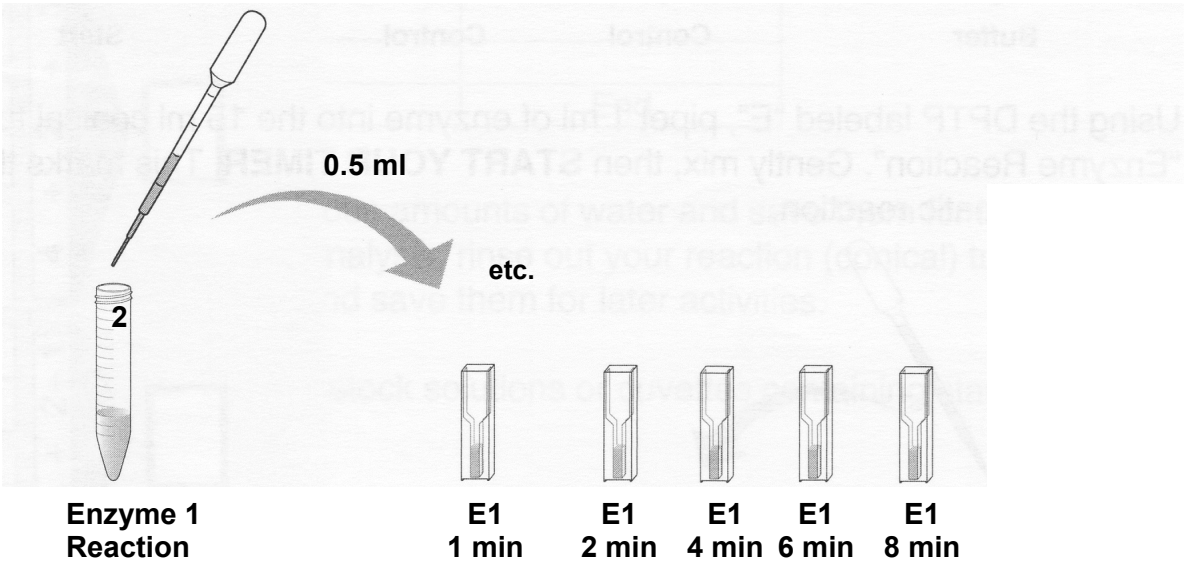
7. **Label one Pasteur transfer pipette "E" for enzyme, and the other one "C" for control.**
   - Only use the "C" transfer pipette for the control reaction tube, and the "E" transfer pipette for transfers into the enzyme reaction tubes

8. **Use the transfer pipette labeled "C" and pipette 1.0 ml of "Buffer" into the 15ml conical tube labeled "Control" and gently mix. Once you have mixed the buffer with the substrate, remove 0.5ml of this solution and transfer it into the cuvette labeled "Start".**

9. **Now, use the transfer pipette labeled "C" and pipette 0.8ml of "Buffer" into the 15ml conical tube labeled "Enzyme 1-Low" (2). Following, use the transfer pipette labeled "E" and pipette 0.2ml of "Enzyme" solution into the 15ml conical tube labeled "Enzyme 1-Low" (2). Gently mix and then START YOUR TIMER. This step marks the beginning of the enzymatic reaction with the low enzyme concentration.**

10. **At the times indicated in the Figure and Results Table below, use the transfer pipette "E" and remove 0.5ml of the solution from the conical tube "Enzyme 1-Low" (2) and transfer it to the corresponding (labeled) cuvette containing the stop solution.**

Pipetting scheme – Enzyme Reaction 1



11. After all the enzyme samples have been transferred at the appropriate times into the corresponding cuvettes (after 8 min), use the transfer pipette 'C" and remove 0.5ml of the solution from the tube labeled "Control" (1). Transfer this volume into the cuvette labeled "End".

- keep the cuvettes with your collected samples on your bench for the later spectrophotometric analysis

12. **Label five new 1.0 ml plastic cuvettes E2-1, E2-2, E2-4, E2-6 and E2-8.**

- label only the upper most part of the cuvette, so that your label does not interfere with the later absorbance reading

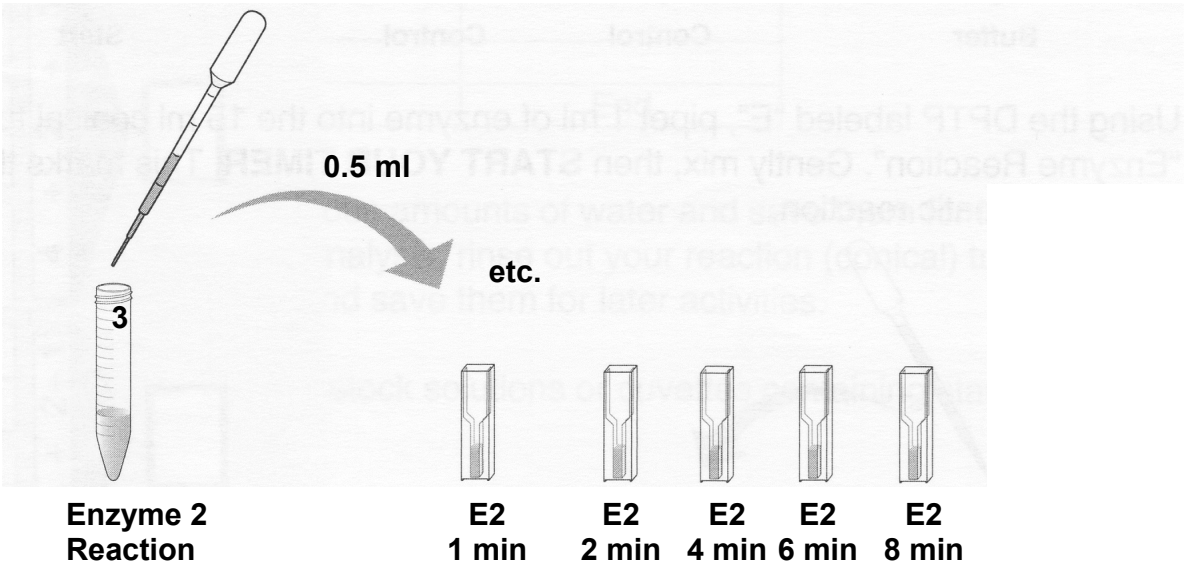
13. **Use a clean Pasteur transfer pipette and pipet 0.5ml of stop solution into each of the five labeled plastic cuvettes.**

- the stop solution is a strong base, so avoid getting this solution onto your skin or your clothes!
- after the pipetting work, rinse the transfer pipette well with distilled water and save it for future transfer activities

14. Now, use the transfer pipette labeled "E" and pipette 1.0ml of "Enzyme" solution into the 15ml conical tube labeled "Enzyme 2-High" (3). Gently mix and then START YOUR TIMER. This step marks the beginning of the enzymatic reaction with the high enzyme concentration.

15. At the times indicated in the Figure and Table below, use the transfer pipette "E" and remove 0.5ml of the solution from the conical tube "Enzyme 2-High" (3) and transfer it to the corresponding (labeled) cuvette containing the stop solution.

Pipetting scheme – Enzyme Reaction 2



16. **After your done with all your sample transfers, rinse out all used transfer pipettes with plenty amounts of distilled water and save them on your bench for later activities.**
    Note: Do NOT discard the unused stock solutions or cuvettes containing the DNP standards, since they will be used for the next activity.

Measurements & Data Collection

In this part you will measure the absorbance of the collected samples from your enzymatic reactions 1 and 2 using a spectrophotometer set at a wavelength of 410nm. The absorbance readings for the individual cuvettes will be written in the Results Table below and the amount of product formed will be quantitatively determined with the help of a standard curve.
NOTE: MAKE SURE THAT THE SPECTROPHOTOMETER IS TURNED ON ABOUT 1—15 MINUTES BEFORE THE START OF THE FOLLOWING MEASUREMENTS!

17. **Locate the spectrophotometer and the five cuvettes of standards labeled S1-S5 on your lab bench. Their concentrations are noted in the Table below.**

Table: Absorbance values for the p-nitrophenol standards

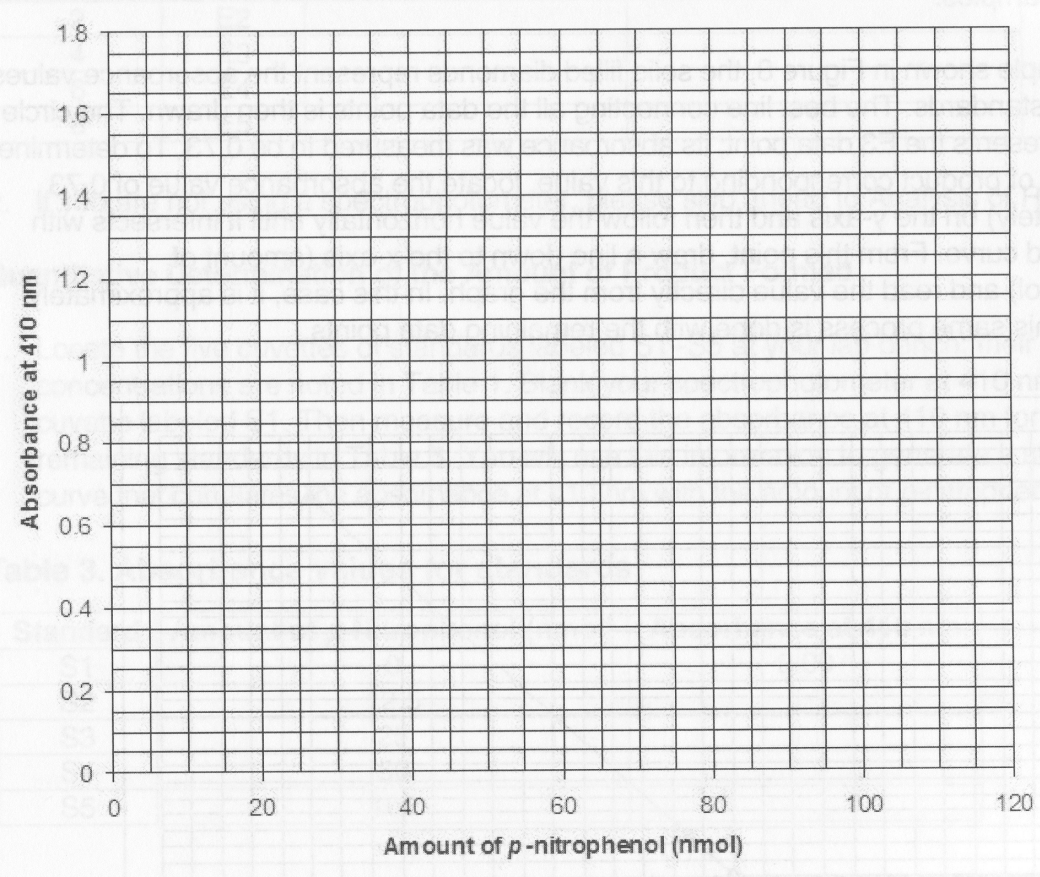
Standard	Amount of p-Nitrophenol (nmol)	Absorbance at 410nm
S1	0	
S2	12.5	
S3	25	
S4	50	
S5	100	

18. **Blank your spectrophotometer spectrophotometer at 410nm with the cuvette labeled S1. Then measure and record the absorbance at 410nm for the remaining standards (S2-S5) in**

the Table above.

19. **Use the data in the Table above and establish a standard curve by plotting the individual amounts of p-nitrophenol (x-axis) against the corresponding absorbance readings at 410nm (y-axis) in the Graph 1 below.**
    - draw a "best line" to connect all the plotted data points in the Figure below
    - this standard curve is then used later to determine the amount of p-nitrophenol product present in your enzyme assay samples

Graph 1: Standard Curve for p-Nitrophenol



20. **Measure the absorbance of your enzyme-catalyzed reaction cuvettes (E1 and E2 time points) and of your two control cuvettes ("Start", "End") at 410nm, and record your absorbance readings in the Results Table below. You will use these data together with your earlier established standard curve to determine the amount of p-nitrophenol product formed at the different time points in your two (high and low) enzyme reactions E1 and E2.**

Results Table: Activity 1
Absorbance readings of the enzyme reactions and amount of p-nitrophenol determined from the standard curve

Time (minutes)	Cuvette	Absorbance at 410nm	Amount of p-nitrophenol*

			(nmol)
0	Start		
8	End		
1	E1-1		
2	E1-2		
4	E1-4		
6	E1-6		
8	E1-8		
1	E2-1		
2	E2-2		
4	E2-4		
6	E2-6		
8	E2-8		

* these data are deduced with the help of your earlier established standard curve

21. **Use your earlier plotted standard curve and determine the nanomoles (nmol) of product existing in each of the cuvettes from the corresponding absorbance readings.**
    - take the absorbance number for a given sample in a cuvette and draw a horizontal line from this data point towards the curve/line of your standard curve
    - exactly where the horizontal line crosses with the curve, draw a second, this time vertical line down towards the x-axis
    - where this line crosses the x-axis, read the corresponding number which is your amount of p-nitrophenol in nanomoles (nmol) in that given sample

Analysis of Results

In this part of the lab you will plot the data from the absorbance measurements of your enzyme reactions. The amount of p-nitrophenol product produced in the enzyme reactions E1 & E2 is plotted over time. From the two graphs you will then determine the initial rate of product formed by the cellobiase enzyme in the two enzyme reactions and give the enzyme activity in nmol/min.

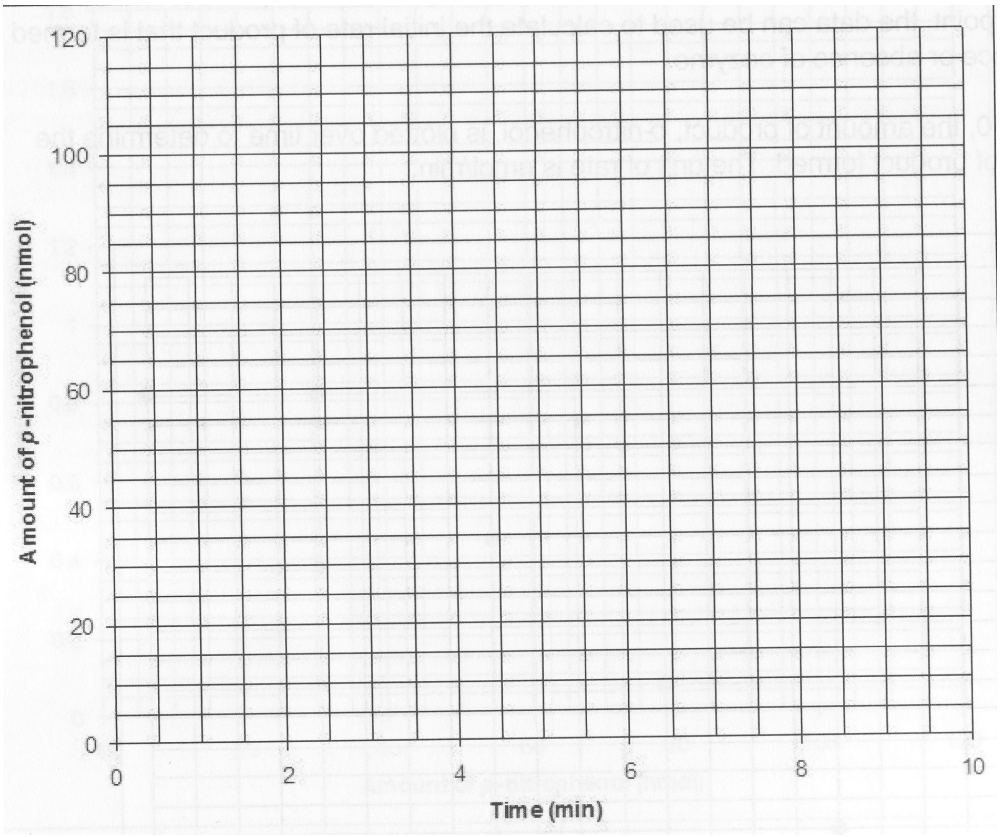
22. **Plot the amount (in nmol) of p-nitrophenol produced (y-axis) against time (x-axis) for the two examined enzyme reactions E1 and E2 in the Graph 2 below.**

23. **Determine the initial rate of reaction for both established graphs (in nmol/min)**
    - find the region in both curves where the amount of product formed over time increases in a linear fashion; that is the time frame of the reaction where there is plenty of substrate is available for the cellobiase enzyme to encounter and to create the enzyme product p-nitrophenol

    - use the following formula to calculate your initial rate of reaction for both enzyme reactions

Initial rate of product formation = slope of the line = change in y / change in x

Graph 2: Reaction rate curves for cellobiase enzyme at low (E1) and high (E2) enzyme amounts in the reaction



Lab Activity 2: Determine the effect of temperature on the reaction rate of the cellobiase enzyme

In this lab activity you will study whether and how the enzymatic activity of the cellobiase enzyme changes at different temperatures. Knowledge of the optimum temperature at which a given enzyme works (catalyzes) best is important to operate commercial enzyme-operated bioreactors efficiently and economically.

Temperature strongly affects chemical reactions. Increased temperatures (i.e. heat) usually speed up chemical reactions, while low temperatures slow chemical processes down. Heat speeds up the kinetic movement of molecules which then increases the probability of successful molecular collisions. For enzyme catalyzed chemical reactions one would therefore expect an increased rate of the enzymatic reaction at higher temperatures due to the increased number of collisions between the enzyme and its substrate molecule. However, at some critical temperature, the forces that allow the enzyme to maintain its crucial functional 3-dimensional shape will eventually be broken and the enzyme becomes denatured. The temperature at which a given enzyme changes its shape, i.e. becomes denatured, depends on the particular properties of the enzyme, such as amino acid sequence and composition. Some enzyme are stable at temperatures close to boiling (100^{o}C), while other are already denatured at room temperature. Most enzymes found in life forms function best, i.e. have the highest enzyme activity, at moderate temperatures between 20 – 40^{o}C. Usually, the environment in which the enzyme functions in nature, e.g. within the cells of a given life form, is a good predictor of the conditions at which it will work best in a laboratory setting. For example, enzyme used by bacteria living in hot springs, thermal pools or compost piles, can still function at high temperatures, while enzymes found in bacteria living in arctic ice function normally at low temperatures.

<u>Procedure</u>

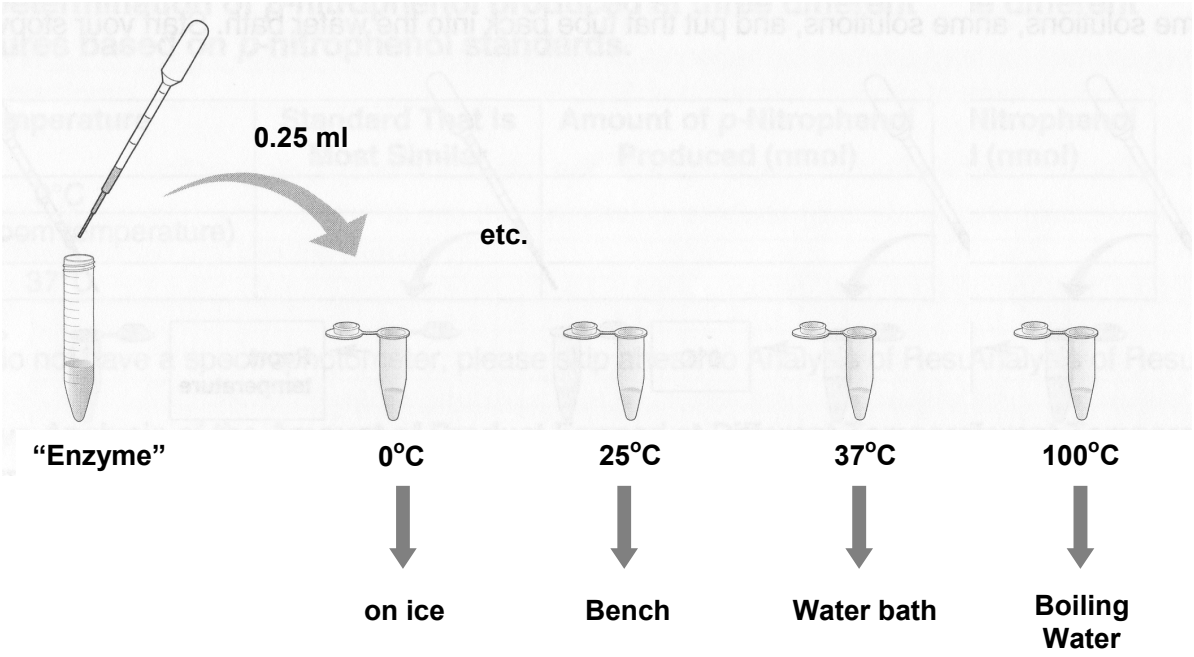
1. **Label four 0.5ml plastic cuvettes "0°C", "25°C", "37°C", and "100°C".**
   - label only the upper part of your cuvettes!

2. **Use a clean Pasteur transfer pipette and pipette 0.5ml of "Stop" solution into each cuvette.**
   - rinse the transfer pipette thoroughly with distilled water after use and save

3. **Label four 1.5ml microcentrifuge tubes with "0°C Enzyme", "25°C Enzyme", "37°C Enzyme", and "100°C Enzyme".**

4. **Use a clean Pasteur transfer pipette and pipette 0.25ml of "Enzyme" solution into each microcentrifuge tube as shown in the Figure below.**
   - rinse the transfer pipette thoroughly with distilled water after use and save

Figure: Pipetting scheme – Enzyme



5. **Label four 1.5ml microcentrifuge tubes with "0°C Substrate", "25°C Substrate", "37°C Substrate", and "100°C Substrate".**

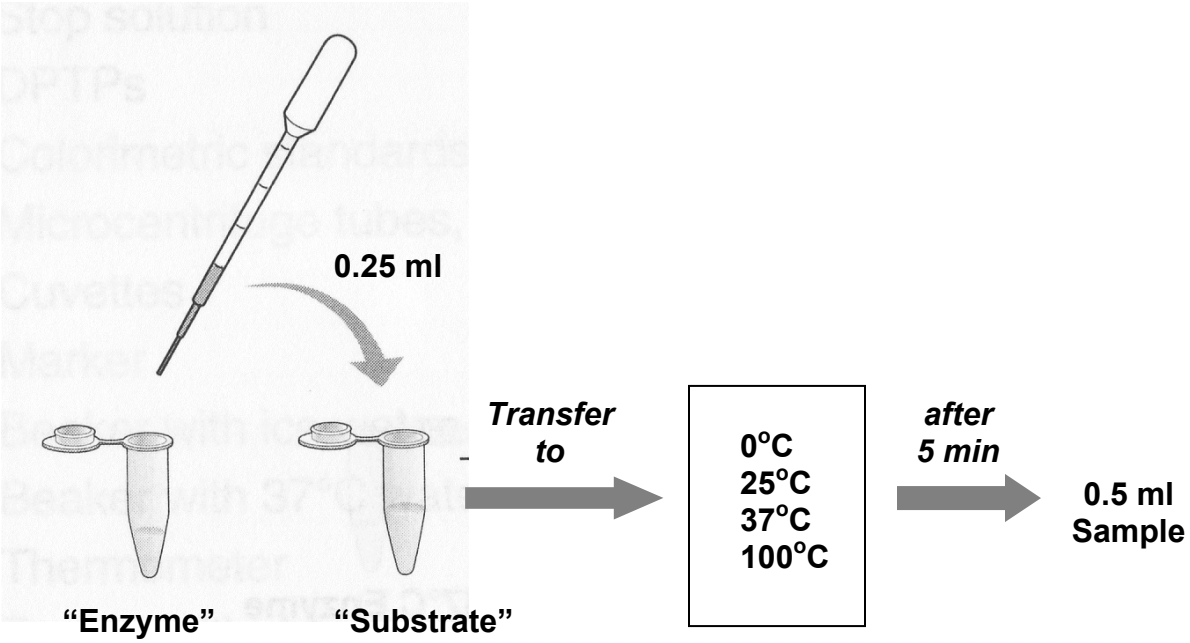
6. **Use a clean Pasteur transfer pipette and pipette 0.5ml of the 1.5mM "Substrate" solution into each microcentrifuge tube.**
   - rinse the transfer pipette thoroughly with distilled water after use and save

7. **Place the microcentrifuge tubes labeled "0°C Enzyme" and "0°C Substrate" on ice in an ice bucket. Place the microcentrifuge tubes labeled "25°C Enzyme" and "25°C Substrate" in a rack on your bench and keep at room temperature. Place the microcentrifuge tubes labeled "37°C Enzyme" and "37°C Substrate" in a floaty and put floaty plus tubes in a water bath adjusted to 37°C. Place the microcentrifuge tubes labeled "100°C Enzyme" and "100°C Substrate" in a floaty and put floaty plus tubes in a beaker with boiling water. Allow the**

tubes to equilibrate at their corresponding temperature for at least 10 minutes.

8. Have a stop watch ready. Now use a clean Pasteur transfer pipette and pipette 0.25ml of enzyme solution from the tube labeled "0°C Enzyme" into the tube labeled "0°C Substrate", and then place the tube now containing your enzyme-substrate mix back on ice. START THE TIMER for this reaction!

Pipetting scheme – Enzyme-Substrate Mix



9. Use the same Pasteur pipette from step 8, and pipette 0.25ml of enzyme solution from the tube labeled "25°C Enzyme" into the tube labeled "25°C Substrate", and then place the tube now containing your enzyme-substrate mix back into the rack on your bench. START THE TIMER for this reaction!

10. Use the same Pasteur pipette from step 8, and pipette 0.25ml of enzyme solution from the tube labeled "37°C Enzyme" into the tube labeled "37°C Substrate", and then place the tube now containing your enzyme-substrate mix back into the 37°C water bath. START THE TIMER for this reaction!

11. Use prongs and thermogloves and take the floaty with the two "100°C tubes out of the boiling water. Place in rack on your bench. Now, use the same Pasteur pipette from step 8, and pipette 0.25ml of enzyme solution from the tube labeled "100°C Enzyme" into the tube labeled "100°C Substrate", and then place the tube now containing your enzyme-substrate

mix back into the beaker with your boiling. START THE TIMER for this reaction!

12. **After 5 minutes for each of the started enzymatic reactions, use a clean Pasteur transfer pipette and pipette 0.5ml of your enzymatic reaction (starting with your "0°C" enzyme-substrate mix) to the correspondingly labeled cuvettes containing the stop solution. Allow all solutions in the cuvettes to reach room temperature before proceeding with the next step.**
    - use a new and clean Pasteur transfer pipette for each of the four different enzyme reactions!

13. **Locate the spectrophotometer. Then blank your spectrophotometer at 410nm with the cuvette labeled S1.**

14. **Measure the absorbance values for your four cuvettes from step 12. and the record the values in the Results Table below.**

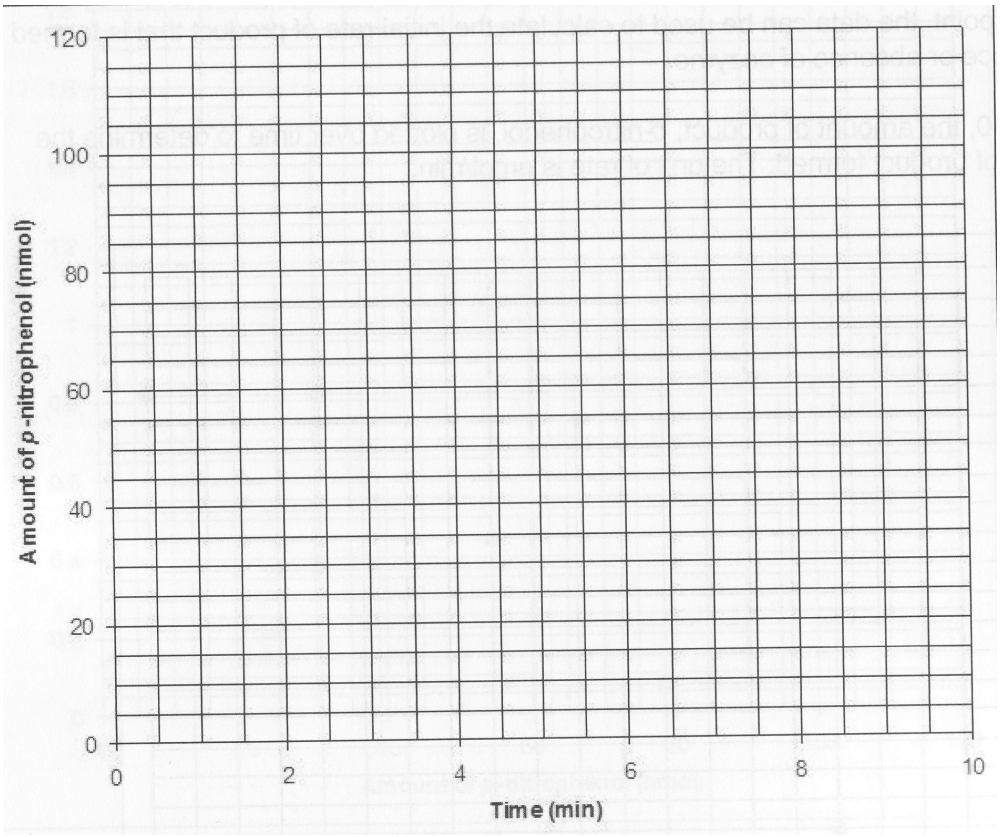
Results Table: Activity 2

Temperature (°C)	**Absorbance at 410nm**	**Amount of p-Nitrophenol** (nmol)
Blank	0	0
0		
25 (room temperature)		
37		
100		

15. **Follow the same protocol used in Activity 1 and convert the absorbance readings into the corresponding amount of p-Nitrophenol product (in nmol) with the help of your previously established standard curve. Write these deduced values in the Result Table above.**

16. **Plot the amount (in nmol) of p-nitrophenol produced (y-axis) after 5 minutes against time (x-axis) for the four examined enzyme reactions in the Graph 3 below.**

Graph 3: Reaction rate curves for cellobiase enzyme at different temperatures



Analysis of Results

In this part of the lab you will plot the data from the absorbance measurements of your four enzyme reactions at the different temperatures. The amount of p-nitrophenol product produced in the enzyme reactions "0°C", "25°C", "37°C", and "100°C", is plotted over time (5min). From the four graphs you will then determine the initial rate of product formed by the cellobiase enzyme in the four different enzyme reactions and give the enzyme activity in nmol/min.

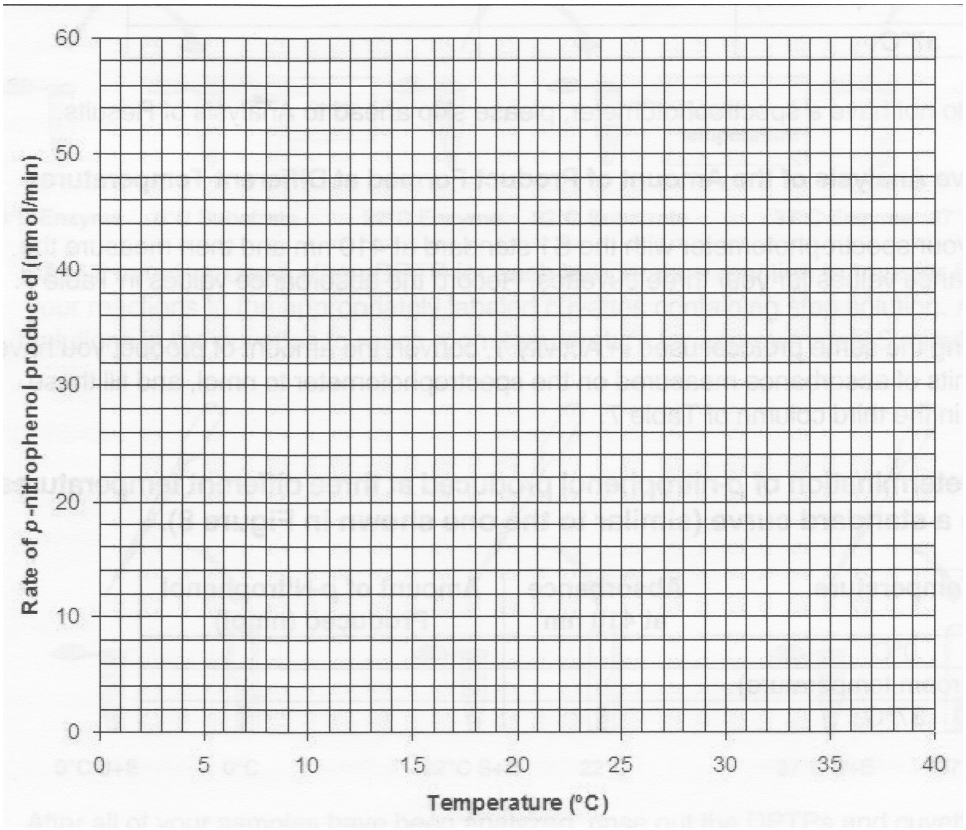
17. **Use the previously established graph and calculate the initial rate for each of the enzymatic reactions at the four different temperatures. Since you only measured the amount of p-Nitrophenol at one time point (= 5min), assume that the amount of p-Nitrophenol at o minutes was 0 nmol.**
    - for example:
      Initial Enzymatic Rate = (35 nmol – 0 nmol) / (5 min – 0 min) = 7.0 nmol/min

Temperature (°C)	**Initial rate of product formation** (nmol/min)
4.0	
25	
37	
100	

18. **Plot the effect of temperature on the activity of the cellobiase enzyme in form of a dot plot in the Graph 4 below.**

- graph enzymatic rate in nmol p-nitrophenol produced per min (y-axis) against the temperature in ^{o}C (x-axis)

<u>Graph 4:</u> Effect of temperature on the enzymatic activity of the cellobiase enzyme



19. Answer the "Analysis Questions" related to this activity at the end of this document.

Lab Activity 3: Determine the effect of pH on the reaction rate of the cellobiase enzyme

In this lab activity you will study whether and how the enzymatic activity of the cellobiase enzyme changes at different pH values. One of the ways with which enzymes interact with and bind their substrates into the active site is by charge groups on one molecule, i.e. the enzyme, attracted to the oppositely charged groups on the other molecule, i.e. the substrate. Consequently, if the pH of the environment in which the enzyme-substrate interaction takes place changes, it is possible that the positively and negatively charged groups on the enzyme and/or the substrate change or even loose their charge due to protonization or deprotonization events. Not only is it possible for the pH to affect the "landscape of charges" on an enzyme, but it can also affect the substrate. The net result of these pH-induced changes that the enzyme and its substrate will no longer interact with each other in an optimized fashion. Similar to enzymes optimized by life forms to work best at either high or low temperatures, different enzymes are optimized to work at different pH values. For example, enzymes that are present

in the acid environment of an animal's stomach are optimized to work at low pH values around pH 3, while pancreatic enzymes that are secrted into the lumen of the small intestine have their highest enzyme activity at neutral or basic pH values (pH 7.2-9.0). Certain bacteria which thrive in alkaline lakes produce enzymes which have the pH optimum of their enzymatic activity at very alkaline pH values.

Procedure

1) **Label three 0.5ml plastic cuvettes "pH 5.0", "pH 6.3", and "pH 8.6".**
   - label only the upper part of your cuvettes!

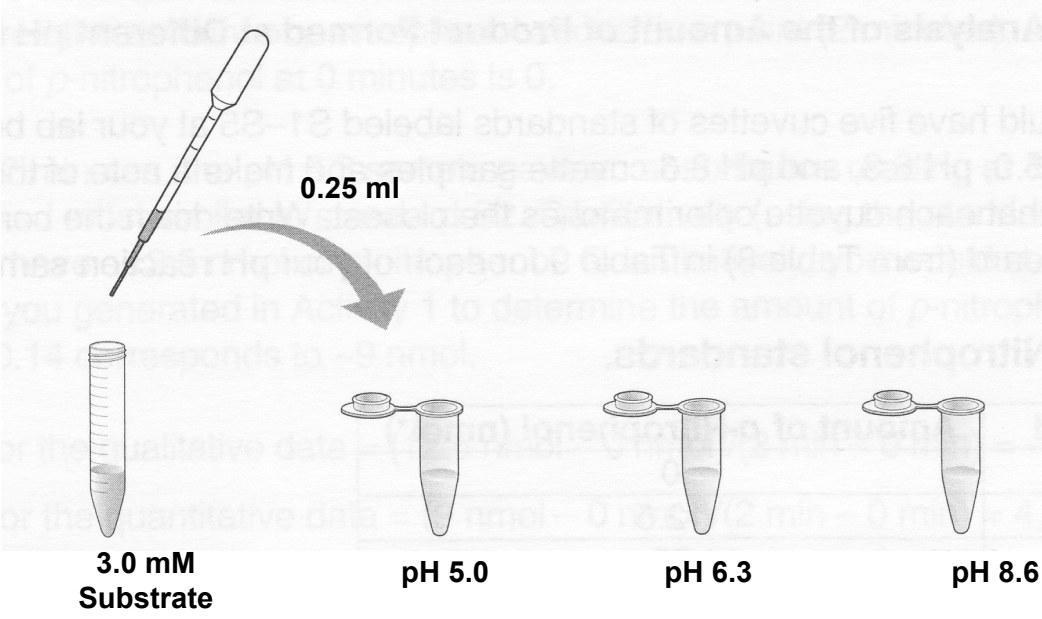
2) **Use a clean Pasteur transfer pipette and pipette 0.5ml of "Stop" solution into each of the three cuvettes.**
   - rinse the transfer pipette thoroughly with distilled water after use and save

3) **Label three 1.5ml microcentrifuge tubes with "pH 5.0", "pH 6.3", and "pH 8.6".Use a clean Pasteur transfer pipette and pipette 0.25ml of a 3.0mM "Substrate" solution into each microcentrifuge tube as shown in the Figure below.**
   - rinse the transfer pipette thoroughly with distilled water after use and save

4) **Use a clean Pasteur transfer pipette and pipette 0.25ml of "Adjustment Buffer pH 5.0" to the microcentrifuge tube "pH 5.0" and mix. Briefly rinse Pasteur pipette in distilled water and use for the next step.**

5) **Use a clean Pasteur transfer pipette and pipette 0.25ml of "Adjustment Buffer pH 6.3" to the microcentrifuge tube "pH 6.3" and mix. Briefly rinse Pasteur pipette in distilled water and use for the next step.**

6) **Use a clean Pasteur transfer pipette and pipette 0.25ml of "Adjustment Buffer pH 8.6" to the microcentrifuge tube "pH 8.6". Briefly rinse Pasteur pipette in distilled water and save.**



7) **Have a stop watch ready. Now use a clean Pasteur transfer pipette and swiftly pipette 0.25ml of "Enzyme" solution to each of the microcentrifuge tubes labeled "pH 5.0", "pH 6.3", and "pH 8.6". IMMEDIATELY start your stop watch!**

8) **After 5 minutes, use a clean Pasteur transfer pipette for each pH reaction and transfer 0.5 ml of your enzymatic reaction in the three reaction tubes to the corresponding cuvettes labeled "pH 5.0", "pH 6.3", and "pH 8.6" containing the stop solution.**

9) **Locate the spectrophotometer. Then blank your spectrophotometer at 410nm with the cuvette labeled S1.**

10) **Measure the absorbance values for your three cuvettes from step 5. and record the values in the Results Table below.**

Results Table: Activity 3

pH	**Absorbance at 410nm**	**Amount of p-Nitrophenol** (nmol)
Blank	0	0
pH 5.0		
pH 6.3		
pH 8.6		

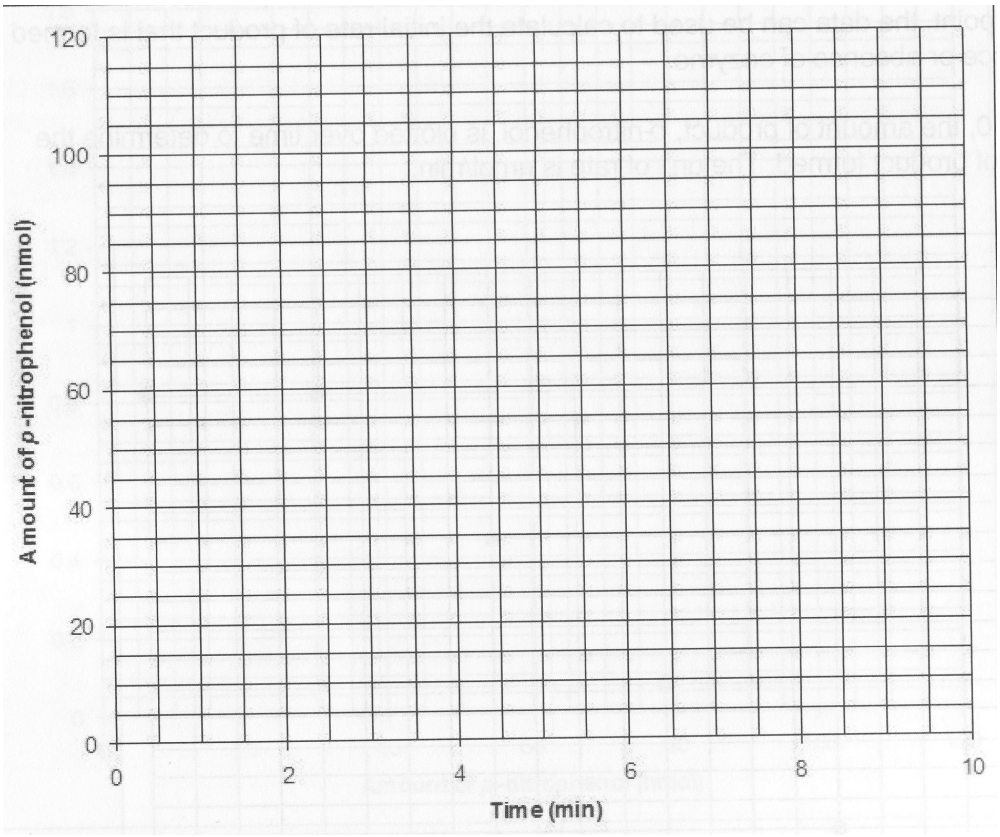
11) **Follow the same protocol used in Activity 1 and convert the absorbance readings into the corresponding amount of p-Nitrophenol product (in nmol) with the help of your previously established standard curve. Write these deduced values in the Result Table above.**

Analysis of Results

In this part of the lab you will plot the data from the absorbance measurements of your three enzyme reactions at the different pH values. The amount of p-nitrophenol product produced in the enzyme reactions "pH 5.0", "pH 6.3", and "pH 8.6", is plotted over time (5min). From the three graphs you will then determine the initial rate of product formed by the cellobiase enzyme in the three different enzyme reactions and give the enzyme activity in nmol/min.

12) **Plot the amount (in nmol) of p-nitrophenol produced (y-axis) after 5 minutes against time (x-axis) for the three examined enzyme reactions in the Graph 5 below.**

Graph 5: Reaction rate curves for cellobiase enzyme at different pH values



13) Use the previously established graph and calculate the initial rate for each of the enzymatic reactions at the four different temperatures. Since you only measured the amount of p-Nitrophenol at one time point (= 5min), assume that the amount of p-Nitrophenol at o minutes was 0 nmol.

- for example:

Initial Enzymatic Rate = (23 nmol – 0 nmol) / (5 min – 0 min) = 4.6 nmol/min

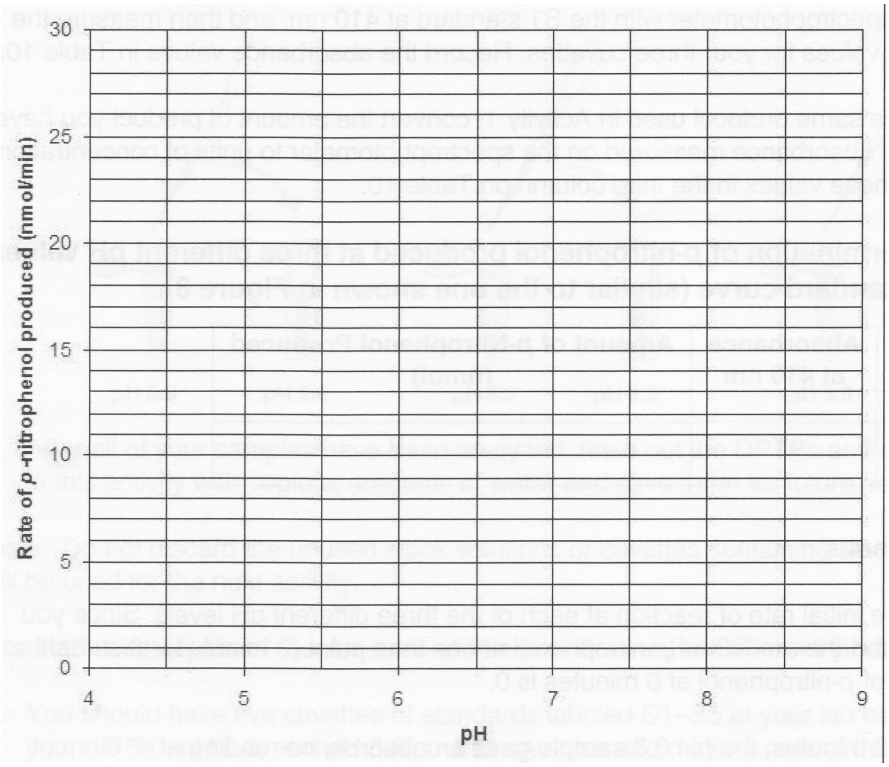
pH	**Initial rate of product formation** (nmol/min)
5.0	
6.3	
8.6	

14) Plot the effect of pH on the activity of the cellobiase enzyme in form of a dot plot in the <u>Graph 6</u> below.

- graph enzymatic rate in nmol p-nitrophenol produced per min (y-axis) against the pH (x-axis)

15) Answer the "Analysis Questions" in the section below

<u>Graph 6:</u> Effect of pH on the enzymatic activity of the cellobiase enzyme



Lab Activity 4: Determine the effect of substrate concentration on the reaction rate of the cellobiase enzyme

In this lab activity you will study whether and how the enzymatic activity of the cellobiase enzyme changes dependent on the concentration of the substrate molecule. You will compare the rate of break down of the substrate p-nitrophenyl glucopyranoside (p-NPG) into glucose and p-nitrophenol in the presence of different amounts of the substrate p-NPG. In other words, you will determine the effect that changing the substrate concentration will have on the initial rate of the enzymatic reaction.

Because enzymes have an active site into which the substrate binds upon successful interaction with an enzyme, one would expect that an enzyme will have less successful "collisions" with its substrate molecule when there are less molecules available, i.e. at a lower substrate concentrations. Since less substrate molecules will be converted into products under these conditions, one would hypothesize a lower initial rate for the enzyme. At much higher substrate concentrations one would expect that – considering a constant amount of enzyme present – that at some point the active sites of all enzymes eventually will be filled with a substrate molecule. Considering a constant speed with which a given enzyme converts its substrate molecule into the corresponding product (or turnover rate), one would hypothesize that at a certain maximum substrate concentration a enzyme will its maximum initial rate. For a more complex, mathematical analysis of the effects of varying substrate concentrations on the initial rate of enzymatic reaction please refer to the "Michaelis-Menton Kinetics" section on the instructor's website.

In this exercise you will compare the cellobiase catalyzed breakdown of p-nitrophenyl glucopyranoside to glucose and p-nitrophenol in the presence of two different (high and low) substrate concentrations.

Procedure

1) **Locate the 15 ml conical tubes labeled "Stop Solution", "1.5mM Substrate", "Enzyme Stock", and "Buffer" on the lab bench.**

- these stock solutions have been previously prepared by the lab technicians

2) **Label one clean 15 ml conical tube with "S-Low" and one clean 15 ml tube with "S-High". Also label each of the tubes with your initials.**

3) **Use a clean Pasteur transfer pipette and pipette 1.5 ml of the "1.5mM Substrate" solution into the 15 ml conical tube labeled "S-High".**
   - rinse the transfer pipette thoroughly with distilled water after use and save

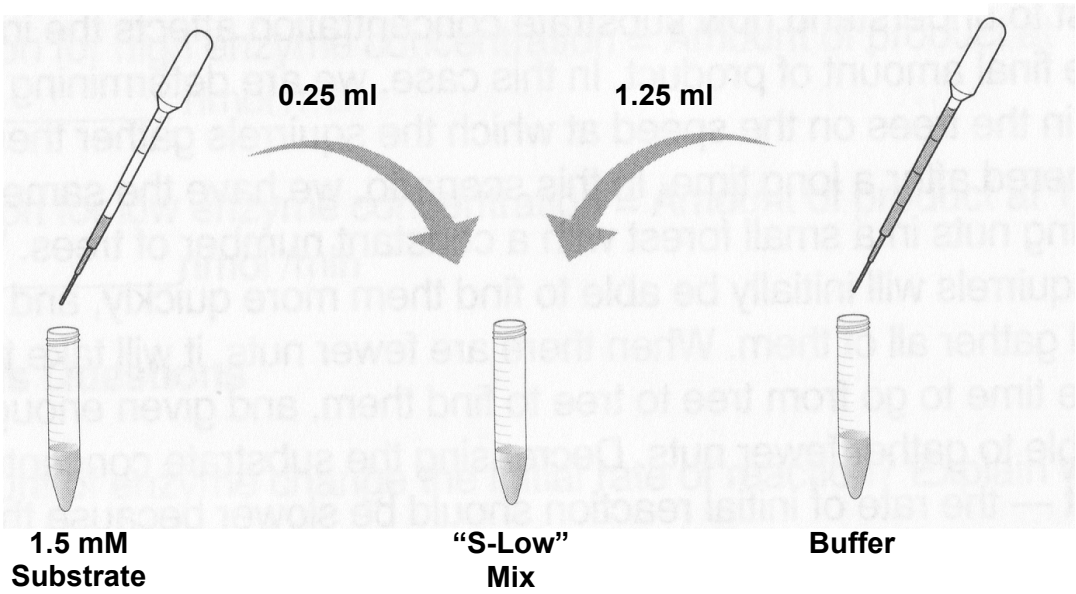
4) **Now, use a clean Pasteur transfer pipette and pipette 1.25 ml of "Buffer" solution into the 15 ml conical tube labeled "S-Low". Rinse the transfer pipette thoroughly with distilled water and then pipette 0.25 ml of the "1.5mM Substrate" solution into the 15ml conical tube labeled "S-Low" and mix by agitation (see Figure below).**
   Questions:
   a. You just prepared a ____ dilution of the 1.5 mM substrate stock
   b. The substrate concentration in your "S-Low" tube is _____ mM.

5) **Label your six cuvettes "H1, H2, and H3" (for high concentration substrate time points) and "L1, L2, and L3" (for low concentration substrate time points).**
   - only label the upper part of each of the cuvettes

6) **Use a clean Pasteur-type transfer pipette and pipette 0.5 ml of "Stop Solution" into each of the six cuvettes.**
   - rinse the transfer pipette thoroughly with distilled water after use and save

7) **Label one clean Pasteur transfer pipette as "H" for high substrate concentration, and a second clean Pasteur transfer pipette as "L" for low substrate concentration.**

Pipetting scheme



8) **Use a clean Pasteur transfer pipette and pipette 0.75 ml of "Enzyme" solution into your 15 ml conical tube labeled "S-High" and mix by gentle agitation of the tube.**

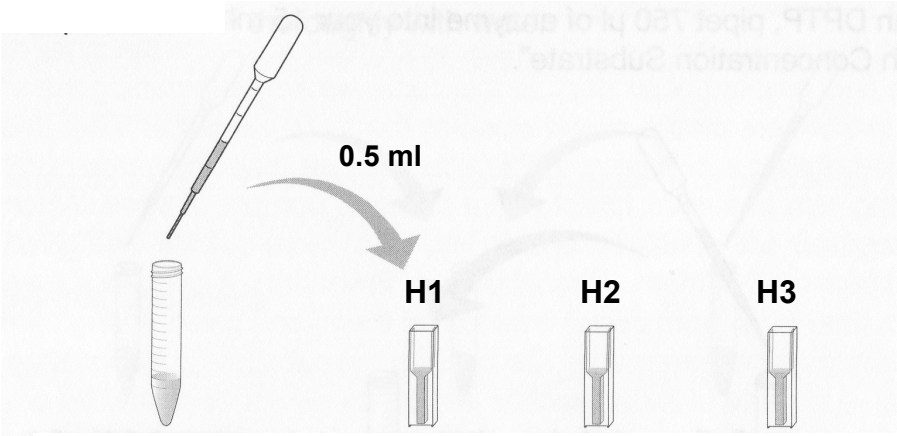
9) **IMMEDIATELY after step 8), use a clean Pasteur transfer pipette and pipette 0.75 ml of "Enzyme" solution into your 15 ml conical tube labeled "S-Low" and mix by gentle agitation of the tube. START YOUR TIMER!**

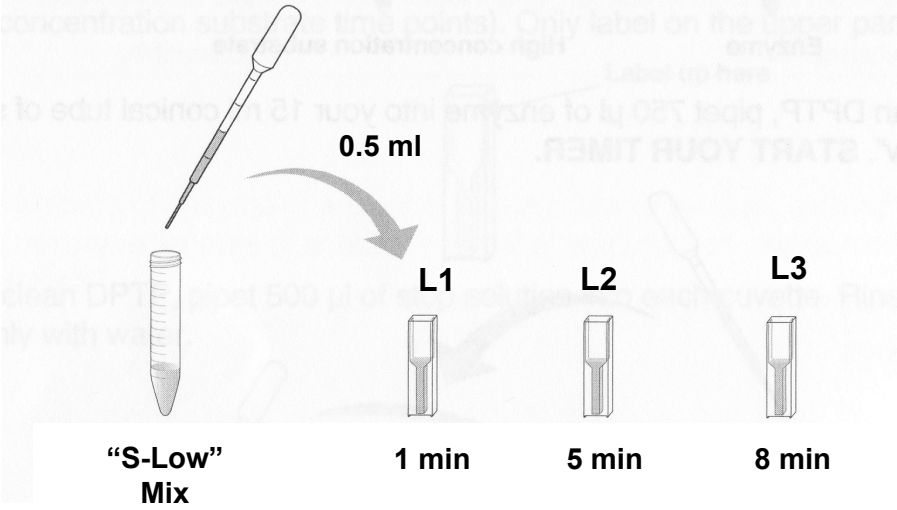
10) **At the times indicated in the Table below (1min, 5min, 8min), use the correctly labeled Pasteur transfer pipette ("H" or "L" → see step 7) and remove 0.5 ml from the conical reaction tube "S-High" and 0.5 ml from the conical reaction tube "S-Low" and transfer this sample into the correspondingly labeled cuvette containing the stop solution (see Figure below).**

Time (min)	Cuvette (High Substrate)	Cuvette (Low Substrate)
1	H1	L1
5	H2	L2
8	H3	L3

11) **When you are done with the last sample removal from your enzymatic reaction (after 8min), rinse out all used Pasteur transfer pipettes with plenty of distilled water and save them for later lab activities. Also rinse out your two 15ml conical (reaction) tubes and save them for later use.**

Pipetting scheme





Locate the spectrophotometer. Then blank your spectrophotometer at 410nm with the standard cuvette labeled S1.

- make sure that the spectrophotometer was turned on about 15 min before this activity
- you should have the 5 cuvettes of standards labeled S1 – S5 from the previous activities on your bench

16) **Measure the absorbance values for your six cuvettes from step 10) and record the values in the <u>Results Table</u> below.**

<u>Results Table: Activity 4</u>

Cuvette	Absorbance at 410nm	Amount of p-Nitrophenol (nmol)
H1 (1 min)		
H2 (5 min)		
H3 (8 min)		
L1 (1 min)		
L2 (5 min)		
L3 (8 min)		

17) **Follow the same protocol used in Activity 1 and convert the absorbance readings into the corresponding amount of p-Nitrophenol product (in nmol) with the help of your previously established standard curve. Write these deduced values in the Result Table above.**

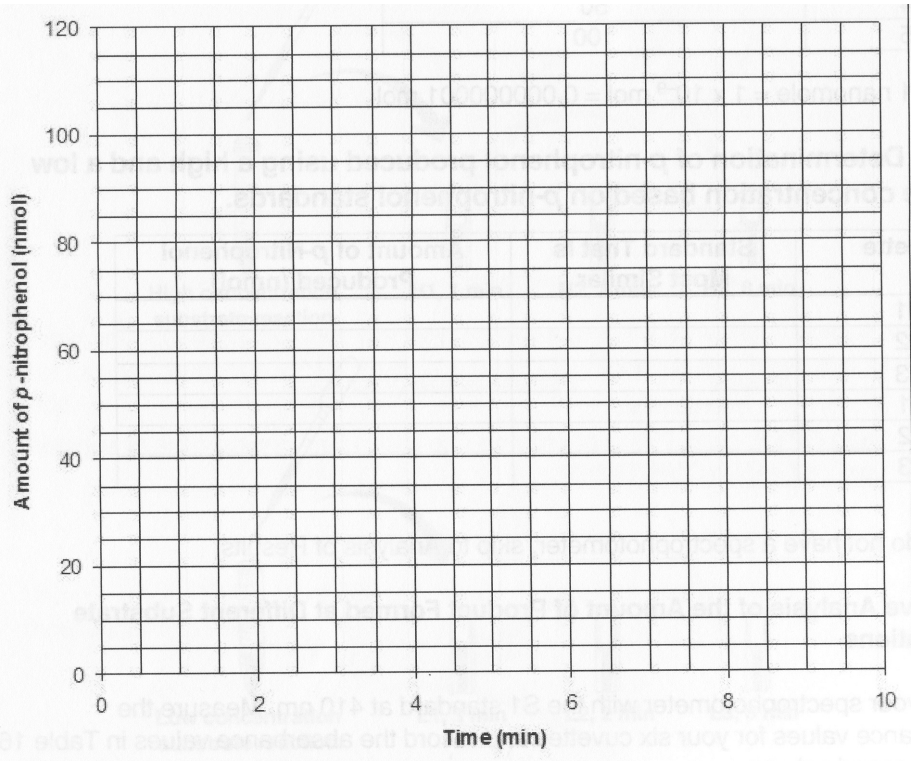
Analysis of Results

In this part of the lab you will plot the data from the absorbance measurements of your enzyme reactions at the two different (high and low) substrate concentrations. The amount of p-nitrophenol product produced over time in the two enzyme reactions "S-High", and "S-Low", is plotted against time (in minutes). From the two graphs you will then determine the initial rate of product formed by the cellobiase enzyme in the two different enzyme reactions and give the enzyme activity in nmol/min.

18) **Plot the amount (in nmol) of p-nitrophenol produced (y-axis) after 5 minutes against time (x-axis) for the three examined enzyme reactions in the Graph 7 below.**

19) **Answer the "Analysis Questions" for this activity at the end of this lab manual.**

<u>Graph 7:</u> Reaction rate curves for cellobiase enzyme at different substrate concentrations



Lab Activity 5: Compare the initial reaction rate of bacterial and fungal cellobiase enzymes with the lab enzyme.

In this lab activity you will study whether the culture supernatants and/or cell extracts of certain cellulose-degrading, i.e. cellulolytic, microbes contain cellobiase enzyme activity. You will compare the initial rate of bacterial and/or fungal cellobiases with that of the cellobiase enzyme studied in the previous activities.

Cellulases, which break down cellulose in smaller fragments and cellobiose, and cellobiases, i.e. enzymes which break down the β1,4-glycosidic bond of cellobiose into two glucose molecules, are produced by many microbial life forms, most namely bacteria and fungi. Certain bacteria, such as members belonging to the genera Bacillus, Clostridium, Pseudomonas, Streptomyces and Cytophaga, and many fungi, most namely molds (Trichoderma, Rhizopus, Aspergillus), yeast and mushrooms, produce cellulases and cellobiases which they usually secrete in form of exo-enzymes into their cell surrounding. There the secreted cellulases break down the cellulose-made cell wall of plant material, and the cellobiases digest the cellulose degradation product cellobiose further down to the final, fermentable glucose molecules. Cellobiases can also be found in the seeds of bitter almonds where it is known as emulsin. Cellulolytic fungi are usually found in rich soils, compost and other decaying organic matter where they play an important role as decomposers. Cellulolytic bacteria have been isolated from very diverse natural environments, including termite guts and the rumen of herbivorous, hoofed animals, such as cows and buffalos.

In this activity a student team will either collect the supernatant of a culture of a cellulolytic bacterium and use this as source of cellobiase enzyme (<u>Team A</u>), or prepare a cellobiase-containing extract of a collected/purchased mushroom and use this extract as source of cellobiase enzyme for further studies (<u>Team B</u>).

YOUR INSTRCUTOR WILL TELL YOU AHEAD OF THE LAB STUDY WITH WHICH CELLOBIASE ENZYME (BACTERIAL OR FUNGAL) YOU WILL BE CONDUCTING YOUR STUDY WITH.

Procedure (Team A – Fungal cellobiase study)

1. **Protocol the name and source of the mushroom you selected for this study.**

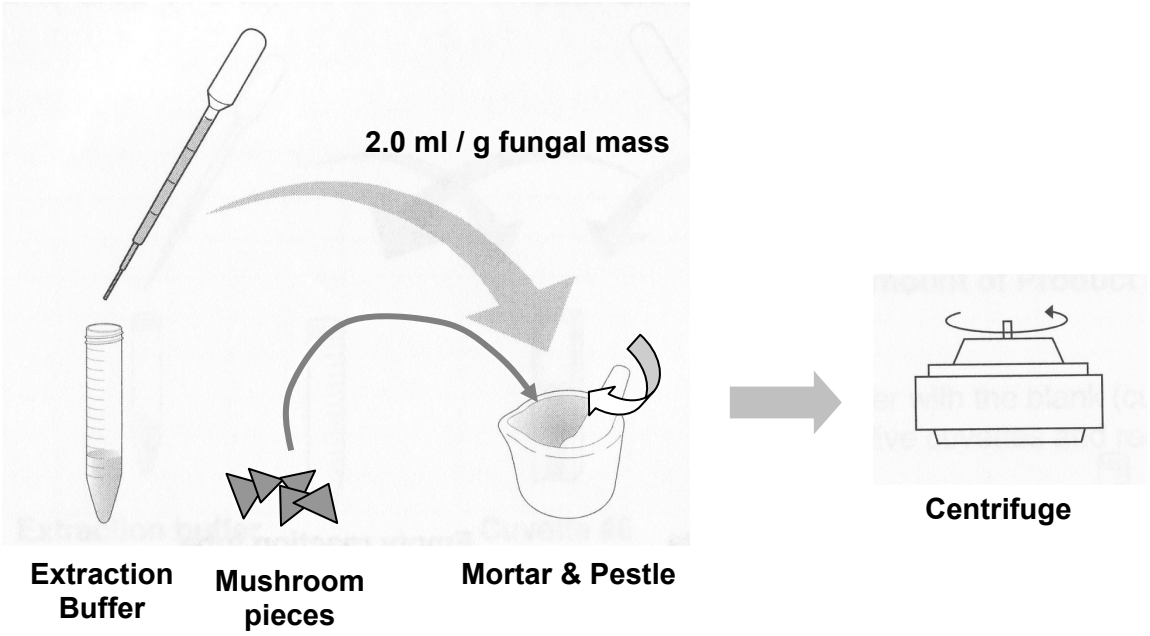
   Mushroom: _____
   Source: _____

2. **Use clean scissors (or a scalpel) and cut the mushroom in small (ca. 5-8 mm) pieces.**

3. **Use a weighing boat and weigh out approximately 1 g of your mushroom pieces on a table balance. Transfer the pieces in a clean mortar.**
   _____ g

4. **Use a clean Pasteur transfer pipette and transfer 2 ml of Extraction buffer for every gram of mushroom into the mortar (see Figure below).**
   _____ ml

Fungal Cellulase Extraction



5. **Use a clean pestle and thoroughly grind the mushroom pieces to produce a homogeneous slurry.**
   - use circulating movements of your pestle under constant pressure

6. **Use a micropipettor and pipette the slurry into a clean 1.5 ml microcentrifuge tube. Place the close-capped tube into a microcentrifuge and spin the sample at top speed (about 10,000 x g) for 2 minutes.**

- MAKE SURE THAT THE ROTOR IS COUNTER-BALANCED BEFORE STARTING THE CENTRIFUGATION!
- this step removes solid particles out of the slurry

7. **Take the microcentrifuge tube out of the centrifuge and carefully transfer the supernatant into a clean microcentrifuge tube. Place the tube with your mushroom extract (supernatant) on ice until further use.**

8. **Use a marker pen and label six plastic cuvettes "1-6". Keep them on your bench.**
   - make sure that you only label the upper part of the cuvette

9. **Use a clean Pasteur transfer pipette and pipette 0.5 ml "Stop" solution into each cuvette.**
   - thoroughly rinse the transfer pipette with distilled water after use and keep for later use

10. **Label a clean 15 ml conical tube with the initials of the type of mushroom you are using for this study, e.g. "YM" ("your mushroom"). Then use a clean Pasteur transfer pipette and pipette 3 ml of the 1.5mM "Substrate" solution into the "YM" tube.**
    HAVE YOUR TIMER READY!

11. **Now use a clean Pasteur transfer pipette and pipette 0.25 ml of your mushroom extract (= supernatant from step 7. on ice) into the 15 ml conical tube "YM" to start the enzymatic reaction. START YOUR TIMER!**

12. **At the times indicated in the Table below, remove 0.5 ml of the mushroom extract/substrate mix in the "YM" tube, and pipette it to the corresponding, previously labeled plastic cuvette ("1-5") that contains the stop solution.**

Time	Cuvette #
1 min	1
2 min	2
5 min	3
6 min	4
8 min	5
see "Blank"	6

13. **Use a clean Pasteur transfer pipette and pipette 0.5 ml "Extraction" buffer to cuvette "6". Rinse the pipette with distilled water and then add one drop of your mushroom extract. This cuvette will serve you as the "blank" for the following spectrophotometric analysis of your samples.**

14. **Rinse out all used Pasteur transfer pipettes with plenty amounts of distilled water and save them on your bench. DO NOT TOSS THEM!**

15. **Locate the spectrophotometer. Then blank your spectrophotometer at 410nm with the cuvette "6".**

16. **Measure the absorbance values for your five cuvettes from step 12. and record the values in the Results Table below.**

Results Table: Activity 5-A

Time (min)	**Absorbance at 410nm**	**Amount of p-Nitrophenol** (nmol)
Blank	0	0
1 min		
2 min		
5 min		
6 min		
8 min		

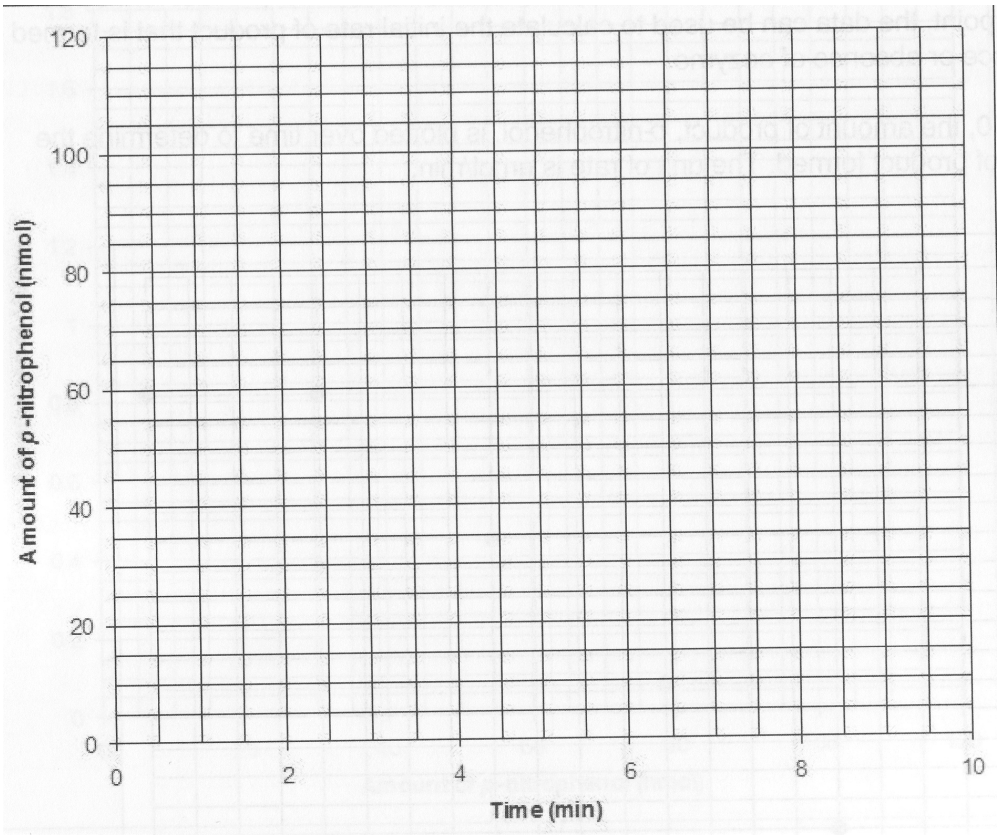
20) **Follow the same protocol used in Activity 1 and convert the absorbance readings into the corresponding amount of p-Nitrophenol product (in nmol) with the help of your previously established standard curve. Write these deduced values in the Result Table above.**

Analysis of Results

In this part of the lab you will plot the data from the absorbance measurements with your mushroom extract. You will determine whether your mushroom contains cellobiase enzyme activity, and – if so – compare the initial rate of the mushroom enzyme with the initial rate of your cellobiase enzyme from activity 1. The amount of p-nitrophenol product produced by both enzymes ("mushroom enzyme" versus lab enzyme), is plotted over time. From the established graph you will then determine the initial rate of product formed by the mushroom cellobiase enzyme and give the enzyme activity in nmol/min.

21) **Plot the amount (in nmol) of p-nitrophenol produced (y-axis) against time (x-axis) for the two enzyme reactions ("mushroom enzyme" & "BioRad enzyme") in the Graph 8 below.**

Graph 8: Reaction rate curve for the mushroom extract and BioRad cellobiase enzyme



22) Use the established Graph 8 and calculate the initial rate for the enzymatic reaction of your mushroom extract.

- for example:

Initial Enzymatic Rate = (39 nmol – 0 nmol) / (6 min – 0 min) = 6.5 nmol/min

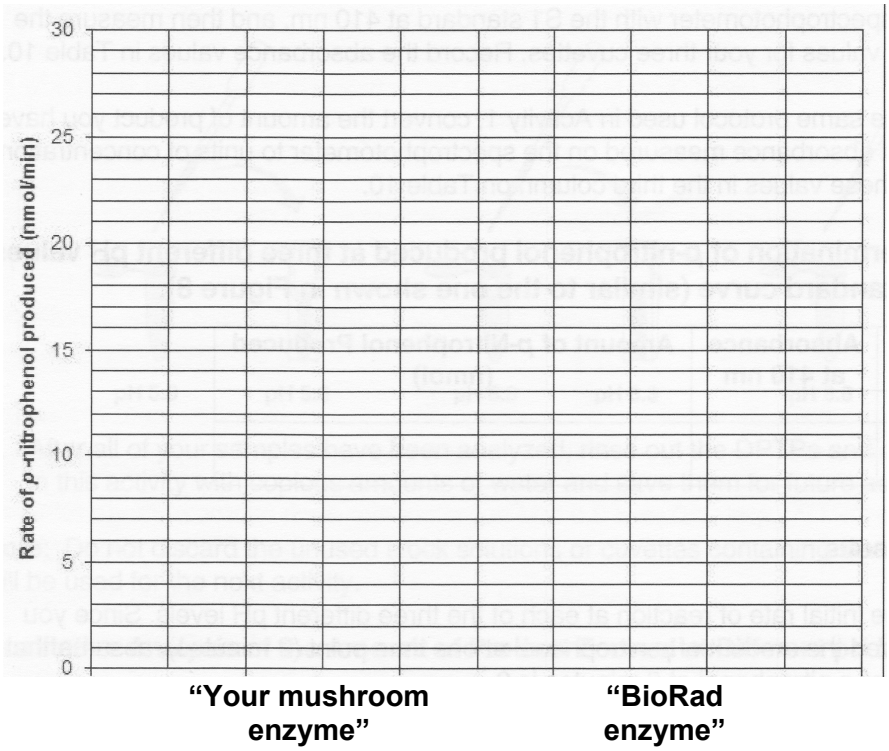
Cellobiase enzyme reaction	**Initial rate of product formation** (nmol/min)
"Your mushroom enzyme"	
BioRad enzyme*	

23) Plot the calculated initial rates for both cellobiase enzymes in form of a <u>bar chart</u> in the <u>Graph 9</u> below.

- graph enzymatic rate in nmol p-nitrophenol produced per min (y-axis) against the two samples

24) Answer the "Analysis Questions" in the section below

<u>Graph 9:</u> Comparative initial rates of a mushroom cellobiase and of the BioRad cellobiase enzyme



<u>Procedure (Team B – Bacterial cellobiase study)</u>

1. **Locate the Erlenmeyer flask containing 50 ml of a 48 or 72 hour culture of a cellulolytic bacterium and all the other solutions on your lab bench top.**
   - your instructor will assign your team a certain bacterial culture, which can be either a species belonging to one of the following genera:
     1. Bacillus sp.
     2. Cellulomonas sp.
     3. Pseudomonas sp.
   - the bacterial culture has been cultivated aerobically at 35^{o}C under continuous shaking in a shaker water bath (set at 175 rpm) for 48 hours or 72 hours

2. **Protocol the name of the bacterium you have been assigned to work with during this lab activity, the name of the growth medium and the cultivation time in the corresponding lines below.**

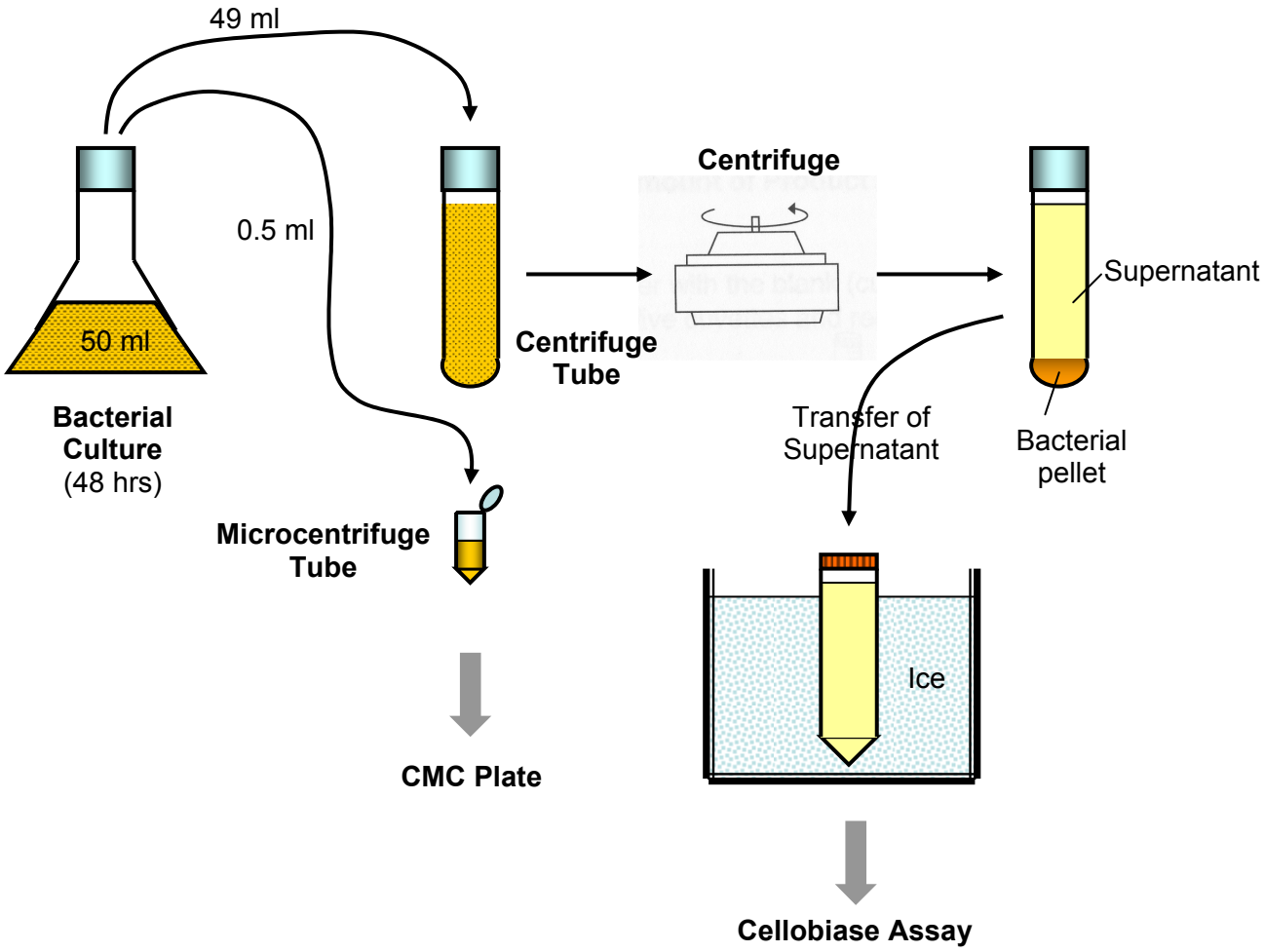
   Bacterium: _____
   Medium: _____
   Cultivation time: _____ hours

3. **Label one sterile 1.5 ml microcentrifuge tube and two clean 50 ml conical centrifuge tubes with your initials and the name/number of the assigned bacterial culture. Use a sterile 1 ml pipette and aseptically transfer 0.5 ml of your bacterial culture into the labeled**

microcentrifuge tube. Now, use a clean 25 ml graduated pipette and pipette the remaining volume of your bacterial culture into one of the labeled centrifugation tubes. Tightly screw cap the tube and place on ice (see Figure below).

- make sure that you swirl the bacterial culture before the transfer to assure a homogeneous cell suspension
- place the microcentrifuge tube with your bacterial sample in a rack and keep on your bench; this sample is later needed in Activity 6

Figure: Preparation of bacterial cellulose enzyme-containing supernatant



4. **Place the tube with your collected bacterial culture in the rotor (e.g. GSA rotor) of a pre-chilled (4°C) cooling centrifuge (e.g. Sorvall RC-5B) and centrifuge the bacterial cells at 3,000 x g (about 8,000 rpm) for 20 minutes.**
   - MAKE SURE THAT THE ROTOR IS PROPERLY COUNTER-BALANCED BEFORE STARTING THE CENTRIFUGE!
   - this step sediments the bacteria from the cellulase enzyme containing medium

5. **After completion of centrifugation, take the centrifuge tube out of the centrifuge and place on ice. Now, carefully transfer the clear supernatant by decanting it into a clean 50 ml conical centrifuge tube, screw cap and then place on ice until further use.**
   - MAKE SURE THAT NO BACTERIAL SEDIMENT (PELLET) IS TRANSFERRED

DURING THIS STEP!

6. **Use a marker pen and label six plastic cuvettes "1-6". Keep them on your bench.**
   - make sure that you only label the upper part of the cuvette

7. **Use a clean Pasteur transfer pipette and pipette 0.5 ml "Stop" solution into each cuvette.**
   - thoroughly rinse the transfer pipette with distilled water after use and keep for later use

8. **Label a clean 15 ml conical tube with the initials of the bacteria which cultural supernatant you are using as cellulase source for this study, e.g. "Bb" ("Bacillus brevis"). Then use a clean Pasteur transfer pipette and pipette 3 ml of the 1.5mM "Substrate" solution into the labeled (e.g. "Bb") conical tube.**
   HAVE YOUR TIMER READY!

9. **Now use a clean Pasteur transfer pipette and pipette 0.25 ml of your bacterial supernatant (= supernatant from step 5. on ice) into the labeled (e.g. "Bb") 15 ml conical tube to start the enzymatic reaction. START YOUR TIMER!**

10. **At the times indicated in the Table below, remove 0.5 ml of the bacterial supernatant/substrate mix in the labeled (e.g. "Bb") conical tube, and pipette it to the corresponding, previously labeled plastic cuvette ("1-5") that contains the stop solution.**

Time	Cuvette #
1 min	1
2 min	2
5 min	3
6 min	4
8 min	5
see "Blank"	6

11. **Use a clean Pasteur transfer pipette and pipette 0.5 ml "1x Resuspension buffer" to cuvette "6". Rinse the pipette with distilled water and then add one drop of your bacterial supernatant. This cuvette will serve you as the "blank" for the following spectrophotometric analysis of your samples.**

12. **Rinse out all used Pasteur transfer pipettes with plenty amounts of distilled water and save them on your bench. DO NOT TOSS THEM!**

13. **Locate the spectrophotometer. Then blank your spectrophotometer at 410nm with the cuvette "6".**

14. **Measure the absorbance values for your five cuvettes from step 12. and record the values in the Results Table below.**

Results Table: Activity 5-B

Time (min)	**Absorbance at 410nm**	**Amount of p-Nitrophenol** (nmol)

Blank	0	0
1 min		
2 min		
5 min		
6 min		
8 min		

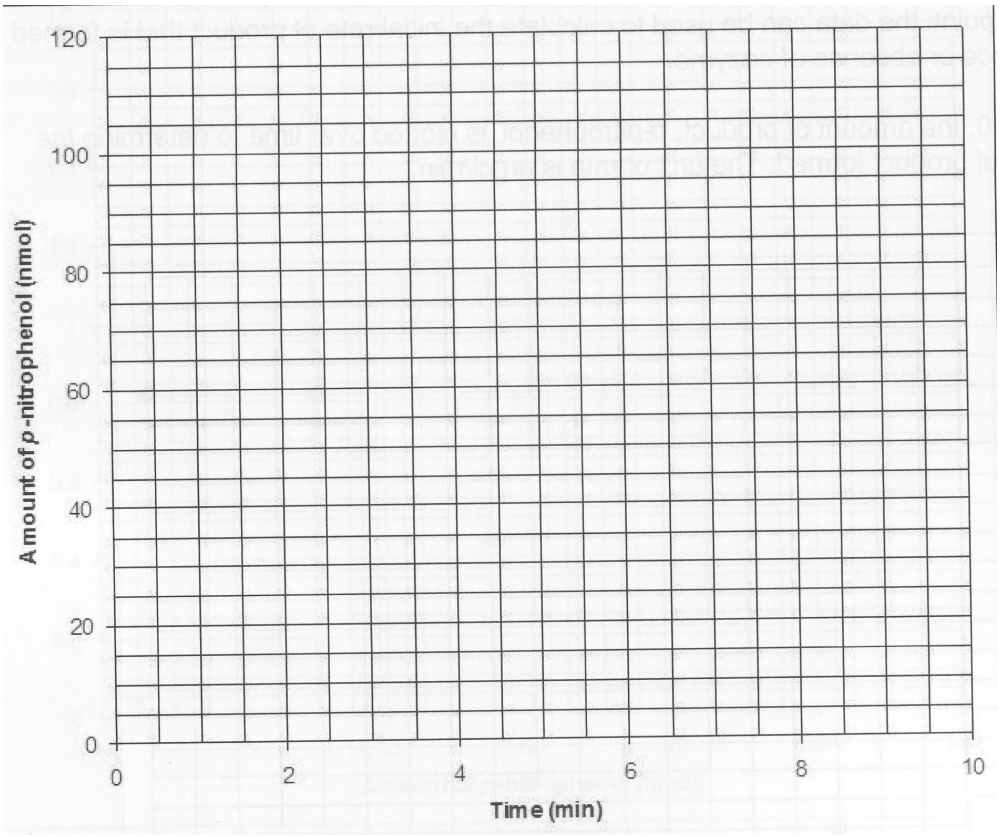
25) Follow the same protocol used in Activity 1 and convert the absorbance readings into the corresponding amount of p-Nitrophenol product (in nmol) with the help of your previously established standard curve. Write these deduced values in the Result Table above.

Analysis of Results

In this part of the lab you will plot the data from the absorbance measurements with your bacterial supernatant. You will determine whether the bacterium which was assigned to you at the beginning of this lab activity contains cellobiase enzyme activity, and – if so – compare the initial rate of the bacterial enzyme with the initial rate of your cellobiase enzyme from activity 1. The amount of p-nitrophenol product produced by both enzymes ("bacterial enzyme" versus lab enzyme), is plotted over time. From the established graph you will then determine the initial rate of product formed by the bacterial cellobiase enzyme and give the enzyme activity in nmol/min.

26) Plot the amount (in nmol) of p-nitrophenol produced (y-axis) against time (x-axis) for the two enzyme reactions ("bacterial enzyme" & "BioRad enzyme") in the Graph 10 below.

Graph 10: Reaction rate curve for the bacterial supernatant and BioRad cellobiase enzyme



27) Use the established Graph 10 above and calculate the initial rate for the enzymatic reaction of your bacterial enzyme.

- for example:

Initial Enzymatic Rate = (39 nmol – 0 nmol) / (6 min – 0 min) = 6.5 nmol/min

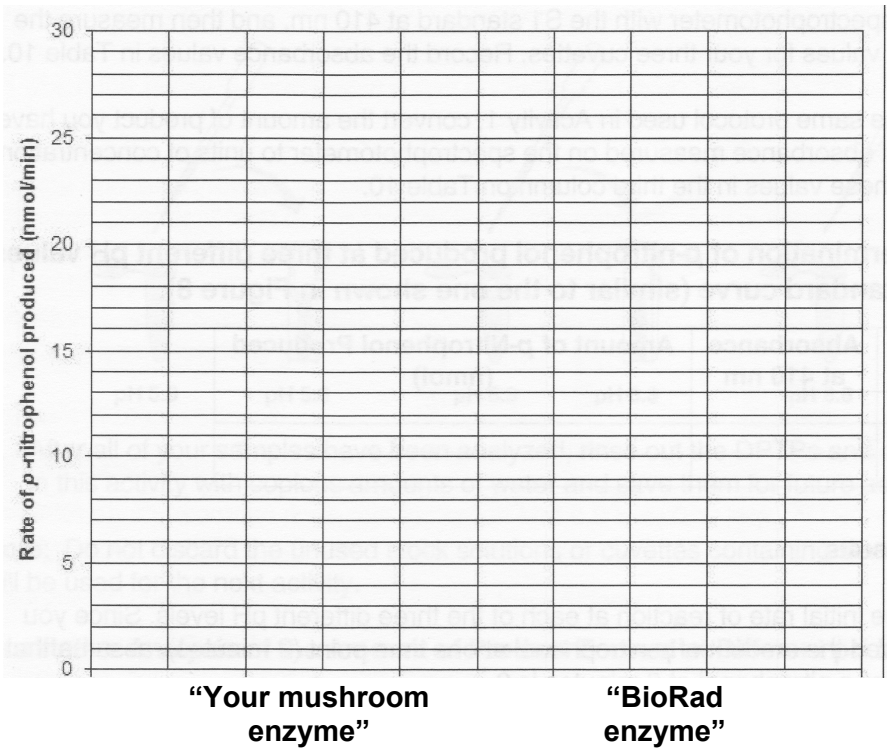
Cellobiase enzyme reaction	**Initial rate of product formation** (nmol/min)
"Your bacterial enzyme"	
BioRad enzyme*	

28) Plot the calculated initial rates for both cellobiase enzymes in form of a <u>bar chart</u> in the <u>Graph 11</u> below.

- graph enzymatic rate in nmol p-nitrophenol produced per min (y-axis) against the two samples

29) Answer the "Analysis Questions" in the section below.

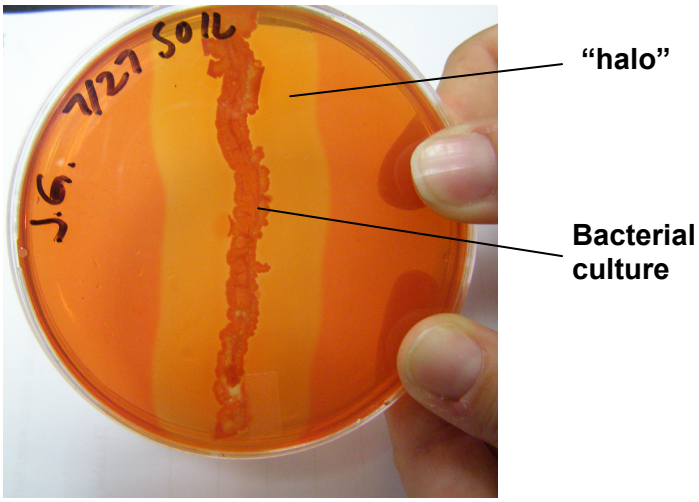
<u>Graph 11:</u> Comparative initial rates of a bacterial cellobiase and of the BioRad cellobiase enzyme



Lab Activity 6: Examination of cellulolytic activity of an assigned bacterium on CMC agar plates.
Certain bacteria found in nature are able to break down the cellulose in their surrounding environment

with the help of cellulose degrading enzymes called cellulases. Some of these cellulolytic bacteria are able to secrete the cellulase enzymes outside the cell where they break down cellulose into smaller, metabolizable sugar components. When plated on special cellulose-containing agar plates, i.e. carboxymethyl cellulose (CMC) plates, these exo-enzyme secreting bacteria form a typical clear zone (or "halo") around the growing bacterial culture (see **Figure** below).

Congo Red Stain on CMC Agar Plates



In this lab activity you will examine whether the bacterium you have been assigned with is able to secrete cellulose degrading enzymes, i.e. cellulase, into the surrounding medium. You will streak the assigned bacterium onto the surface of a carboxymethyl cellulose (CMC) agar plate, cultivate the bacterium over 2-3 days and perform the Congo Red staining procedure to analyze the plates for cellulose degradation. You will compare the cellulolytic activity of your bacterium with that of other student teams.

Procedure

Generally: STRICTLY ADHERE TO THE RULES OF THE ASEPTIC TECHNIQUE AS YOU WORK ON THIS ACTIVITY.

1. DISINFECT YOUR WORK AREA WITH 70% ALCOHOL (OR OTHER DISINFECTING AGENT) BEFORE AND AFTER THE PROCEDURE.
2. HEAT FLAME THE PLATINUM LOOP AND WIRE WITH A BUNSEN BURNER FLAME BEFORE AND AFTER TRANSFERING THE BACTERIA ONTO THE AGAR PLATE
3. TURN OFF THE GAS AFTER COMPLETION OF THIS ACTIVITY!

1. **Locate the microcentrifuge tube containing 0.5 ml of a 48 or 72 hour culture of YOUR assigned bacterium (see step 3 of Activity 5), the Bunsen burner, a platinum loop and a CMC agar plate on your lab bench top.**
   - your instructor will have assigned your team a certain bacterial culture

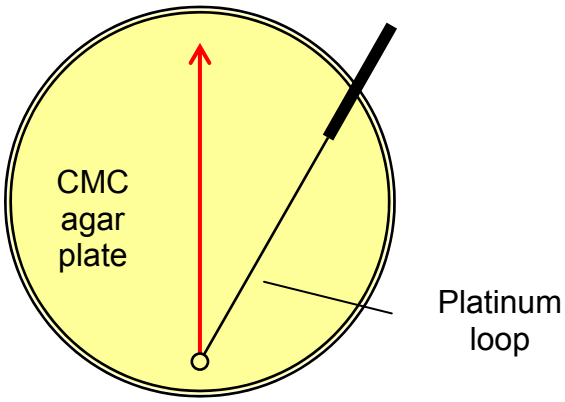
2. **Label the bottom of the CMC plate with your initials and the name of the bacterium you have been assigned to work with. Write the genus/species of the assigned bacterium, the name of the growth medium and the cultivation time in the corresponding blanks below.**

   Bacterium: _____

Medium: _____
Cultivation time: _____ hours

3. **Open the gas valve and lit up the Bunsen burner. Flame the loop and the platinum wire over the Bunsen burner flame until red-hot. Let the heat sterilized loop briefly (< 1min) cool down in air and then pick up one loopful of your bacterial culture in the microcentrifuge tube.**

4. **Aseptically streak the loopful of bacterial culture onto the surface of the CMC agar plate.**
   - make one single straight line as shown in the Figure below
   - your instructor will give you a demonstration how to do this step aseptically



5. **Incubate the (closed lid) CMC agar plate in an incubator at 35^{o}C for 48 hours.**
   - the bacterial culture on the plate can be stored in a fridge until the next lab meeting

6. **Take the CMC agar plate out of the incubator and draw the growth margins of your bacterial culture on the bottom of the plate with the help of a fine pointed marker pen.**
   - this documentation is important since the bacterial culture on the agar surface can become detached during the following staining and washing steps.

7. **Flood the surface of the CMC agar plate with an aqueous solution of 0.1% Congo red (1mg/ml) and keep the closed lid and non-agitated agar plate for 30 min at room temperature on your lab bench.**
   - the Congo Red dye will stain the cellulose material in the agar matrix
   DO NOT GET IN SKIN CONTACT WITH THE CONGO RED SOLUTION! WEAR GLOVES DURING THIS AND THE FOLLOWING STEP!

8. **Pour off the excess Congo red solution into a Biohazard container and then flood the CMC agar plate surface with 1M NaCl for 15 min at room temperature.**

9. **Immediately document the visible "light orange-colored" zone of cellulose hydrolysis ("halo") with the help of a digital camera. Alternatively, the halo can be stabilized for at least 2 weeks by flooding the CMC agar plate with 1 M HCl, which changes the dye color to bluish-purple and inhibits further enzyme activity.**

10. **Attach or paste the photo of your Congo Red stained CMC agar plate with your bacterial culture into the section below. Label the photo indicating the halo and the bacterial culture.**

Photo documentation of CMC agar plate results

(cut and paste your photo of the CMC plate with your bacterial culture after Congo Red staining procedure into the frame below and label)

Student Name/Team: _____ Date: _____

Bacterium: _____

Medium: _____

Biofuels Lab #3 - Microbial Hydrogen Production

Objectives

1. Learn how to set up a bioreactor for batch cultivation of bacteria for hydrogen production
2. Learn how to measure bacterial hydrogen production
3. Learn how to measure pH value using a pH electrode
4. Learn how to take bacterial samples for measurement of optical density
5. Learn how to calculate and plot biohydrogen production rate and yield
6. Know the principle of a hydrogen fuel cell

Introduction

Molecular hydrogen or hydrogen gas (H_2) is considered an important fuel alternative with the potential to replace currently used fossil fuels in the near future. Currently, most of the hydrogen gas produced on an industrial scale is not used as a fuel but consumed in diverse industrial processes, most namely petroleum oil and food oil hydrogenation and fertilizer production. The industrially produced hydrogen gas is to the vast extent produced from the gasification of fossil fuels, most namely coal and natural gas. Moreover, hydrogen production via gasification is accompanied with the generation of noxious and poisonous waste products, such as carbon monoxide. Therefore, this type of hydrogen gas production for future fuel use can not be considered as a sustainable and clean solution to the world energy demand problem.

Alternative hydrogen production strategies are therefore of high interest and biological hydrogen production is investigated by many scientists as one of the possible hydrogen production alternatives. Biological hydrogen or biohydrogen production is the synthesis of hydrogen gas with the help of living organisms in a controlled bioreactor environment. Many microorganisms most importantly algae and bacteria are able to produce hydrogen gas from renewable resources, most importantly sunlight and biomass. While certain algae are able to generate hydrogen gas directly from sunlight in the presence of water, several genera of bacteria are known to produce hydrogen gas from diverse renewable biomass-derived molecules, most namely carbohydrates such as glucose, sucrose and xylose, under fermentative conditions.
Bacterial hydrogen gas (H_2) production (see **Figure** below) is the consequence of a transfer of cellular reduction equivalents, i.e. electrons (= e^-), onto protons (H^+). The necessary reduction equivalents derive from the metabolic breakdown of hydrogen-rich food molecules, such as glucose. The general formation of H_2 is shown in the chemical reaction below.

$$\mathbf{2\ H^+ + 4\ e^- \longrightarrow H_2}$$

Three different H_2 production strategies and pathways have been identified in different types of bacteria, each with a unique arsenal of enzymes and reduction equivalent donors.
Strictly anaerobic bacteria, such as *Clostridium sp*., oxidize the glycolysis end product pyruvate to acetyl-CoA and carbon dioxide. This chemical reaction which requires presence of Coenzyme A (HS-CoA) and ferredoxin (Fd) is catalyzed by the pyruvate:ferredoxin-oxidoreductase enzyme as shown in the chemical reaction below.

$$\mathbf{Pyruvate + HS\text{-}CoA + 2\ Fd \longrightarrow Acetyl\text{-}CoA + 2\ FdH + CO_2}$$

Hydrogen gas is generated from reduced ferredoxin (FdH) in a follow up reaction (see below) which is catalyzed by a hydrogenase enzyme.

$$\mathbf{2\ FdH \rightarrow 2\ Fd + H_2}$$

Many facultative anaerobic bacteria, most namely *Escherichia coli* and *Enterobacter sp*. generate biohydrogen gas from hydrogen-containing feedstock, such as glucose or sucrose, with the help of the enzyme pyruvate:formate-lyase (PFL) and the formate:hydrogen-lyase (FHL)/hydrogenase (HYD) complex according to the following chemical reactions.

$$\mathbf{PFL:\ \ Pyruvate + HS\text{-}CoA \longrightarrow Acetyl\text{-}CoA + Formate\ (HCOOH)}$$

$$\mathbf{FHL:\ \ HCOOH + X\ \text{-}\ (Ni, Se) \longrightarrow\ CO_2 + XH_2}$$

$$\mathbf{HYD:\ \ XH_2\ \text{-}\ (Mg^{2+}) \longrightarrow\ X + H_2}$$

The pyruvate:formate-lyase enzyme catalyzed (or Enterobacteriaceae-type) pathway is characterized by the formation of the intermediate product formic acid (HCOOH or formate). In this pathway pyruvate is first cleaved into acetyl-CoA and formate with the help of the enzyme pyruvate:formate-lyase (PFL). In a subsequent reaction formate then is cleaved by the nickel (Ni)/Selenium (Se)-dependent formate:hydrogen-lyase (FHL) enzyme system via a short-lived XH_2 intermediate to the final fermentation end product H_2. The formate:hydrogen lysase complex is comprised of a selenium (Se)-dependent formate-dehydrogenase enzyme subunit and a magnesium (Mg^{2+})- dependent hydrogenase component, which catalyzes the final formation of hydrogen gas (H_2) from an unknown XH_2 intermediate molecule.
A third bacterial hydrogen production pathway generates hydrogen gas with the help of the $NADH_2$:Ferredoxin-oxidoreductase enzyme. This enzyme which is widely found in anaerobic bacteria is able to move electrons and protons derived from NADH + H^+ onto ferredoxin (Fd) as shown in the chemical reaction below.

$$\mathbf{NADH + H^+ + 2\ Fd \longrightarrow NAD+ + 2\ FdH}$$

$$\mathbf{2\ FdH \longrightarrow 2\ Fd + H_2}$$

Bacteria which are able to utilize the NAD hydrogen for the generation of hydrogen gas are known to be acetate producers, such as *Ruminococcus albus*, a symbiotic bacterium which lives in the rumen of herbivorous animals.

Hydrogen producing bacteria are able to yield a theoretical maximum of 4 moles of H_2 for each mol of glucose fermented. Since under realistic conditions bacteria produce other fermentation by-products, other than acetate, the yield achieved in fermenters is usually much lower and somewhere between 1.1 – 3.0 moles H_2 per mol glucose. The molar yield of hydrogen is also dependent on the type of feedstock.

Bacterial hydrogen production has been reported in many natural and surprisingly unusual environments, such as termite guts, in the rumen of cows, and in teeth cavities. Molecular hydrogen (H_2) has been shown to be generated as a fermentation by-product in the large intestine of animals and it is suspected that certain bacteria use the hydrogen gas as energy source.
H_2 is also released during the natural fermentative degradation of plant-derived cellulose and cellulose-containing detritus by anaerobic bacteria, such as *Clostridium thermocellum* or *C. cellulolyticum*. Cellulose-degrading bacteria - which play an eminent role at the foundation of the global nutrient cycles - thrive in the anaerobic sediments of bodies of stagnant water and deeper, oxygen-deprived layers of soil. These cellulolytic bacteria are able to break down the polysaccharide

cellulose into smaller fragments, e.g. cellobiose, and into the monosugar component glucose, with the help of cellulose-degrading enzymes called cellulases and beta-glucosidases. Fermentation of these cellulose-derived mono- or disaccharides in these bacteria is often accompanied with the generation of significant amounts of hydrogen gas. The presence of hydrogen producing bacteria is crucial for the production of biogas (= methane) in the anaerobic digester units of biogas plants.
The key enzymes used by different hydrogen producing microbes are hydrogenases. Most hydrogenases studied to date are nickel-iron-selenium [NiFeSe]- of nickel-iron [NiFe]-containing enzymes. While [NiFe]-dependent uptake hydrogenases catalyze the reversible heterolytic cleavage of molecular hydrogen ($H_2 \leftrightarrow 2\ H^+ + 2\ e^-$), other hydrogenases, especially the formate:hydrogen-lyase-associated hydrogenases and the Ech hydrogenases (which are present in a variety of bacteria, e.g. Desulfovibrio & Frankia species) are responsible for the ability of certain bacteria to generate significant amounts of molecular hydrogen (H_2) under anaerobic growth conditions.
Hydrogenases are extremely oxygen-sensitive enzymes and become rapidly inactivated in the presence of molecular oxygen (O_2). This requires the establishment of anaerobic conditions in fermenters to assure biohydrogen production. To date, three different types of hydrogenases, i.e. membrane- bound uptake hydrogenases, H_2-evolving hydrogenases and the so called bidirectional hydrogenases, have been identified in different bacteria.

Visible H_2 production by a hydrogen producing microbe

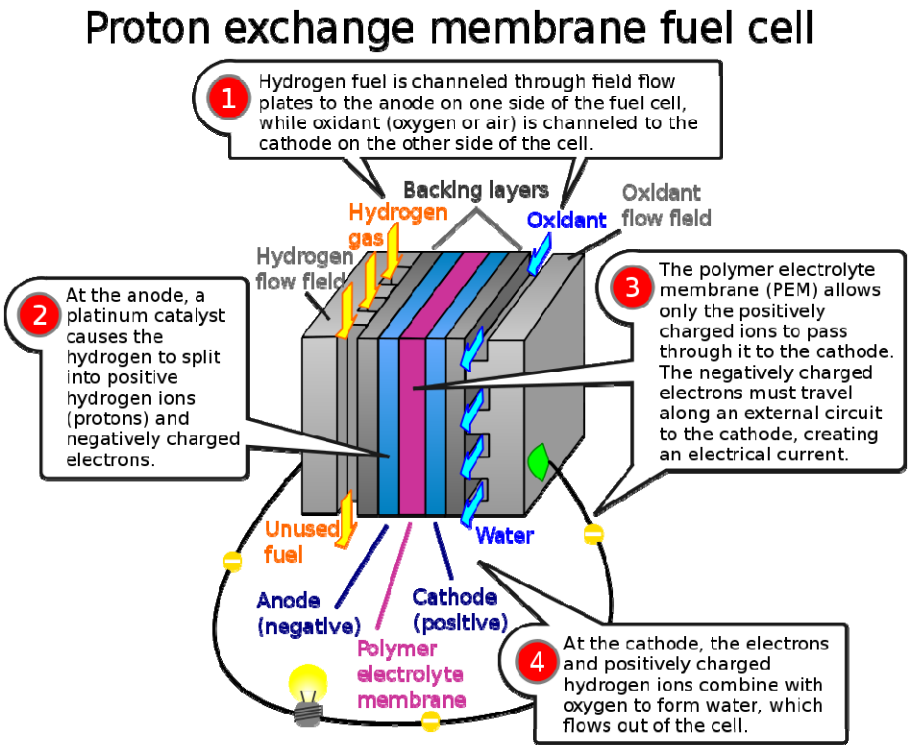
The biological function of hydrogen gas production by hydrogen-generating bacteria is not completely understood yet. However, H_2 seems to be more than just an electron acceptor and mere metabolic waste product in these bacteria. It is suspected that the produced hydrogen gas plays an important role as a biological energy molecule. There is evidence that certain bacteria, especially bacteria which are in possession of a bidirectional hydrogenase enzyme (see chemical reaction below), are able to use the electrons and protons derived from the oxidation of hydrogen gas. The

resulting electrons are feed into a membranous electron transport chain while the generated protons are used to establish a proton gradient which proton motive force (pmf) is used to generate the cell fuel molecule ATP.

$$\mathbf{H_2} \longrightarrow \mathbf{2\,H^+ + 2\,e^-} \longrightarrow \text{pmf} \longrightarrow \mathbf{ATP}$$

For example, the human pathogenic bacterium *Helicobacter pylori* has the ability to use H_2 as a crucial energy molecule by tapping its electrons and protons with the help of the catalytic (oxidative) activity of a respiratory hydrogenase. While bacteria seem to rely on hydrogen energy since billions of years, humans just recently discovered the great potential of hydrogen as a clean energy source when utilized with the help of special hydrogen conversion device called a fuel cell. While the same chemical reaction as shown above takes place within a hydrogen operated fuel cell, this modern devise does not generate chemical energy in form of ATP but electrical energy and (due to the second law of thermodynamics) also significant amounts of heat.

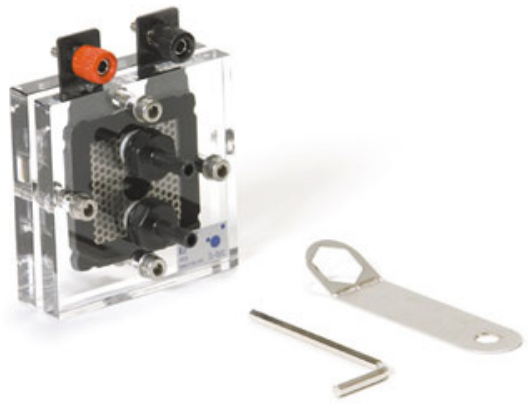
Hydrogen fuel cells are technical devises which convert the energy of hydrogen gas into electrical energy. Hydrogen fuel cells reach with about 30-45% comparatively high energy conversion efficiencies. They are made up of three segments which are sandwiched together: the anode, the catalytic polymer electrolyte membrane, and the cathode (see **Figure** below).



Two chemical reactions occur at the interfaces of the three different segments. At the anode a membrane bound catalyst, usually platinum or palladium, oxidizes the incoming hydrogen gas, usually hydrogen, yielding positively charged protons and negatively charged electrons. The electrolyte is a substance specifically designed to selectively allow the passage of the protons but not of the resulting electrons. A common electrolyte material in hydrogen fuel cells is a polymer membrane called polyether sulfone. The hydrogen gas-derived freed electrons travel through a wire creating the electrical DC current. The protons travel through the electrolyte to the cathode. Once

reaching the cathode, the protons are reunited with the electrons and the two react with a third chemical, usually oxygen gas, to create water.

The average energy conversion efficiency of a hydrogen fuel cell is much higher than the one of internal combustion engines. A typical fuel cell running at 0.7 V has an energy conversion efficiency of about 50%, meaning that 50% of the energy content of the hydrogen gas is converted into electrical energy. The remaining 50% will be converted into heat. For a hydrogen fuel cell operating at standard conditions with no reactant leaks, the efficiency is equal to the cell voltage divided by 1.48 V, based on the enthalpy, or heating value, of the reaction. Consequently, they can have very high efficiencies in converting chemical energy to electrical energy, especially when they are operated at low power density, and using pure hydrogen and oxygen as reactants. In this lab you will couple a small experimental PEM hydrogen fuel cell (see **Figure** below) with a nominal power of 1 W to the fermenter outlet to demonstrate the production of hydrogen gas (H_2) by a hydrogen producing bacterium.



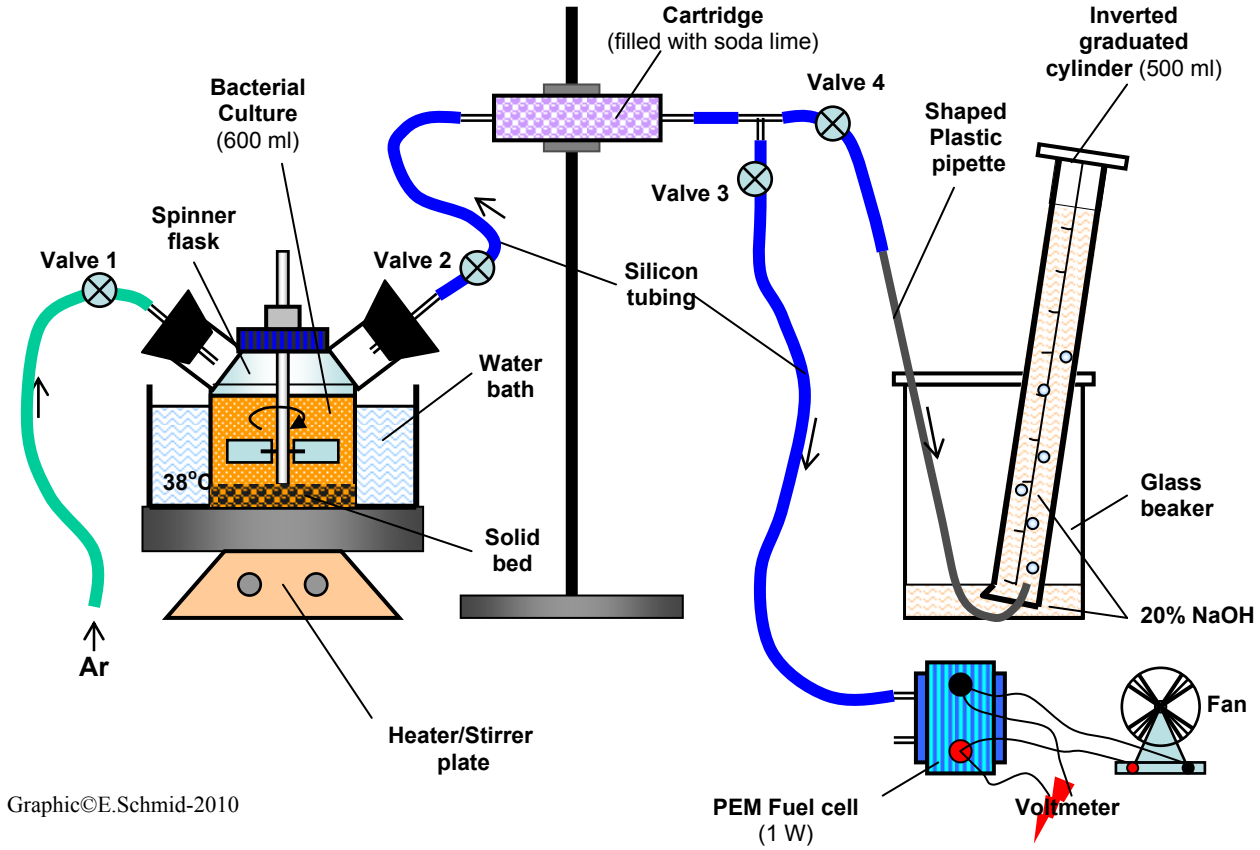
From: http://www.fuelcellstore.com/

H_2 is the lightest gas known to man and it has a low solubility in water. It therefore easily escapes the aqueous environment of a fermenter after its production by a suitable hydrogen producing microbe from where it reaches the gas space above the fermentation broth. From there the accumulated hydrogen gas can be siphoned off for further utilization for example in a hydrogen fuel cell.

Prominent hydrogen gas-producing bacteria are: different Clostridium species, *e.g. C. thermocellum,* different Enterobacter species, e.g. *E. cloacae* and *E. aerogenes, Escherichia coli, Citrobacter freundii, and Proteus sp.* In this lab section you will learn how to cultivate the hydrogen producing bacterium *Enterobacter aerogenes* under anaerobic conditions in a closed bioreactor environment. *E. aerogenes* is a gram negative, rod shaped, facultative anaerobic bacterium which can be isolated from very different natural environments, most prominently fresh water, soil, sewage, plant surfaces, animal and human fecal matter. *Enterobacter aerogenes* is metabolically very versatile and it can produce hydrogen gas from different carbohydrates including glucose, sucrose, xylose, L-arabinose, maltose and D-mannose. With glucose as carbon feedstock, *E. aerogenes* not only produces hydrogen gas but also significant amounts of carbon dioxide, ethanol and 2,3-butanediol as a major fermentation end products. You will cultivate this hydrogen producer under batch conditions using a lab set up as shown in the **Figure** below and study its hydrogen production rate and yield in the presence of different carbon molecules as feedstock. You will further learn how to analyze important

cultivation parameters, most namely pH, hydrogen production rate and optical density with different carbon sources as feedstock.

Lab Set Up – Biological Hydrogen Production under Batch Conditions



Lab Activity 1: Set up a biohydrogen reactor platform and measure fuel cell voltage and the production of hydrogen gas of a gas producing bacterium over time.
In this lab activity you will learn how to set up a bioreactor to study the hydrogen production of a gas producing bacterium under batch conditions. Each student team will work with the same bacterium but study the hydrogen production with different carbohydrates as bacterial feedstock. You will measure the amount of hydrogen gas generated over time and calculate the hydrogen production rate and yield of the bacterium for the assigned carbohydrate under study.

HYDROGEN GAS IS A HIGHLY FLAMMABLE GAS WHICH EASILY IGNITES IN A NORMAL OXYGEN ATMOSPHERE IN THE PRESENCE OF OPEN FLAMES. THEREFORE, ABSOLUTELY NO OPEN FLAMES, E.G. BUNSEN BURNER, OR IGNITION SPARKS DURING THIS EXPERIMENT!

Procedure

1. **Locate your assigned bacterial culture-containing bioreactor (spinner flask) which is placed in a water bath on a heater/stirrer plate. The clamps of the inlet and outlet tubing should be tightly closed at this point.**
   - this 600 ml bacterial mono-culture, containing a hydrogen gas producing bacterium, was inoculated about 4 hours before the beginning of this lab activity
   - record the start time of your culture (which will be written on your flask) below:

   Culture start time: _____ (t = 0 hours)

   - record the type of carbon feedstock of your bacterium in the assigned bioreactor.

   Bacterial feedstock: _____ , e.g. glucose, maltose, etc.

   - record the starting concentration c_F of the bacterial feedstock at the beginning of the inoculation.

   c_F (t=0 hours) = _____, e.g. 1.5%, 110 mM, etc.

2. **Use a thermometer and measure the temperature in the water bath. If the temperature is not at 38°C increase the temperature by changing the setting of the temperature control know of the heater stirrer plate. Make sure that the rotational speed of the impeller in the spinner flask is set at 60 rpm.**
   - MAKE SURE THAT THE IMPELLER DOES NOT DISTURB THE SOLID BED LAYER IN YOUR SPINNER FLASK!

3. **Locate the capped soda lime-filled plastic cartridge on your bench. Horizontally carry it in its capped condition to a table balance, place on balance and then remove both caps. Weigh the cartridge (without the caps) and record this "before" weight below.**

   Cartridge weight (before): CW(t = 0 h) = _____ g

   Cap the cartridge again and horizontally carry it back to your bench.

4. **Connect one spinner flask outlet (valve 1) to the tubing of (at this point) closed gas line carrying argon gas and leave valve 1 closed. Connect the second spinner flask outlet (valve 2) with one end of the soda lime cartridge using a silicone tube. Leave valve 2 closed.**
   Sparging gas: _____

5. **Locate the "three-way" silicone tubing and connect the short, valve-less end to the opposite end of the soda lime cartridge. Make sure that both valves (valve 3 and valve 4) of the silicone tubing still remain closed at this point.**
   - make sure that the tubing is slip-proof and does not easily come off when pulled; eventually use a strip of Para film to make a tight, slip-proof connection!

6. **Locate the glass beaker with the NaOH-filled inverted 500 ml graduated plastic cylinder and the gas inlet tubing (curved plastic pipette) on your bench. Make sure that the NaOH level in the beaker is just above the bottom margin of the cylinder. Write down the initial level of the NaOH solution in your inverted graduated cylinder.**

   Start level (t = 0 min) = _____ ml

BE EXTREMELY CAREFUL WHILE WORKING WITH THE SODIUM HYDROXIDE (NaOH) COMPONENT OF YOUR EXPERIMENTAL SET UP!

NaOH (which is the clear liquid in your beaker and graduated cylinder) is very caustic and causes serious damage to skin, fabric and other materials when spilled. Wear eye protection (goggles), gloves and a lab coat while working with or close to this unit! In case of a spill immediately wipe off the area with water soaked paper tissue and discard in biohazard container!

7. **Connect the end of the valve 4-connected silicone tubing to the free (upper) ending of the gas inlet tubing (curved plastic cuvette) of the glass beaker/inverted cylinder assembly. Keep valve 4 closed.**
   - make sure that the tube connection is slip-proof and does not easily come off when pulled; eventually use a strip of Para film to make a tight, slip-proof connection!

8. **Connect the end of the valve 3-connected silicone tubing to the hydrogen gas inlet of your fuel cell stack. Keep valve 3 closed.**
   - make sure that the tube connection is slip-proof and does not easily come off when pulled; eventually use a strip of Para film to make a tight, slip-proof connection!

9. **Connect the fuel cell stack with the mini fan using the corresponding electrical cords, and make a parallel wire connection to your voltmeter.**

10. **Now, first open valve 3 of your biohydrogen reactor assembly and then slowly open valve 2 to release the eventually accumulated gas in the bioreactor (spinner flask).**
    Describe your observations in the section below.
    What happens to the fan? _____

    What is your total incubation time of your bacterial culture at this time point? _____ hours

11. **Now open valve 1 of your biohydrogen reactor assembly and sparge the vessel interior with argon gas for 10 minutes at an adjusted flow rate of 20 ml/min. Leave valves 2 and 3 open, but valve 4 closed during this sparging process.**
    (Your instructor will explain to you how to use the gas meter to adjust the gas flow rate.)

    What happens to the fan? _____

    Wait until you have a steady voltage reading on your voltmeter. Write this voltage reading and the time it took to reach this stable reading down below.

    Voltage: _____ V
    Time: _____ min

    Measure the voltage of your fuel cell at each of the five stacks and record your readings in the Results Table 1 below.

Results Table 1

Fuel Stack #	Voltage (V)	Current (mA)	Power (mW)*
1			
2			
3			
4			
5			

* Calculated electrical power (P) of fuel cell = Voltage (U) times Current (I)
P (in mW) = U (in V) x I (in mA)

12. **HAVE YOUR TIMER READY. Now close valve 3 and immediately open valve 4. Wait until the first gas bubbles appear at the bottom of the graduated cylinder and then start your timer.**

13. **Read the level of the NaOH meniscus in your inverted graduated cylinder every 30 min and write the numbers down in the Results Table 2 below.**

Results Table 2

Time "t" (min)	NaOH level in graduated cylinder (ml)	Net Volume Change* (ml)
0	_____ (Start level)	0
30		
60		
90		
120		

* Net volume change = Level at time "t" - Start Level

14. **Make sure that the NaOH level in the glass beaker does not become higher than 15-25 mm. Use a drain tubing and plastic syringe to drain the rising NaOH level in the glass beaker into an empty glass bottle from time to time.**
- your instructor will give you a ***demo*** to show you how to perform this step!

15. **Before the NaOH in the graduated cylinder becomes completely emptied into the glass beaker by the accumulating hydrogen gas, close valve 4 and keep valve 3 closed. Now quickly but savely refill the graduated cylinder with 20% NaOH solution again (*) and write down the meniscus of your new NaOH level in the cylinder in the section below.**
- **PERFORM STEP 14 OVER A SINK AND WEAR GOGGLES, A LAB COAT AND GLOVES DURING THIS STEP!** Keep in mind that NaOH is an extremely caustic

chemical! Immediately wipe off eventual spills on your bench or on other materials with plenty of water and wet tissues!

- * your instructor will give you a demo to show you how to perform this step!

New level (t = _____ min) = _____ ml

16. **Once you have re-assembled your refilled inverted graduated cylinder into your glass beaker, slowly open valve 4 again. Continue with your hydrogen gas measurements as described in step 13.**
    - make sure that you add this new hydrogen gas volume to the hydrogen gas generated in the previous cylinder set up within the 30 min time period

17. **Once you observe that the calculated net volume change in your inverted cylinder is declining, i.e. that the maximum hydrogen production has been reached, close valve 4 and immediately open valve 3. Wait a couple of minute until your fan starts spinning again. Record the total incubation time for the bacterial culture below.**

Total incubation time : _____ hours

18. **Now use your voltmeter and measure the voltage and current on your fuel cell assembly at the 5 fuel cells stacks again (see step 12) and record these numbers in the Results Table 3 below.**

Results Table 3

Fuel Stack #	Voltage (V)	Current (mA)	Power (mW)*
1			
2			
3			
4			
5			

* Calculated electrical power (P) of fuel cell = Voltage (U) times Current (I)

P (in mW) = U (in V) x I (in mA)

19. **Carefully disconnect the soda lime-filled plastic cartridge of your biohydrogen work station and close cap both ends. Now carry the cartridge in horizontal position over to a table balance. Remove both caps, weigh the cartridge (without caps) and record the weight and the total incubation time of the bacterial culture below.**

Cartridge weight (after _____ hours): CW = _____ g

Total bacterial cultivation time: _____ hours

20. **Open one of the side caps of the spinner flask and pipette 1 ml of the bacterial culture into a clean 1ml plastic cuvette. Go to the spectrophotometer and measure the absorption of this culture at a wavelength of 550 nm. Record the absorbance (A_{550}) below.**

- use a second plastic cuvette filled with 1 ml of TYP medium as blank

A_{550} (after ___ hours incubation time) = _____

21. **Label an empty 15 ml conical centrifugation tube with your name's/team's initials and then weigh this tube (with the screw cap on) on a fine balance. Record the empty weight W_e (tube) of the tube below.**

    W_e (tube) = _____ g

22. **Go back to your bioreactor, open one of the side caps of the spinner flask again and then pipette 10 ml of you bacterial culture into the previously weighed 15 ml centrifugation tube (step 20). Tightly screw cap and then centrifuge this tube at 3,300 x g for 10 min in a table centrifuge.**
    - Make sure that you properly counter balance the rotor before starting the centrifuge!

23. **Take the tube out of the rotor and decant the supernatant into a biohazard container (liquids). Remove residual liquid above the bacterial pellet with a pipette and discard this liquid as well. Weigh the tube with you bacterial pellet (with the screw cap on) on a fine balance. Record the wet weight W_w (tube) of the tube below.**

    W_w (tube) = _____ g

24. **Hand this tube to your instructor who will dry the bacterial pellet in the tube in a heated incubator until the next lab meeting.**

25. **Tightly close cap the spinner flask again and leave the spinner flask with your bacterial culture in the 37°C water bath with valve 1 closed and valve 2 open.**
    - the assembly will be dismantled by a lab technician after the end of the lab!

Lab Activity 2: Determine the maximum hydrogen production rate of the bacterium used in your biohydrogen reactor platform and calculate the maximum power output of your fuel cell.
In this lab activity you will analyze and evaluate the experimental results measured during lab activity 1. You will calculate the hydrogen production rate (in ml H_2/l/h and mmol H_2/g dry/h) for

the assigned carbon feedstock under study, determine the amount of carbon dioxide absorbed by the CO_2 cartridge during the time period of the experimental run, and calculate the maximum power output of your hydrogen fuel cell. You will further familiarize yourself with the concept of hydrogen yield calculation based on provided lab data.

Day 0

1. **Use the hydrogen gas data collected in Results Table 2 and calculate the hydrogen production rate in millimeters hydrogen gas produced per liter volume per hour (ml H_2/l/h) for each of the measured 30 min time intervals. Record these calculated numbers in the Results Table 4 below.**
   - use following example calculation and formula below.
   - assume we observed a volume change from 87 ml to 154 ml in the inverted NaOH cylinder over a time period of 30 min with a 600 ml (0.6 l) bacterial culture then the hydrogen production rate (HPR) can be calculated as following.

   HPR = 154 ml – 87 ml / (0.5 h x 0.6 l) = 223.3 ml H_2 / h / l

2. **Determine at which time point (t_{Tot}), starting from the time of bacterial inoculation, the bacterial culture reached its maximum hydrogen production rate (HPR_{max}).**

   t_{Tot} (HPR_{max}) = _____ hours

Results Table 4

Total time "t_{Tot}" (h)	**Time "t"** (min)	**NaOH level (cylinder)** (ml)	**Net Volume Change*** (ml)	**Hydrogen Production Rate** (ml H_2/h/l)
0				
____	0	_____ (Start level)		0
____	30			
____	60			
____	90			
____	120			

* Net volume change = Level at time "t" - Measurement Start Level

3. **Determine the amount of carbon dioxide captured (absorbed) by the soda lime cartridge over the time period of the experiment (= "ΔCO_2").**
   - see Steps 3) and Steps 18) for the CW numbers

   ΔCO_2 = CW (after ___ h) – CW (t=0) = _____ g

4. **Calculate the number of moles carbon dioxide generated by the fermenting bacterium over the time period of the experiment. Show your work below.**
   - Molecular weight (CO_2) = 44 g/mol

_____ moles CO_2

5. **Calculate the volume of CO_2 gas (in milli-meters) the bacterium produced over the experimental time period. Show your work below.**
   - Hint: 1 mol of an ideal gas (at NPT) has a volume of 22.4 liters

_____ ml CO_2

Day 7

6. **Receive the 15 ml conical tube with the dried pellet of your previously collected and sedimented bacterial sample (see Lab Activity 1; Step 23) from your instructor. Weigh this tube (with the screw cap on) on a fine balance and record the weight of the tube (W_D) below.**

W_D (tube) = _____ g

7. **Calculate the bacteria dry weight "$DW_{Bacteria}$" by subtracting the empty weight of the tube (W_E) from the "bacteria dry weight" tube (W_D) and record this number below.**

$DW_{Bacteria} = W_D - W_E$ = _____ g (per 10ml)

8. **Convert the number received in Step 7 into "bacteria dry weight per liter" (g dry/l) and record below.**

$DWL_{Bacteria} = DW_{Bacteria} \times 100$ = _____ g / l

9. **Now use the maximum hydrogen production rate (in ml H_2/h/l) you previously calculated (see Activity 2; Step 2) and convert this number into the maximum hydrogen production rate in mmol hydrogen gas produced per gram dry weight per hour (mmol H_2/g_{dry}/h) for your biohydrogen experiment. Show your work below.**

10.

HPR_{Max} = _____ mmol H_2 / g dry / h

11. **Compare this number with other hydrogen production rates published in literature and critically evaluate the outcome of your biohydrogen production experiment. Discuss your findings below.**
   - your instructor will supply you with relevant scientific literature and/or relevant links to retrieve this information

12. **Assuming you were assigned to work with glycerol (220mM) as bacterial feedstock during the biohydrogen experiment as described above. You collected a total amount of 2.3 liters of hydrogen gas (H_2) over a time period of 24 hours with a 600ml bacterial culture. What was your biohydrogen yield (in mol H_2 per mol glycerol) for this experiment? What is the theoretical maximum hydrogen gas yield for glycerol? Show your work.**
(Molecular weight for glycerol = 92.09 g/mol; Molecular weight for H_2 = 2 g/mol;
1 g H_2 (at 26^{o}C, 1 atm) = 11.3 liter)

13. **Assuming you were assigned to work with glucose (1%) as bacterial feedstock during the biohydrogen experiment as described above. You collected a total amount of 1.1 liters of hydrogen gas (H_2) over a time period of 24 hours with a 600ml bacterial culture. What was your biohydrogen yield (in mol H_2 per mol glucose) for this experiment? What is the theoretical maximum hydrogen gas yield for glucose for your bacterium? Show your work.**
(Molecular weight for glucose = 180 g/mol; Molecular weight for H_2 = 2 g/mol;
1 g H_2 (at 26^{o}C, 1 atm) = 11.3 liter)

Lab #4 - Microalgae Cultivation & Testing

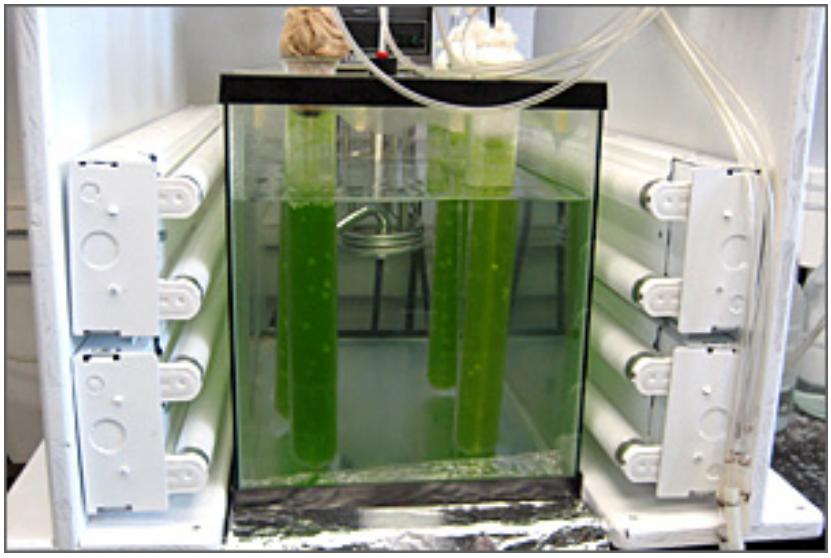
Objectives

1. Learn how to set up and operate a vertical bubble column algae photobioreactor
2. Learn how to measure irradiance (light intensity)
3. Learn how to measure oxygen and carbon dioxide concentrations
4. Learn how to measure pH value
5. Learn how to take algae samples for measurement of:
   - Optical density
   - Dry algae biomass
   - Algae oil content
6. Learn how to calculate and plot photosynthesis rate and algae biomass productivity

Introduction

Alternative energy sources in the form of biofuels will play a critical role in the near future to achieve a carbon neutral and sustainable liquid fuel economy with less dependency on currently used fossil fuels. Most current biofuel production strategies, most namely bioethanol from plant starch and biodiesel from plant oils, are highly land and other resource, e.g. fertilizer, intensive and therefore not sustainable. However, biofuels derived from microalgae biomass represent an attractive and viable alternative to agricultural crops for biomass and biofuels production. Approximately 50% of all primary production of biomass on planet earth is performed by photosynthetic algae. Even though many challenges remain to be addressed before large scale production of algal biofuels will become a reality, cultivation of microalgae in controlled environments of photobioreactors and other cultivation vessels offer important advantages over conventional agriculture of crops (see **Figure** below).

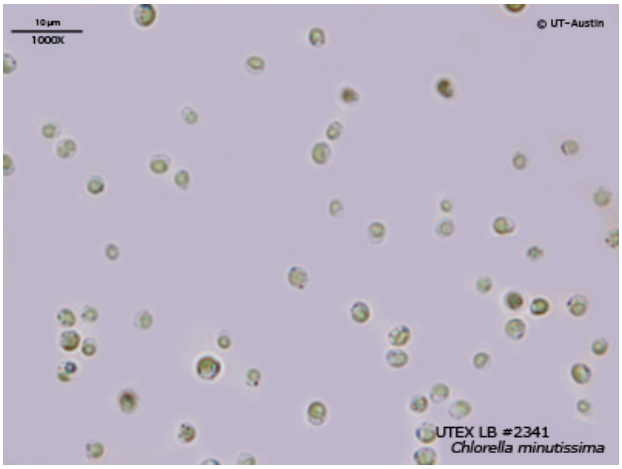
Vertical bubble column tubular photobioreactor



From: http://web.biosci.utexas.edu/utex/bulkcultures.aspx

Microalgae are microscopically small photosynthesizing life forms. The average cell size of these single celled life forms is usually around 5-10 µm (see **Figure** below).Due to their small size they can divide very rapidly under suitable growth conditions. Therefore they show very high growth rates leading to high biomass within short periods of time.

The microalgae *Chlorella minutissima*



Doubling times of algae can be on the order of only 4 to 24 hours. In comparison to agricultural plants, microalgae have a much higher photosynthesis efficiency. Lastly, many microalgae respond with a very high lipid (oil) content under certain growth conditions, which make them good candidate organisms for liquid fuel production. The oil content of some algae can exceed 50% (see **Table** below), while most agricultural crops currently used for biodiesel production, such as canola or soy, produce oil yields of less than 5%.

Table: Oil content of selected microalgae species

Species	**Oil Content** (% of dry weight)	**Reference**
Botryococcus braunii	29-75	Sheehan et al. (1998) Metzger and Largeau (2005)
Chlorella sp.	29	Sheehan et al. (1998) Chisti Y. (2007)
Chlorella protothecoides	15 (phototrophic) 55 (heterotrophic)	Xu et al. (2006) Miao et al. (2006)
Chlorella minutissima	23-45	Chisti Y. (2007)
Nannochloris sp.	31 (6-63)	Ben-Arrotz and Tomabene (1985) Negoro et al. (1991) & Sheehan et al. (1998)
Nannochloropsis sp.	46 (31-68)	Sheehan et al. (1998) Hu et al. (2006)
Scenedesmus sp.	46	Sheehan et al. (1998)
Phaeodactylum tricornutum	31 5.4-10.7% 9.4%	Sheehan et al. (1998) Siron et al. (1989) Wawrik et al. (2010)

Microalgae are microscopically small members of the Subkingdom algae. Algae (sing. alga) can be described as eukaryotic life forms that perform a light-dependent, carbon dioxide gas-consuming and oxygen gas (O_2)-producing process called photosynthesis. During photosynthesis water (H_2O) is consumed and the carbon dioxide gas (CO_2) taken up by algae is used as carbon source to synthesize 6-carbon sugar molecules, most importantly glucose and fructose ($C_6H_{12}O_6$).

Net equation of photosynthesis
(Write down the net equation of photosynthesis below)

_____ + _____ + light ⟶ _____ + _____

The single celled microalgae rely on the photosynthesis pigment chlorophyll a and accessory pigments to collect the energy of light and (with the exception of the Euglenophyta) have a cell wall made up from the glucose-made polymer cellulose. Despite great similarities with green plants, they differ from these photosynthetic eukaryotic life forms in several important aspects:

1. they are single celled life forms which are lacking true tissue differentiation;
2. they do NOT have a well-structured vascular system (no xylem/phloem);
3. they only express very simple reproductive structures;

- depending on the algae species either the whole organism serves as gamete or uni-cellular structures (= gametangia) may produce the gametes;

Some microalgae, for example *Chlamydomonas reinhardtii* and *Euglena*, show one or more **flagella**, which are long cell protrusions important for motility and movement, while others such as *Chlorella sp.* and *Monodus subterraneus* are non-flagellated. Microalgae contain one or more **chloroplasts** with enclosed thylacoids which is the cellular place of photosynthesis. Many microalgae contain a often reddish colored spot within their cells called the **pyrenoid**. The pyrenoid is a dense, proteinaceous area that is associated with synthesis storage of the glucose polymer starch. Microalgae show differently shaped **mitochondria**, which can show lamellar, discoid or tubular cristae. Microalgae are metabolically very versatile and their metabolism can be either **phototrophic or heterotrophic**. Although most algae are phototrophs, microalgae, such as Chlorella, can survive with external organic compounds as carbon- and/or energy source. The uni-cellular microalgae reproduce via a bacteria-like, rapid cell division process called binary fission. Algae are **classified into seven divisions** based on several important cellular properties which are:

a. type of cell wall
b. chemistry and composition of cell wall
c. type of metabolism and storage products
d. type of chlorophyll molecules and accessory pigments
e. presence or absence of flagella (motility)
f. cell or body morphology
g. reproductive structures

The **seven algae divisions** are:

I. **Chlorophyta** (Green algae)
- contain chlorophylls *a* and *b*
- starch is stored inside the chloroplast
- the mitochondria have flattened cristae
- some members have flagella
- conservatively, between 9,000 and 12,000 species
- includes the BTEC293-relevant classes:

Class: Chlorophyceae
- for example: *Chlorella minutissima*

Class: Eustigmatophyceae
- for example: *Nannochloropsis sp.* & *Monodus subterraneus*

II. **Charophyta** (Stoneworts or brittleworts)
III. **Euglenophycophyta** (Euglenoids)

IV. **Chrysophycophyta** (Golden-brown algae, diatoms)
V. **Phaeophycophyta** (Brown algae, kelp)
VI. **Rhodophyta** (Red algae)
VII. **Pyrrhophyta** (Dinoflagellates)

In this lab section you will learn how to cultivate the microalgae *Chlorella minutissima* and *Monodus subterraneus* in a closed bioreactor environment called a bubble column photobioreactor. You will further learn how to analyze important cultivation parameters, most namely optical density, dry algae biomass, oil content, and to correlate these with nutrients, pH and/or CO_2 concentration. Different student teams will be assigned by the instructor to examine the impact of different cultivation parameters, most namely nitrogen content and CO_2 concentration, on algae biomass productivity.

Procedure

Lab Activity 1: Prepare a bubble column photobioreactor and inoculate with an assigned microalgae culture

In this lab activity each student team will prepare a 1,000ml algae culture with their assigned algae species by inoculating the algae in a 1 liter tubular bubble reactor tube following the steps below. Your instructor will tell with which microalgae species you will be conducting your experiments.

Team A: *Monodus subterraneus*
Team B: *Chlorella minutissima*

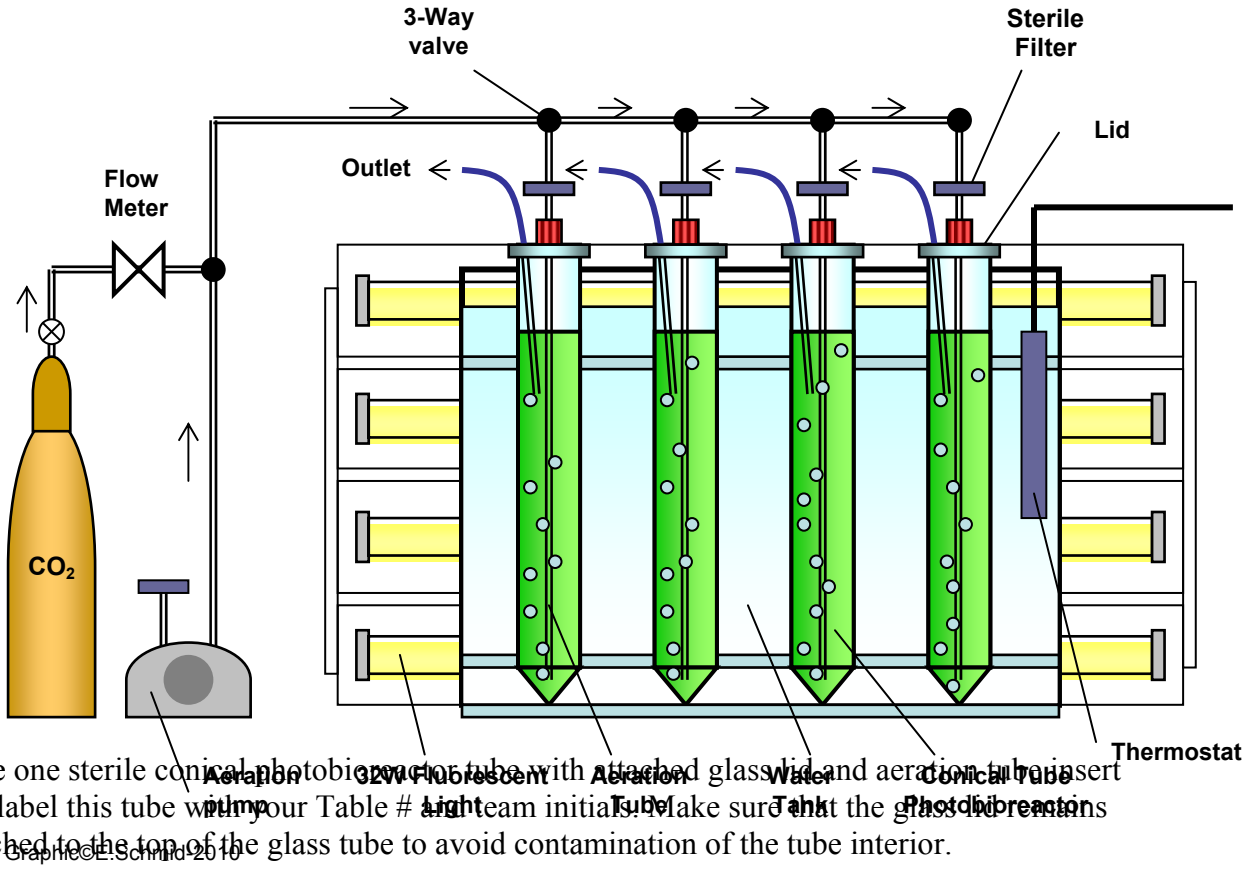
Wear latex or vinyl gloves while performing following steps to minimize the risk of cross contamination of your algae culture!

Day 0

1) Spray and wipe the surface of the work area with 70% alcohol. Wait 10-15 minutes before starting your bench work.

2) Familiarize yourself with the individual components of the bubble column photobioreactor assembly (see **Figure** below) with you will be working with in this lab section, including conical glass tubes (with glass lids and inserted aeration tube), gas inlet, thermostat, CO_2 flow meter and aeration pump.

Vertical bubble column tubular photobioreactor set-up



Graphic©E.Schmid-2010

3) Take one sterile conical photobioreactor tube with attached glass lid and aeration tube insert and label this tube with your Table # and team initials. Make sure that the glass lid remains attached to the top of the glass tube to avoid contamination of the tube interior.

4) Remove the aluminum cap from the silicone tube inlet of the aeration tube and attach one sterile Acrodisc filter (0.22m) to the end of this tube. Make sure that you perform this step under sterile conditions, i.e. do not touch the tube ending with dirty hands! Wear alcohol-disinfected vinyl or latex gloves while doing this!

5) Carefully pour 800 ml of sterile algae growth medium into the sterile 1 liter conical photobioreactor tube. Close lid the tube again as soon as you are done pouring the medium in to avoid contamination. Team work during this important step!

6) Inoculate your assigned algae by pouring 200 ml of your assigned algae stock culture (Chlorella or Monodus) into the 1 liter conical photobioreactor tube. Immediately close lid the photobioreactor tube to avoid cross contamination during this inoculation step.
   - TEAM WORK DURING THIS IMPORTANT STEP!
   - the inoculation density in your tube should be between 5 – 10 x 10^6 cells/ml

7) Gently mix the closed lid algae cultivation tube content by swirling (without spilling over).

8) Label one of the three 50ml plastic centrifugation tubes with your team's initials and with "Day 0". Then weigh the empty 50 ml collection tube (with screw cap on) on a table balance. Record the empty weight of the tube (W_{emtpy}) in your lab journal.

$$W_{emtpy} = \_\_\_\_\_ \text{ g}$$

9) Following, remove 2 x 25 ml of the freshly inoculated algae culture from the tube with the help of a sterile 25 ml pipette (plus pipettor) and transfer the algae sample into the labeled 50 ml plastic tube. Place tube in plastic rack for later analysis (see section below)
   - USE A NEW STERILE 25 ML PIPETTE FOR THE SECOND REMOVAL OF THE ALGAE SAMPLE!

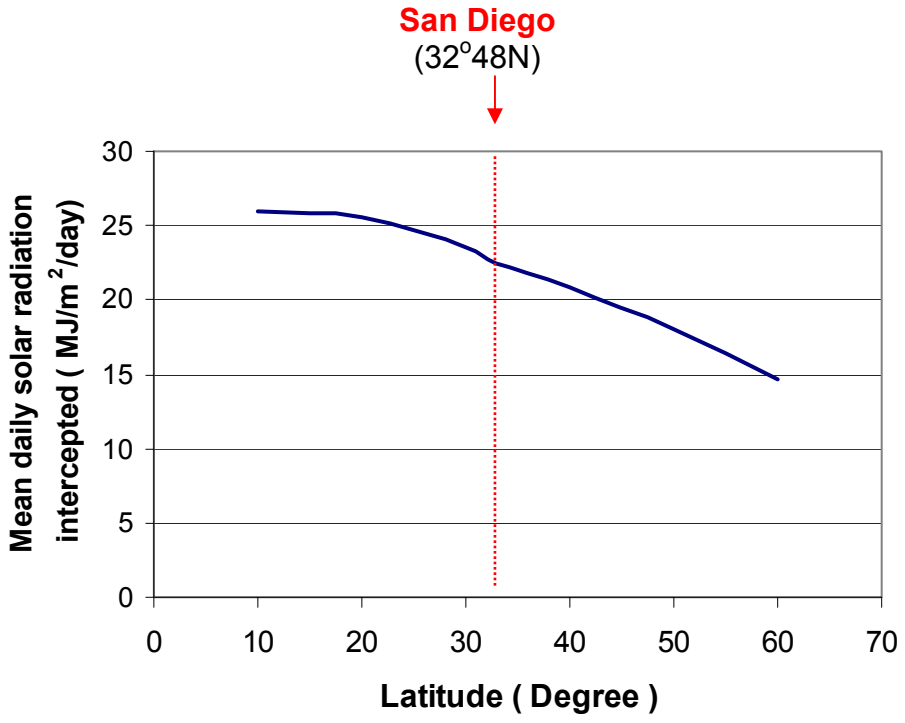
10) Carefully insert the lid closed photobioreactor tube into the allocated slot of the water-filled tank of the photobioreactor assembly. Make sure that the temperature of the thermostat of the water tank is adjusted to 25°C.

11) Connect the end of the (silicone) tubing of the carbon dioxide-air mixture aeration line to the aeration tube-mounted Acro-disc filter. Turn on the aeration pump and start aerating the interior of the photobioreactor tube with filter sterilized 5% CO_2 in air at an adjusted aeration rate of about 50 ml/min.
   - YOUR INSTRUCTOR WILL SHOW YOU HOW TO SET UP AND ADJUST THE SETTINGS OF THE CONTROLLER UNIT

12) Turn on the fluorescent light assembly next to the photobioreactor tubes. Use a Quantum scalar irradiance meter and measure the irradiance ("light intensity") at the surface of the photobioreactor tank. Record this number and the unit below.

Irradiance: _____ W/m^2

13) Compare this irradiance number measured on the surface of the photobioreactor tank assembly with the mean daily solar radiation of San Diego, California (Latitude: 32°48'N). Use the **Figure** below to find the correct answer.
   - Solar radiation is often referred to as insolation



14) Write the "Mean Daily Solar radiation intercepted (MDSR)" number for the San Diego region down in the blank space below.

$$MDSR_{SanDiego} = \_\_\_\_\_ \ MJ/m^2/d$$

15) Calculate the "Mean Simulated Solar Irradiance (MSSI)" number for San Diego in "W/m^2" from the mean daily solar radiation number using the following information below. Show your work in the section below.
- 1 $MJ/m^2/d = 0.01157\ kJ/m^2/s$
- 1 J = 1 Ws

$$MSSI_{SanDiego} = \_\_\_\_\_ \ W/m^2$$

16) Now, calculate the "Mean Light Irradiance" number for San Diego in "$\mu E/m^2/s$" from the MSSI number above using the following information below. Show your work in the section below.
- 1 Einstein (1 E) = 2.72×10^5 Ws

Mean light irradiance (San Diego) = _____ $\mu E/m^2/s$

In biology and especially in algae cultivation, the most common units of light irradiance are "*u*Einstein $/m^2$ /s." An "Einstein" (1 E) is equivalent to 1 mole of photons, the fundamental unit of light. You will recall from chemistry that one "mole" is equivalent to 6.02×10^{23} particles of a substance, such as atoms, molecules or photons. In this case the units are "microEinstein". One *u*Einstein is the millionth part of a mole of photons (1 *u*Einstein = 6.02×10^{17} photons).

17) Incubate the inoculated algae cultures in the tubes at the "Mean Simulated San Diego" irradiance ("light intensity") of 250 W/m^2 for 7 days. Program the timer for the fluorescent light switch to a "natural" day-night cycle, which is: 16 hours light – 8 hours night.

Lab Activity 2: Algae Cell Counting, Optical Density & Algae Dry Biomass Productivity Analysis

Day 0

1) Take a small (15ul) aliquot of the collected algae sample from Step 9) and pipette the sample into the gap of a previously assembled Neubauer counting chamber (hemocytometer). Place the chamber on the stage of a compound light microscope and count the cells at a total magnification of 100 times (TM: 100x). Calculate the cell number (in cells per liter) and write it into the Results Table below.
   - make sure that you gently mixed the algae culture before removing the aliquot

2) Pipette 1 ml of the collected algae sample from Step 9) into a clean 1 ml plastic cuvette and measure the optical density of this (undiluted) sample in a spectrophotometer at a set wavelength of 625nm. Use 1 ml of the corresponding algae culture medium as blank. Record the absorbance reading as "Day 0" in your lab journal.
   - in case your absorbance reading is higher than 1.8, dilute your collected algae sample by a factor of 5 in distilled water (= 0.2 ml sample + 0.8 ml dH_2O)
   - repeat Step 2)

3) Place the screw capped 50 ml plastic collection tube "Day 0" into the rotor of a table centrifuge, counter balance the rotor with another (identical weight) collection tube and centrifuge the algae for 5 minutes at 3,000 x g.

4) After centrifugation decant the supernatant and add 50 ml of physiological saline solution to the algae pellet.

5) Gently resuspend the algae pellet in the saline solution with a Pasteur pipette and repeat Step 3).

6) After this washing step, decant the supernatant and place the collection tube in a heated incubator (adjusted to 42°C). Dry the algae pellet over the next 2-3 days.
   - at the next lab meeting, weigh this tube with your dried algae sample (with screw cap on) on a table balance and record the weight of the tube (W_{algae}) below.

$$W_{algae} = \_\_\_\_\_ \text{ g}$$

- now calculate the algae dry biomass weight (BM_{dry}) using the formula below and write this number in the corresponding column of the Results Table 1 below.

$$BM_{dry} = W_{algae} - W_{emtpy*} \text{ (in g)} \qquad * \text{(see lab activity 1, step 8)}$$

Results Table 1 – Bubble Column Algae Photobioreactor Experiment

Time (Days)	**Cell Count** (cells/liter)	**Absorbance** (A_{625nm})	**Algae Dry Biomass Weight (BM_{dry})** (g)
0			
7			

Day 7

1) At the beginning of the next lab meeting (Day 7), go to your conical algae cultivation tube inserted in the bubble column photobioreactor assembly and observe the culture changes since last week, especially regarding color change and density. Check the aeration flow rate and irradiance (light intensity).
   - Did the color of the algae culture change? Yes _____ No _____

2) Mist spray the aluminum foil-covered lid area of your algae cultivation tube with a 70% ethanol solution. Wait 10 minutes.

3) In the meantime, use a marker pen and label one empty 50 ml centrifugation tube with your team's initials and with 'Day 7". Then weigh this tube (with screw cap on) on a table balance. Record the empty weight of the tube in your lab journal.

4) Now, open the lid of your algae cultivation tube and remove one 2 x 25.5 ml of the algae culture using a sterile 25 ml pipette (with pipette pump) and transfer both withdrawn algae culture samples into the previously weighed 50 ml Plastic collection tube "Day 7". Tightly screw cap the centrifugation tube and place in rack.
   - USE A NEW STERILE 25 ML PIPETTE FOR THE SECOND REMOVAL OF THE ALGAE SAMPLE!

5) Immediately close lid the photobioreactor tube again to avoid cross contamination during this sample collection step.

6) Take a small (15ul) aliquot of the collected algae sample from Step 4 above) and pipette the sample into the gap of a previously assembled Neubauer counting chamber (hemocytometer). Place the chamber on the stage of a compound light microscope and count the cells at a total magnification of 100 times (TM: 100x). Calculate the cell number (in cells per liter) and write it into the Results Table above.
   - make sure that you gently mixed the algae culture before removing the aliquot

7) Pipette 1 ml of the collected algae sample from Step 7) into a clean 1 ml plastic cuvette and measure the optical density of this (undiluted) sample in a spectrophotometer at a set wavelength of 625nm. Use distilled water as blank. Record the absorbance reading as "Day 1" in your lab journal.
   - make sure that you gently mixed the algae culture before removing the aliquot
   - in case your absorbance reading is higher than 1.8, dilute your algae sample by a factor of 5 (or higher) in distilled water (= 0.2 ml sample + 0.8 ml dH_2O)
   - repeat Step 7)

8) Place the screw capped 50 ml plastic collection tube "Day 7" into the rotor of a table centrifuge, counter balance the rotor with another (identical weight) collection tube and centrifuge the algae for 5 minutes at 3,000 x g.

9) After centrifugation decant the supernatant and add 50 ml of physiological saline solution.

10) Gently resuspend the algae pellet in the saline solution with a Pasteur pipette and repeat Step 8).

11) After this washing step, decant the supernatant and place the collection tube in a heated incubator (adjusted to 42-45^{o}C). Dry the algae pellet over the next 2-3 days.
   - leave the screw cap very loose to allow evaporation during the drying process

12) At the next lab meeting, weigh this tube with your dried algae sample (with screw cap on) on a table balance and record the weight of the tube (W_{algae}) below.

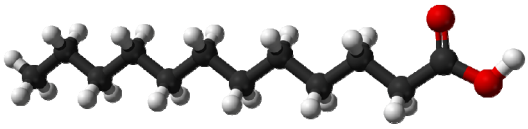
$$W_{algae} = \_\_\_\_\_ \text{ g}$$

   - now calculate the algae dry biomass weight (BM_{dry}) for Day 7 using the formula below and write this number in the corresponding column of the Results Table above.

$BM_{dry} = W_{algae} - W_{emtpy*}$ (in g) * (see lab activity 1, step 8)

Lab Activity 3: Spectrophotometric Algae Oil Content Analysis

In this lab section you will analyze the oil content of a sample of an algae culture growing in a closed bubble column photobioreactor. You will withdraw a small sample after 7 days of culturing an algal strain, treat the harvested algae cells to release the algae oil content, chemically treat the oils to release the fatty acids (sapinification) and use a spectrophotometric method to measure the amount of fatty acids stored by the algae under the chosen cultivation conditions. You will use a standard curve established with the C12 fatty acid lauric acid (dodecaneoic acid → see Figure below) to determine the approximate amount of oils present in your cultivated algae species.



Day 7

1) Go to the bubble column photobioreactor assembly and mist spray the aluminum foil-covered lid area of your algae cultivation tube with a 70% ethanol solution. Wait 10 minutes.

2) In the meantime, use a marker pen and label one clean 15 ml conical centrifuge tube with "A-7d", a second 15 ml centrifuge tube with "S1-7d"and a third centrifuge tube with "S2-7d". Place the labeled tubes in a plastic rack on your bench.

3) Now, open the lid of your algae cultivation tube and remove 10 ml of your algae culture using a sterile 10 ml pipette (with pipette pump) and transfer the sample into the 15 ml conical centrifuge tube labeled "A-7d". Close cap and place this tube into the rotor of a table centrifuge.
   - MAKE SURE THAT YOU COUNTER BALANCE THIS TUBE BEFORE START OF CENTRIFUGATION!
   - Immediately close lid the photobioreactor tube again after sample removal to avoid cross contamination during this sample collection step.

4) Centrifuge the collected algae sample in reaction tube "A-7d" at 3,500 x g for 5 minutes.

5) Decant the supernatant and place the tube with the algae pellet back into the tube rack.

6) Now pipette 1.0 ml of Saponification Buffer to the algae pellet in the "A-7d" tube and also to the 15 ml centrifuge tubes previously labeled as "S1-7d" and "S2-7d".
   - use and thoroughly resuspend the algae pellet in the buffer

7) Pipette 0.05 ml of canola oil into the 15 ml centrifuge tube "S1-7d" and 0.2 ml of canola oil into the tube labeled "S2-7d". Mix the contents by gently flicking the tubes and place both tubes back into the rack.

8) Take tube "A-7d" and quickly resuspend the algae pellet in the saponification buffer with the help of a fine pointed Pasteur pipette by repeated "up-and-down" pipetting. Place the algae suspension on ice.

9) Add 50 mg of dry diatomaceous earth (DE) powder to the tube "A-7d" and mix by agitating the tube.

10) Use the Pasteur pipette from step 8 and transfer the algae cell suspension into a clean porcelain mortar. Now use a porcelain pestle and disrupt the cell integrity of the algae suspension by strongly grinding the suspension with the help of the pestle for 3-5 minutes.

11) Confirm successful algae cell lysis by observing wet mounts of a small aliquot of the ground algae suspension (from tube "A-7d") under a compound microscope at 400x total magnification. Successful cell lysis is indicated by cell debris and loss of the (green) chlorophyll in the cells ("cell bleaching").
- in case of incomplete or failed cell disintegration (cell lysis) repeat Step 10 for another 3-5 minutes.

12) Transfer the lysed algae cell suspension from the mortar into a clean 15 ml conical centrifuge tube labeled ("A-7d"). Place this tube and the two other centrifuge tubes labeled "S1-7d" and "S2-7d" into a rack and incubate all three tubes in a boiling water bath for for 30 min. Remove the tubes from the water bath every 5 minutes and vortex them for 30 seconds.
- this "saponification" step (think of soap) hydrolyzes the ester bonds of the membrane lipids and of the stored triglycerides (oils) into glycerol and free fatty acids

13) Take the samples out of the water bath, place them in your tube rack and let them cool down on the bench.

14) Pipette 1.0 ml of Neutralization Reagent and 1.0 ml of Copper Reagent to each of the saponified samples in your three 15 ml centrifuge tubes.

15) Vortex all three tubes for 2 minutes and then transfer the tube contents into three super-clean 15 ml glass tubes (with screw cap), and label them "A-7d", "S1-7d" and "S2-7d". Place the rack with your three labeled glass tube under a fume hood.

16) Use a glass pipette (with pipettor) and pipette 2.5 ml of chloroform to each glass tube. Vigorously vortex each tube (WITHOUT screw caps on!) for 2 minutes under the fume hood. **Important: This and the following steps have to be performed under a running fume hood! Chloroform is a suspected human carcinogen! So please work with extreme caution and with utmost safety on your mind!**

17) Tightly screw-cap the tubes under the hood and then centrifuge the tubes at maximum speed for 5 minutes in a table centrifuge.
- MAKE SURE THAT THE TUBES ARE COUNTER BALANCED BEFORE STARTING THE CENTRIFUGE!
- this steps separates the organic (chloroform) phase from the aqueous phase

18) Take the tubes out of the centrifuge, place back in rack and place under the fume hood. Now carefully transfer 2.0 ml of the organic phase (AT THE BOTTOM OF THE TUBE !) into a new clean 15 ml glass tube. Make sure that you do NOT transfer any of the aqueous (upper) phase during this step.
- use a clean, fine pointed glass Pasteur pipette for this critical step!
- the aqueous phase contains the remaining (unreacted) copper ions which will produce false positive signals during the following detection step

19) From each tube containing the collected organic phases of samples "A-7d", "S1-7d" and "S2-7d", pipette 0.5 ml into two separate new 15 ml glass tubes. Label them and add 0.5 ml 2-butanol or 0.5 ml Developing Reagent according to the pipetting scheme below.
- use a clean 0.5 ml Hamilton pipette for these volume transfers
- a yellow color should appear over time in the samples with the developing reagent proportional to the amount of free fatty acids present in the sample.
- keep the remaining volume of the chloroform fraction in each tube for later further analysis of the algae fatty acids using different methods.

	A-7d (0.5ml)	A-7d (0.5ml)	S1-7d (0.5ml)	S1-7d (0.5ml)	S2-7d (0.5ml)	S2-7d (0.5ml)
2-Butanol (0.5ml)	+	-	+	-	+	-
Developing Reagent (0.5ml)	-	+	-	+	-	+
Sample #	**1**	**2**	**3**	**4**	**5**	**6**

- Generally: the 2-butanol samples #1, 3 and 5 serve as blank during the spectrophotometric analysis of the samples #2, 4 and 6

20) Wait 10 minutes for the chemical reaction with the free acids to take place.

21) Now start with sample #1 and transfer the content of this tube into a clean 1.0 ml glass cuvette using a clean glass Pasteur pipette and blank the spectrophotometer with this sample at a wavelength of 440nm. Decant the cuvette content into a biohazard container for organic wastes.

22) Transfer the tube content of sample #2 into the empty glass cuvette from Step 21 (again using a clean glass Pasteur pipette) and measure the absorbance of this sample in a spectrophotometer at a wavelength of 440nm.

23) Repeat Steps 21 and 22 for the rest of the samples, i.e. samples 3-6.

24) Record your absorbance readings in the Results Table 2 below.

Results Table 2 – Absorbance readings of oil samples

Tube	**Absorbance (440 nm)**
A-7d	
S1-7d	
S2-7d	

25) Plot the data shown below with the absorbance at 440nm (A_{440}) as a function of different fatty acid (sodium laurate) concentrations, and use the established graph as a standard curve to determine the µmoles of fatty acids present in your algae (A-7d) and two Oil Standards (S1/S2-7d).
- make sure that you use the correct headings and appropriate units.

A_{440}	0						
FA Conc. [µmoles]	0	0.06	0.1	0.195	0.34	0.51	0.75

Per 1.0 ml

26) Record your retrieved experimental data in the table below and calculate the oil content of your algae in mg oil per liter per day (mg oil/l/d). Compare your oil productivity number with published data with the same or other algae.
- When doing your calculations consider following conversion factors and units:
  Molecular weight of average fatty acid = 250 g/mol
  Molecular weight of average oil = 858 g/mol

Algae Species	***Chlorella minutissima***	***Monodus subterraneus***
µmoles FA/2 ml sample		
mmoles FA/l culture		
mg oil/l culture		
mg oil/l/d		

Evaluation

1. How effective were the methods which you used?
   a. Algae cultivation? _____
   b. Cell Counting? _____
   c. Optical Density? _____
   d. Oil Analysis? _____

2. What is the significance of using dry algae mass as the measurement of concentration?

3. What were the limitations of the used equipments?
   a. Photobioreactor? _____
   b. Spectrophotometer? _____

4. What were the sources of error?

5. What possible improvements could be made to the experiment?

6. What ideas do you have for further work?

7. What is the economic importance of the process which you are studying and the calculations which you will make?

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