

## **Growing Cells and DNA Extraction**

### **Goals**

1. Become familiar with the process of growing bacteria
2. Get to know the DNA extraction process
3. Perform miniprep in the lab

### **Keywords**

1. Growth stages
2. Growth rate and generation time
3. Growth curve
4. Macronutrients and micronutrients
5. Defined and complex mediums
6. Techniques for measuring bacteria
7. Spectrophotometer
8. Inhibitors
9. Activators
10. Antibiotics

### **Summary**

Extensive description of the stages required for microbial growth: lag phase, log phase, stationary phase, and death. Generation time and growth rate calculations in the log phase. Description of the types of factors which influence growth, with an emphasis on necessary nutrients and oxygen. Techniques for measuring bacterial concentration- direct and indirect, pros and cons of each technique. Going over the mini-prep protocol.

### **Lesson Plan**

Part 1- Theoretical lecture: growth stages, bacterial growth curve, influence of different factors on growth, measuring bacterial concentration, extraction of DNA from bacteria (after transformation).

-allow for about 50 minutes

Part 2- Lab activity: the students will perform mini-prep

-allow for about 1 1/2 hours

### **Description**

#### **Microbial growth stages:**

- Bacterial growth refers to an increase in the number of bacteria in the culture
- The growth occurs as a result of the replication of single cells
- A single cell gets energy from a food source, increases its volume, and when its DNA is replicated, the cell splits into two daughter cells
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- The process takes between 20 minutes and one month, depending on the type of bacteria and the environmental conditions in which its found
- The change in the number of bacteria can be seen on the **growth curve** graph, which includes the following stages
  - Lag phase
  - Log phase
  - Stationary phase
  - Cell death
- **Lag phase-** during this phase, there is no change in the number of bacteria. The cells are acclimating to their new surroundings by changing their membrane, producing new proteins, etc. In the transition from one medium to another container with the same medium, the lag phase will usually be very short. When transitioning from one medium to a different medium, the lag phase tends to be longer.

The transition from a poor medium to a rich medium, there is no need for the bacteria to develop new systems to generate energy, since there are nutrients all around them. On the other hand, when transferred from a rich medium to a poor medium, bacteria must express whole new systems which will enable them to grow in the new medium, causing a longer lag phase.
- **Log phase- exponential growth phase-** after the bacteria have acclimated to their new surroundings, they start to multiply at a constant rate. For every given interval of time, the number of cells multiplies by two. This is called the **generation time** and is defined by the amount of nutrients available, the type of bacteria, and its genome.
- **Stationary phase-** during this phase, there is a set number of bacteria. In other words, there is a balance between the number of live and dead bacteria. Cells are metabolically active, using up nutrients in their surrounding and excreting various chemicals. This phase can go on for a few hours, days, or even years. Most bacteria in nature are found in the stationary phase, since the energy sources are limited and since there are inhibitors in their surroundings. Therefore, the bacteria mostly don't multiply at a high rate, but rather stay at a constant number in most cases in nature.
- **Death phase-** cell death also occurs at a constant rate (exponential). Cell death can occur very fast or very slowly.

Keywords:

g- generation time: the time needed for the population to multiply

$\mu$ - growth rate: the number of multiplication in a given amount of time

$\mu=1/g$

N- final number of cells

n- number of generations

No- original number of cells

t- growth time

**Factors effecting growth:**

Carbon sources- needed for cell growth

Repressors- inhibit the expression of certain genes

Inducers- induce the expression of genes

Antibiotics- suppress the growth of bacteria, usually by damaging the ability to synthesize proteins by interfering with the translation process.

- Make-up of the cells:
  - 50% carbon
  - 20% oxygen
  - 14% nitrogen
  - 8% hydrogen
  - 3% phosphate
  - 1% Sulphur, potassium, sodium
  - <1% calcium, magnesium, chloride, iron, etc.
  
- Carbon sources: needed for the cell in relatively big amounts. One of the main, basic building blocks of the cell. Usually bacteria prefer to use glucose, but if not present, they can use more complex carbohydrates. Only certain bacteria can break down complex hydrocarbons (complex molecules made up of only carbon and hydrogen atoms)
  
- Macronutrients (needed in large quantities)

*Nitrogen-* an important component of proteins, nucleic acids, etc, which is found in nature in organic and inorganic components

*Oxygen*- important for building the components of cells and as the final electron receptor in cell respiration

*Phosphate*- mostly used for the synthesis of phospholipids and nucleic acids

*Sulfur*- needed for protein synthesis and synthesis of some vitamins

*Potassium*- needed for all organisms for enzymatic processes

*Magnesium*- important for ribosome stability, cell membrane, nucleic acids, and enzyme activity

*Calcium*- necessary only for some organisms. Important for membrane stability

*Sodium*- important for some types of bacteria

*Iron*- a very important part of the respiration process. Most organisms have special systems for integrating iron

- Micronutrients (needed in small quantities)

Though they are only needed in very small quantities, they are necessary for the growth of some organisms. Some examples of these elements include nickel, zinc, manganese, chromium, copper, cobalt, and selenium. Some necessary organic compounds are vitamins (mainly B<sub>12</sub>, B<sub>6</sub>, and B<sub>1</sub>) and amino acids.

These metals are usually found in high enough concentration in water.

- **Growth Mediums:** Growth mediums provide the nutrients and energy which bacteria need in order to grow. Types:
  - Liquid or solid
  - Defined medium (synthetic): contains component with chemically known make-up such as salts, sugars, alcohols, amino acids, fatty acids, etc. When the nutrients are found in low concentrations, the medium is considered a poor medium (as opposed to a rich medium)
  - Complex medium (non-synthetic): contains a mixture of components whose chemical make-up is not necessarily known such as beef extract, yeast extract, blood, peptone (protein hydrolysate), etc.
- **Effect of Oxygen:** microorganisms differ in terms of their need or sensitivity to oxygen. They can be divided into 3 categories:
  - **Obligatory aerobes:** require oxygen in order to grow (example: *Mycobacterium tuberculosis*)
  - **Facultative aerobes:** grow better in the presence of oxygen, but can grow without it as well (example: *Escherichia coli*)

- **Obligatory anaerobes:** die in the presence of oxygen (example: *Clostridium Botulinum*)  
In aerobic bacteria, oxygen serves as the final electron receptor in the cell respiration process.  
In anaerobes, the electrons are passed to other receptors such as gold, iron, manganese, and sulfate, or they may use an alternative respiratory process such as fermentation.  
Obligatory anaerobes are sensitive to metabolic byproducts of oxygen. On the other hand, aerobics synthesize enzymes which know how to break down these byproducts.

### **Techniques for measuring bacteria:**

- **Direct-** techniques which measure the cells themselves
  - **Direct microscopic count-** a known volume of culture sample is placed on a calibrated slide and the microorganisms are counted under a microscope. The number of cells per milliliter of medium can then be estimated
    - Pros:**
      - Quick
      - Inexpensive
    - Cons:**
      - Impossible to differentiate between viable and dead cells
      - It may be hard to see small cells
      - Can't be used to count cells in high concentrations
      - Inaccurate: counting mistakes and moving cells
  - **Coulter counter-** the cells are placed in an electrolytic solution and passed through a thin passageway which allows only one cell to pass at a time. An electric current is passed through the passageway, and each bacteria that passes through changes the solution resistance, causing a pulse. The number of pulses represent the number of cells in the sample.
    - Pros:**
      - Requires minimal effort
      - Very accurate and sensitive
    - Cons:**
      - Both live and dead cells are counted
      - It is impossible to count cells in clusters, since they won't pass through the passageway
      - If there are other molecules in the sample, they may be counted as well

- **Live Cell Count-** cells are plated by spreading a known volume on a petri dish containing a solid medium (agar gel), which serves as an energy source for the bacteria. The plates are incubated overnight. We assume that each cell forms one colony and that each colony originates from a single cell. The number of colonies, together with the known volume plated allows us to deduce the concentration of cells in the sample.

**Pros:**

- Easy
- Relatively accurate at low concentrations

**Cons:**

- Since we assume each colony originates from one cell, the concentration units are given in **colony forming units= CFU**
- Only bacteria which can be grown in the lab will be able to be counted

- **Indirect methods-** hints at the number of bacteria in a sample

- **Dry cell weight-** a known volume of the sample of the culture can be dried, and the weight can provide an indication of the number of bacteria. As the cells multiply, the total weight of the bacteria increases. As a general rule:

$$1 \text{ mg} = 10^9 \text{ cells}$$

- **Following the change in one of the cell components-** based on the fact that usually the make-up of a cell is constant and known (proteins, membrane, DNA, etc.). A sample of cells is taken and one of the component of the solution is measured. From the amount measure, the amount of cells in the solution can be estimated.
- **Optical density measurement-** the most popular technique. The culture becomes turbid over time because each cell diffracts light. The optical density is proportional to the concentration of bacteria, but the proportion constant changes from bacteria to bacteria because of size and shape differences.

**Pros:**

- Relatively accurate
- Simple
- Quick
- Does not ruin the sample

**Cons:**

- Both dead and live cells may be counted
- Samples must have high concentrations (around  $10^7$  cells per ml)

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- Can't be used in medium which absorbs light

### **DNA extraction:**

In the first step, the cells are grown to a high concentration in order to increase the DNA which can be extracted. Therefore, the cells are usually grown in 5 ml of LB medium containing the appropriate antibiotic (this is important since without the proper antibiotics, the cells will likely get rid of the plasmid, and other types of cells may grow in the medium as well). The growth is done at optimal temperature for cell growth- usually 37°C, and shaken at 200-250 rpm (in order to allow the oxygen to incorporate into the medium). The ideal growth time is 16 to 20 hours (this allows maximal growth with minimal cell death as a result of lack of nutrients in the medium, which may lead to plasmid breakdown).

After growth, the cells are centrifuged in order to separate the bacteria from the medium.

A lysis buffer containing SDS, a detergent, is added to destroy the cells by ruining the membrane. This step is followed by the addition of neutralization buffer, containing ethanol, to neutralize the lysis effect by washing away SDS and salts which would otherwise interfere with future processes done with the DNA (such as cutting with restriction enzymes). The sample is centrifuged in order to separate the proteins, cell fragments, fats, and sugars from the DNA.

The supernatant is bound to a column and washed, in order to ensure a pure product without macromolecules other than DNA. Centrifugation during the binding and washing steps causes the liquid to pass through the column membrane. The column is dried by centrifugation in order to remove any remaining ethanol before elution of the DNA (ethanol would otherwise interfere with processes performed on the DNA at a later stage).

Lastly, the column is transferred to a new eppendorf, and the DNA is eluted from it using hot water.

### **Nanodrop:**

A peak at 260nm signifies DNA in the sample. The instrument shows us the concentration of DNA in units of ng/ml.

### **Important ratios:**

**260/280-** a ratio which indicates the amount of protein in the sample. A high ratio is preferred, since DNA absorbs at 260nm and protein absorbs 280 nm.

**260/230-** a ratio which indicates the amount of salts, phenol compounds, and thiocyanate in the sample

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*The desired ratio is about 1.8*