**اعداد: م. م. مروة شاكر محمود**

# Dilution and Plating of Bacteria and Growth Curve

## Definition of microorganism :

Any organism too small to be seen by the naked eye, e.g. bacteria, viruses, protozoa, some fungi, and some algae. Microorganisms can be divided into four nutritional groupings: photoautotrophs, photoheterotrophs, chemoautotrophs, and chemoheterotrophs.

## Definition of Bacteria

**Bacteria:** Single-celled microorganisms which can exist either as independent (free-living) organisms or as parasites (dependent upon another organism for life). Its categorized as one of three types of microorganisms -- bacteria (short rods), bacilli (longer rods), and spirilla (spiral forms).

**Examples of bacteria include:**

: A normal inhabitant of yogurt, **Acidophilus**

. gonorrhea: Which causes an infection very similar to **Chlamydia**

**:** Most common cause of the dreaded gas **Clostridium welchii**gangrene

: The common peaceful citizen of our colon and, upon occasion, ***E. coli*** of adangerous disease.

***Streptococcus*** : the bacterium that causes the important infection of the Strep throat.

## Growth

**Definition**: (biology) the process of an individual organism growing organically; a purely biological unfolding of events involved in an organism changing gradually from a simple to a more complex level.

## Theory and Significance

Perhaps the most widely used technique for the study of bacteria is the growth of a microbe of interest in a liquid nutrient medium, followed by dilution and plating on a solid agar medium. Here the theory is that onecolony arises from one organism. Each colony is then referred to as a colony forming unit (CFU). In addition to providing an estimate of bacterial -numbers, this procedure allows the opportunity to obtain pure culture isolates. Often times, researchers will measure the turbidity of the liquid culture at different time intervals using a spectrophotometer. The comparison of turbidity with plating results allows for a quick estimation of bacteria numbers in future studies. These techniques are used in all

aspects of microbiology including clinical and environmental microbiology. Because of its importance .This topic is introduced here as the first exercise in this laboratory manual. The growth of a bacteria isolate will be followed as a function of time to illustrate the various phases of growth that occur in liquid culture. Intuitively one can recognize that bacterial growth (via cell division) in liquid media will continue to occur until: a) nutrients become limiting; or b) microbial waste products accumulate and inhibit growth. To understand and define the growth of a particular microorganism, cells are placed in a flask in which the nutrient supply and environmental conditions are controlled. If the liquid medium supplies all the nutrients required for growth and environmental parameters are conducive to growth, the increase in numbers can be measured as a function of time to obtain a growth curve Several distinct growth phases can be observed within a growth curve (Figure 1-1). These include the lag phase, the exponential or log phase, stationary phase, and the death phase. These phases correspond to distinct periods of growth and associated physiological changes (Table 1-1).

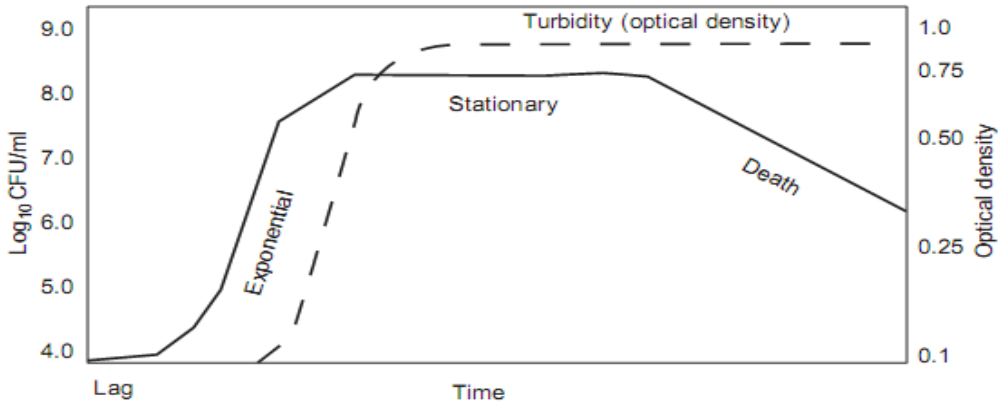


**Figure (1-1)** A typical growth curve for a bacterial population. Compare the difference in the shape of the curves in the death phase (colony-forming units (CFUs) versus optical density).The difference is due to the fact that dead cells still result in turbidity.

**Table 1-1** The Four Phases of Bacterial Growth

| Phase | Characteristics |
|---|---|
| 1. Lag Phase | Slow growth or lack of growth due to physiological adaptation of cells to culture conditions or dilution of exoenzymes due to initial low cell densities. |
| 2. Exponential or Log Phase | Optimal growth rates during which cell numbers double at discrete time intervals known as the mean generation time (Fig. 1-2). |
| 3. Stationary Phase | Growth (cell division) and death of cells counterbalance each other resulting in no net increase in cell numbers. |
| 4. Death Phase | Death rate exceeds growth rate resulting in a net loss of viable cells. |

Theoretically, the time taken for cell division to occur is the mean generation time or doubling time. The mean generation time can be calculated through the use of a dilution and plating experiment .

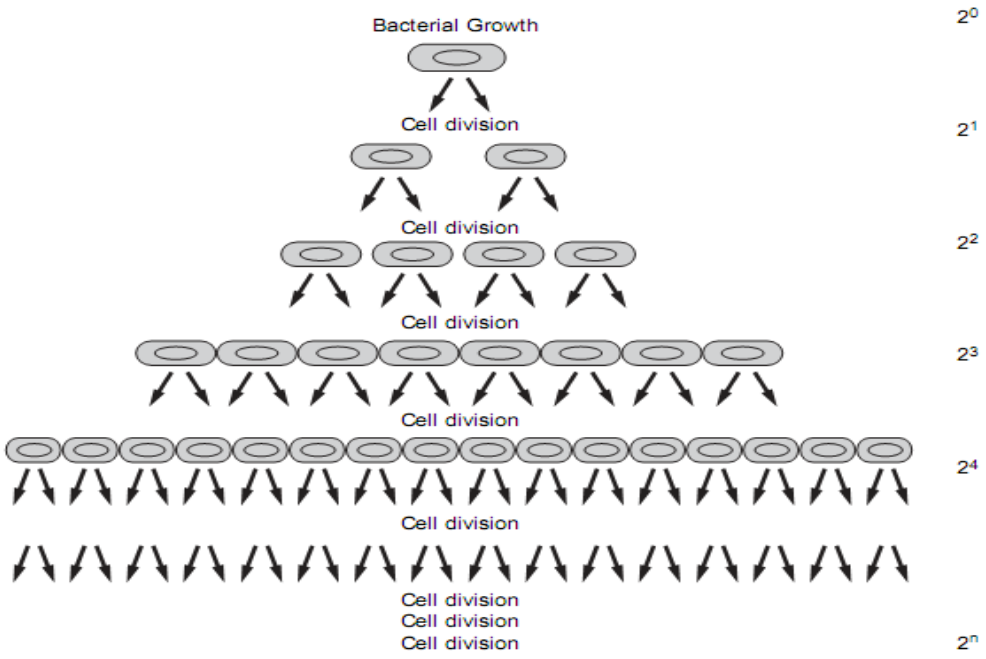


**Figure (1-2)** Exponential cell division. Each cell division results in a doubling of the cell number. At low cell numbers the increase is not very large, however after a few generations, cell numbers increase explosively. After divisions we have 2n cells .

## Procedure (1)

1- Activated ***E.coli*** culture with trypticase soy broth medium.

2- Incubated a 50 ml flask of trypticase soy broth (TSB) medium with ***E.coli*** over night at 37C. This will yield $10^9$ CFU/ml .

3- Use 100ml of the prepared culture to inoculate 250ml of TSB (in a 500ml flask). Mix thoroughly and remove 5ml and refrigerate immediately. This is T = 0 and will yield approximately 5 x $10^5$ CFU/ml. Place the flask of ***E. coli*** in a 37°C shaking incubator. Remove 5ml aliquots of culture every hour upto 8 hours. Store each aliquot at 4°C.These cultures should be designated T0 through T8.

## Procedure (2)

### First Period

1. Set up a series of dilution tubes to obtain dilutions of $10^{-1}$ through $10^{-7}$ of the E. coli cultures. Microfuge tubes are convenient to do this (see Figure 1-3). Each dilution tube will have 900ml of dilution fluid (sterile saline). A dilution series will be needed for each E. coli culture (T0 thru T8).
2. Begin dilutions by adding 100ml of E. coli from the tube labeled T0 which is the initial E. coli culture to tube A. Tube A is the 10-1 dilution of T0.
3. Vortex the 10-1 tube for 5 seconds.
4. Follow this by subsequently adding 100ml of Tube A to the next tube of saline (Tube B).Tube B is a 10-2 dilution of T0. Repeat until completing the dilution series, referring to Table 1-2 to see how far you will need to make dilutions for each E. coli culture. Remember to vortex each tube prior to transfer. It is also important to use a new pipette tip for each transfer.
5. Repeat dilutions for T1 through T8 or for whatever samples were assigned to you. Again refer to Table 1-2 to see how far you need to make your dilutions.
6. Plate according to the regiment specified in Table (1-2).
7. Label plates with the dilution and volume to be added to the plate, make sure the label contains the time point plated (T1 thru T8) identification.
   Use triplicate plates for each dilution.
8. Pipette 100ml from each of the three dilutions to be plated. Add 100 ml of each dilution tube to be plated by pipetting the amount to the center of the agar plate (Figure 1-3).
9. Immediately spread the aliquot by utilizing a flame sterilized "L" shaped glass rod. If the aliquot is not spread immediately, it will sorb

in situ in the plate resulting in bacterial overgrowth at the spot of initial inoculation.

10. Repeat the plating for each dilution series for T1 through T8 cultures, remember to sterilize the rod in between plates and especiallybetween different dilutions.
11. Once plates have dried for a few minutes, invert and place in 37°C incubator overnight. Following this, store plates in refrigerator until the next class period.

### Second Period

1. Examine plates for uniformity of colonies and lack of contamination (see Figure 1-4).
2. For each culture (T0 through T8), count triplicate plates at one dilution that contains between 30 and 300 colonies.
3. Calculate the number of cells per ml of original culture for T0 through T8 cultures .
4. For example, the number of colonies resulting from a 10-4 dilution is 30,28, and 32.Mean number of colonies = 30 colonies These arose from 0.1 ml of a 10-4 dilution.
5. Plot log10 CFU/ml versus time (hours).
6. From the graph, identify the exponential phase of growth. Using two time points within the exponential phase of growth and corresponding cell numbers, calculate the mean generation time.

**Table 1-2** Plating protocol for *E. coli* cultures

| *E. coli* culture | Dilutions to be plated | | |
|---|---|---|---|
| $T_0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
| $T_1$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
| $T_2$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| $T_3$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ |
| $T_4$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| $T_5$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| $T_6$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ |
| $T_7$* | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| $T_8$* | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |

*Lower dilutions take into account lower populations due to death phase.

## Tricks of The trade

DO:

- Keep broth cultures on ice until you dilute and plate.
- Use multiple dilutions to ensure you get countable plates.
- Change pipette tips to prevent contamination.
- Label the Petri plate bottoms, not the tops.

DO NOT:

- Place ethanol jars next to Bunsen flames since it may cause a fire.

• Leave Petri plates exposed without lids since this will allow for.
• Fires that result from ethanol jars.
• Inhalation of ethanol.

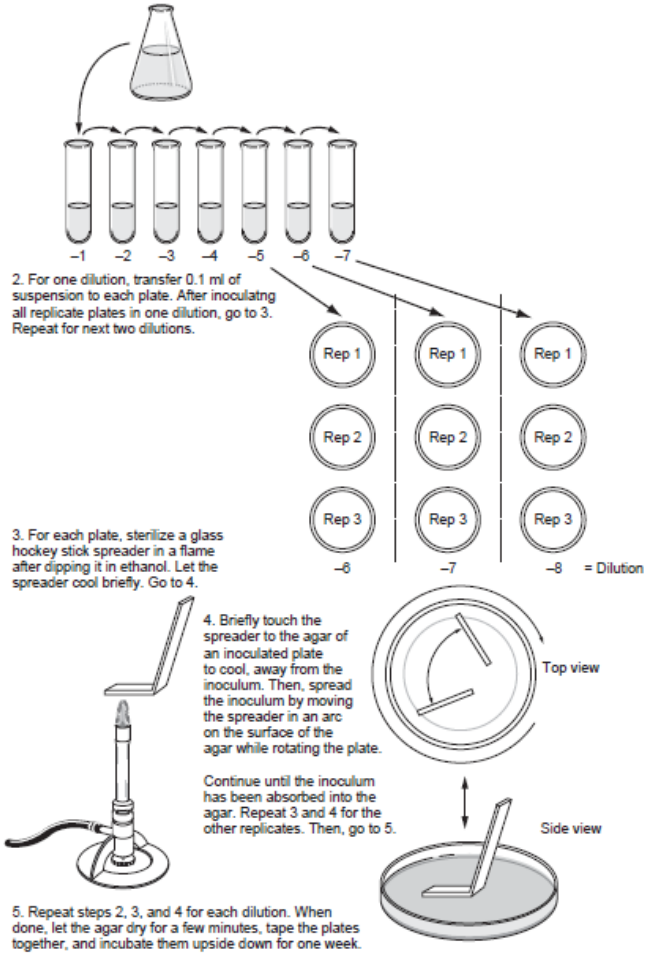


**Figure 1-3** Schematic showing the procedure for counts of *E. coli*.

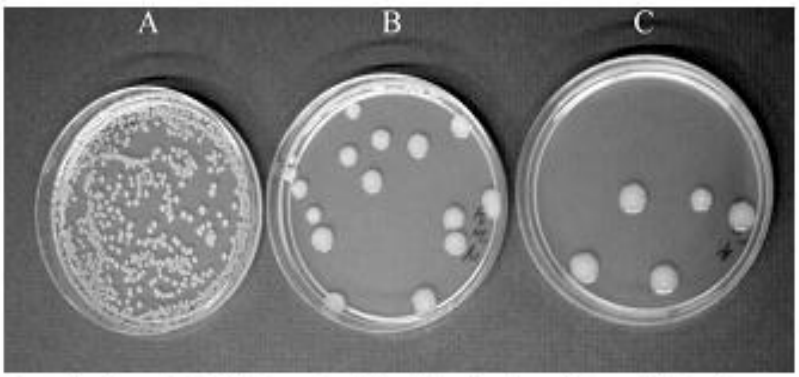


**Figure 1-4** Example of a dilution series of *E. coli* plated at three dilutions. Dilutions decrease from left to right. Here, plate A is the one which should be counted (Photo courtesy K.L. Josephson).

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# Detection of coliform group bacteria

## Introduction

The safety of drinking water is dependent on their being no sewage contamination of potable water, and this is tested using the MPN method and membrane filter (MF) technique. Indicator bacteria called coliform, which are found in the intestine of warm-blooded animals. The presence of these bacteria in water suggests the potential of disease such as cholera (***Vibrio cholera***), dysentery (***Shigella dysenteriae***), and typhoid fever (***Salmonella typhi***).

## Coliform group bacteria:

Consists of several genera of bacteria belonging to the family ***Enterobacteriaceae*.** They may be defined as (1) aerobic and facultative anaerobic, (2) non-spore forming, (3) Gram-negative rods (4) that ferment lactose with gas production within 48 hours of incubation at 35°C.

### 1) MPN method

Most Probable Number (MPN) is a qualitative method for coliform count. It is a very simple and inexpensive method, which is suitable for the routine monitoring of drinking water and treated waste water effluents that contain around 1000 or fewer fecal coliforms per 100 ml of water sample.

## Equipment Required

- Test tubes, Durham tubes (a very small test-tube) and cotton wool.
- Sterile screw-capped bottle.
- Pipettes and test-tube rack.
- Bunsen burner.
- Incubator and autoclave.
- MacConkey broth medium.

## Procedure

**1.** Set up the tubes and label 3 of them with 10ml , 3 with 1ml and the other 3 with 0.1ml .

2. Collect a sample in a sterile screw-capped bottle.

3. Shake the sample bottle thoroughly, and aseptically with draw 10ml to each tube of 10 ml, 1ml to each tube of 1ml and 0.1ml to the tubes of the third set.

4. Incubate at 35°C for 24-48 hours.

5. Examine the tube for gas production.

6. Determine the MPN from the given table.

### 1. Presumptive test

In the presumptive test 9 tubes of MacConkey broth are inoculated with measured amount of water sample and incubated. After 24 hours, the fermentation tube is examined for gas production. If there is no gas production, the samples are incubated for another 24 hours and reexamined. If gas production is observed by the end of 48 hours, the presumptive test is positive; coliform bacteria are present in the sample. The results are reported as most probable number (MPN) of coliform per 100 ml.

### 2. Confirmed test

In this test, a plate of EMB agar is inoculated from positive tubes. This medium inhibits the growth of Gram positive bacteria and causes the colonies of coliform distinguishable from non-coliforms. On this medium coliforms produce small colonies with dark centers and this will confirms the presence of lactose-fermenting, Gram negative bacteria. However, ***E. coli*** produces small colonies with green metallic sheen.

### 3. Completed test

In the completed test, a Durham tube of MacConkey broth and a nutrient agar slant are included. If gas produced in the tube and a slide from the agar slant reveals Gram negative, non-spore forming rods, we can be certain that we have is coliform bacteria.

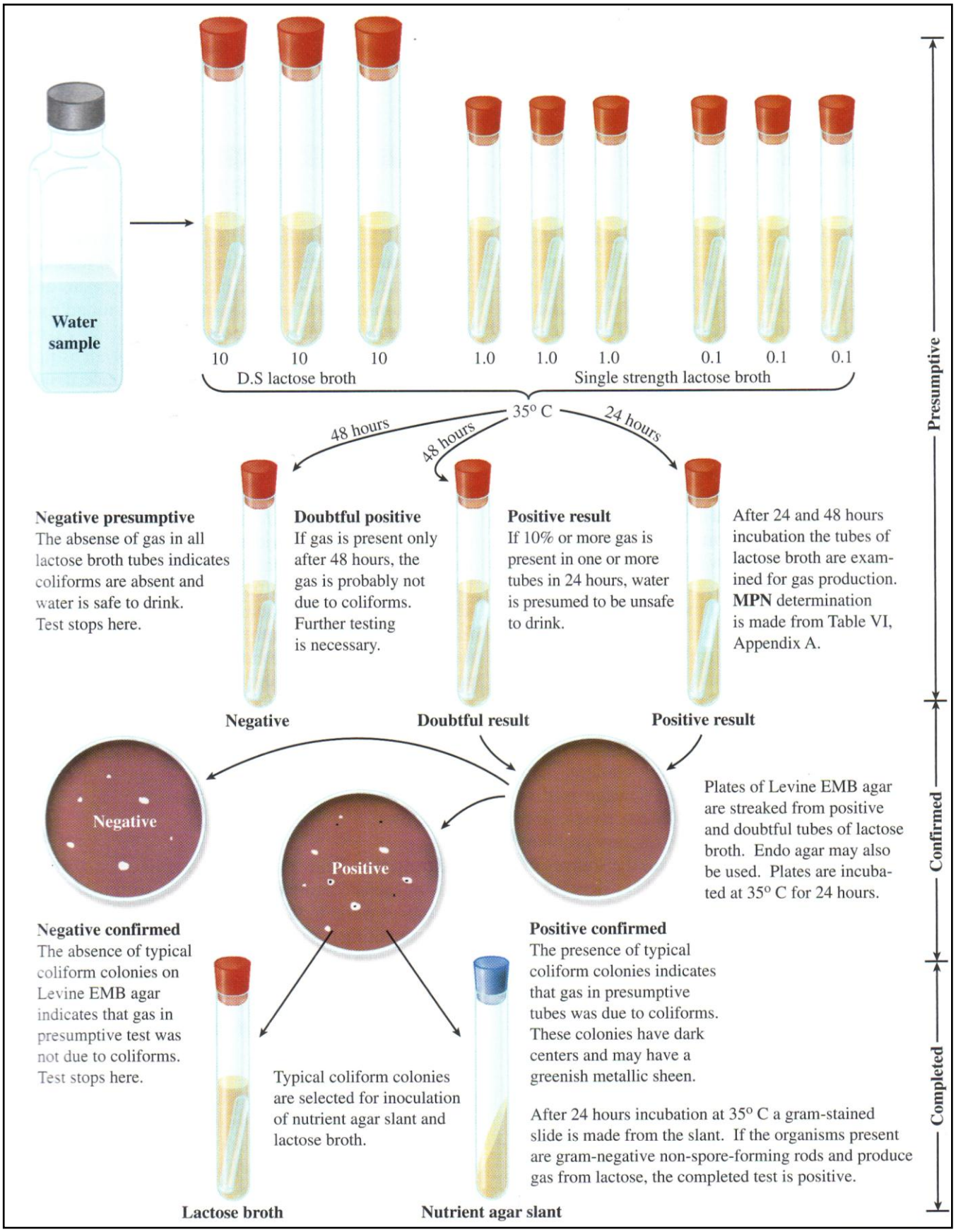
Water sample
10
10
10
1.0
1.0
1.0
0.1
0.1
0.1
D.S lactose broth
Single strength lactose broth
35° C
48 hours
48 hours
24 hours
Presumptive
Negative presumptive
The absense of gas in all lactose broth tubes indicates coliforms are absent and water is safe to drink. Test stops here.
Doubtful positive
If gas is present only after 48 hours, the gas is probably not due to coliforms. Further testing is necessary.
Positive result
If 10% or more gas is present in one or more tubes in 24 hours, water is presumed to be unsafe to drink.
After 24 and 48 hours incubation the tubes of lactose broth are examined for gas production. MPN determination is made from Table VI, Appendix A.
Negative
Doubtful result
Positive result
Negative
Positive
Confirmed
Plates of Levine EMB agar are streaked from positive and doubtful tubes of lactose broth. Endo agar may also be used. Plates are incubated at 35° C for 24 hours.
Negative confirmed
The absence of typical coliform colonies on Levine EMB agar indicates that gas in presumptive test was not due to coliforms. Test stops here.
Positive confirmed
The presence of typical coliform colonies indicates that gas in presumptive tubes was due to coliforms. These colonies have dark centers and may have a greenish metallic sheen.
Typical coliform colonies are selected for inoculation of nutrient agar slant and lactose broth.
After 24 hours incubation at 35° C a gram-stained slide is made from the slant. If the organisms present are gram-negative non-spore-forming rods and produce gas from lactose, the completed test is positive.
Completed
Lactose broth
Nutrient agar slant

**TABLE VI** MPN Determination from Multiple Tube Test

| NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF | | | MPN Index per 100 ml | 95 PERCENT CONFIDENCE LIMITS | |
|---|---|---|---|---|---|
| 3 of 10 ml each | 3 of 1 ml each | 3 of 0.1 ml each | | Lower | Upper |
| 0 | 0 | 1 | 3 | <0.5 | 9 |
| 0 | 1 | 0 | 3 | <0.5 | 13 |
| 1 | 0 | 0 | 4 | <0.5 | 20 |
| 1 | 0 | 1 | 7 | 1 | 21 |
| 1 | 1 | 0 | 7 | 1 | 23 |
| 1 | 1 | 1 | 11 | 3 | 36 |
| 1 | 2 | 0 | 11 | 3 | 36 |
| 2 | 0 | 0 | 9 | 1 | 36 |
| 2 | 0 | 1 | 14 | 3 | 37 |
| 2 | 1 | 0 | 15 | 3 | 44 |
| 2 | 1 | 1 | 20 | 7 | 89 |
| 2 | 2 | 0 | 21 | 4 | 47 |
| 2 | 2 | 1 | 28 | 10 | 150 |
| 3 | 0 | 0 | 23 | 4 | 120 |
| 3 | 0 | 1 | 39 | 7 | 130 |
| 3 | 0 | 2 | 64 | 15 | 380 |
| 3 | 1 | 0 | 43 | 7 | 210 |
| 3 | 1 | 1 | 75 | 14 | 230 |
| 3 | 1 | 2 | 120 | 30 | 380 |
| 3 | 2 | 0 | 93 | 15 | 380 |
| 3 | 2 | 1 | 150 | 30 | 440 |
| 3 | 2 | 2 | 210 | 35 | 470 |
| 3 | 3 | 0 | 240 | 36 | 1,300 |
| 3 | 3 | 1 | 460 | 71 | 2,400 |
| 3 | 3 | 2 | 1,100 | 150 | 4,800 |

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## Biological Oxygen Demand measurement (BOD):

**Biological Oxygen Demand (BOD)** is the amount of dissolved oxygen needed by aerobic microorganisms to break down organic pollutants present in a liter of the sample by the action of the microorganism, under specified condition, within a certain period of time (after 5 days).

## Importance:

**BOD** is an important water quality parameter because it provides an index to assess the effect discharged wastewater will have on the receiving environment. The higher the BOD value, the greater the amount of organic matter or "food" available for oxygen consuming bacteria.

**BOD** measurement can be used to evaluate the impact of biodegradable substances in water & waste by measuring the quality of water & treatment result in waste water. Depending on the measurement site and type waste water the BOD value can lie between a few mg/ L & several thousand mg/ L. .

**The BOD serves as a bulk parameter indicating:**

1- Level of organic pollution of the waste water.
2- The quantity of oxygen needed for the respiration of the organism contain in the BOD bottle during the incubation period.
3- Evolution the efficiency of the treatment system through the reduction in BOD value.

.

**The BOD is made up from tow reaction during 5 days:**

1- Oxygen demand for decomposition & metabolism of carbon & hydrogen compounds.
2- Oxygen demand for endogenous respiration.

## Collection of sample

Collect sample very carefully, do not let the sample remain in contact with air or be agitated, because either condition causes a change in its gaseous content. Samples from any depth is steam lakes & or reservoirs, need special precautions to eliminate changes in pressure and temperature.

Collect surface water samples in narrow –mouth glass-stopper BOD bottles of 300 ml capacity with tapered & pointing ground – glass stoppers and flared mouth. Avoid entraining or dissolving atmospheric oxygen .Attack a glass or rubber tube to the tap and extend to bottom of bottle Let bottle overflow tow or three times its value and replace the stopper so that no air bubbles are entrained.

**Special Reagents:**

A- Manganese sulfate solution: Dissolved 450g $MnSO_4$.4H2O or 400 $MnSO_4.2H_2O$ or 364g$MnSO_4$.H2O in distilled water. Diluted to 1L.

B- Alkali-iodode-azide reagent: Ddissolved 500g NaOH (or 700g KOH) and 135g (or 150g KI) in distilled water and diluted to 1L.

C- Sulfuric acid: Concentrated $H_2SO_4$(sp.gr.1.84): one milliliters in equivalent to about 3 ml Alkali-iodode-azide reagent.

D- Starch indicator solution : Dissolved 2g laboratory- grade solutuion (at 80$^oC$ , stir vigorously untile solution is clear and allow stand for 1-2 hr.

**Procedure:**

1- Remove BOD bottle (250-300ml) stopper: Add 1ml $MnSO_4$ solution and at once add 1ml of alkaline iodide solution . Restop -per leaning no air bubbles and then mix by inverting.

2- After sligh settling of the precipitate, shake and allow to settle one third the BOD or precipitate dowen.

3- Add one ml $H2SO_4$, restoper and mix to dissolve all precipitate.

4-Within one hour, transfer 20 ml of solution into the conical flask and titrate with the 0.01N thiosulfate untile color is very pale. Add 5 ml starch indicator and conclude titration until blue color disappears and solution is colorless. Recored burette reading .

5- Calculate oxygen content (mg O2/L):

$$mg\ (O_2/L) = \frac{Volume\ thiosulfate * 200 * K}{Volume\ of\ sample}$$

K=Volume of bottle /Volume of bottle -2 =1.01

**General Reaction**:

$Mn^{+2} + 2OH^- \rightarrow 2Mn\ (OH)_2$

$2Mn(OH)_2 + O2 + H2O \quad 2Mn(OH)_3$ .

$Na_2S_2O_3 + I_3^- \longrightarrow S_4O_6 + 3I^-$ .

$2Mn(OH)_3 + 3I^- + 6H^+ \longrightarrow 2Mn^{+2} + I3^- + 6H2O$

**Calculation of BOD value**:

1- Procedure for determination $BOD_5$ value similar to that of DO.
2- Some BOD bottles used for measurement of initial dissolved oxygen.
3- The remained BOD bottles are incubated for 5 days in dark container at 20 $^oC$ . Then the value of dissolved oxygen determined after 5 days.
4- The BOD5 value were calculate according to the equation:
BOD5 =DO1-DO2

Where:

DO1=Dissolved oxygen (mg/L) on the first day incubation.
DO2= Dissolved oxygen (mg/L) of Sample after 5 day incubation.

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## Biodegradation of hydrocarbon pollutants:

Petroleum hydrocarbons contain many different types of organic compounds, namely alkanes (normal, iso-, and cycle-), aromatics, polycyclic aromatics, and asphalt compounds. Each of these compounds has a different biodegradation rate in the natural environment. The chemical structure of each compound determines its biodegradation potential, but other factors such as solubility, toxicity, and interaction with other molecules present also affect the rate and extent of biodegradation.

Microorganisms (bacteria, yeasts, and fungi) that use petroleum hydrocarbons as a source of cell carbon and energy are widely distributed in nature. More than 100 strains of bacteria have been identified that degrade petroleum hydrocarbons. Some of the more common bacterial species belong to the following genera: ***Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Bacillus, Vibrio, Brevibacterium, Corynebacterium***, and ***Flavobacterium*.**

In an environment that is not under stress, bacteria are generally believed to be primarily responsible for the degradation of petroleum hydrocarbons. They generally are in greater numbers compared to yeasts and fungi in an environment contaminated with petroleum hydrocarbon compounds.
Fungi and yeasts perform better in degrading these compounds in conditions which preclude bacterial growth.

## Alkane biodegradation

Straight chain n-alkanes usually are more easily degraded compound to other hydrocarbons, but smaller chain length alkanes ($C_1$ to $C_{10}$) are inhibitory to some of the hydrocarbon degrading microorganisms. These molecules act as a solvent disrupting the membrane structure of the cell. Intermediate chain length ($C_{10}$ to $C_{20}$) degrades most readily by many microorganisms. Alkenes with longer chain length ($>C_{20}$), often referred to as "waxes" are quite hydrophobic in nature and thus degrade very slowly. Branching of the alkane chain inhibits degradation. Thus, n- alkanes are easily degraded by microorganisms while their branched chain is slow to degrade. The cyclic alkanes also degrade quite slowly. Some of the monocyclic compounds such as cyclopentane, cyclohexane, and cycloheptane have a solvent effect on the lipid membranes of microbial cells and thus are toxic to most of the hydrocarbon degrading bacteria.

The primary attack on the hydrocarbon molecule in the presence of oxygen is through the action of oxygenates. The mono-oxygenase reaction with the alkane results in an alcohol product. The alcohol product is then oxidized to an

aldehyde and, finally, to an acid product. The acid product can be further degraded by beta-oxidation. Presence of branching interferes with the beta-oxidation process. Cycloalkanes are susceptible to degradation by co-metabolism in the presence of other easily degradable compounds.

## Procedure:

### Isolation of Microorganisms from polluted hydrocarbons:

The polluted hydrocarbons used in this experiment were collected from various locations which include spilled oily , contaminated soil and from waste water treatment.

**A**. Testing the capability of isolates to degrade hydrocarbons in solid media:

1. The mineral salt medium, with the following composition (g/l): $MgSO_4.7H_2O$, 0.2; $CaCl_2$. $2H_2O$, 0.02; $KH_2PO_4$, 1; $K_2HPO_4$, 1; $NH_4NO_3.6H_2O$, 1 and $FeCl_3$, 0.05 was used as the enrichment media with 1% (v/v) crude oil, as the sole carbon source to isolate oil-degrading microorganisms.
2. One percent (1% w/v) of the contaminated soil and 1% (v/v) of the liquid waste samples were added to the 250 ml conical flasks containing 50 ml mineral salt medium. The flasks incubated at 30°C at 120 rpm in shaker incubator.
3. After 5 days of incubation, 1 ml of enriched media was transferred into serial dilutions of (1/10) NaCl solution ten sex fold.
4. Then 0.1 ml from these dilutions transferred onto nutrient agar plates, which were covered with 0.1 ml of crude oil and incubates at 30°C. oil degrading bacteria appear as colonies surrounded by zones of clearing on the surface of plates. Colonies of the hydrocarbon-utilizing bacteria picked, isolated and purified by streaking on nutrient agar. The isolated colonies were then stored on nutrient agar plates and slants and later were examined for further screening in liquid and stored at 4°C until further experiments.

### B. Testing the capability of isolates to degrade the hydrocarbons in liquid media:

For testing the isolates for biodegradation capabilities the following procedure were applied:

## Procedure:

1. The screened isolates were grown in nutrient broth for 16–18 h at 30 ˚C. This culture was used as stock culture inoculums at the 1% (v/v) level.

2. The cultivations condition were performed in 250 ml flasks containing: A 50 ml mineral medium (mentioned above), yeast extract 0.5 (g/l), and 1%(v\v)of hydrocarbon source, pH adjusted to 7. Autoclaved and then 1% ml of inoculums, were adding, and then incubated in shaker incubator at 30 ˚C and at 120 rpm, for 96 h.
3. After incubation period the following measurements were determines:

**a. <u>Measurement the optical density (OD) of the culture:</u>**

1. Transfer 2 ml of culture medium into the spectrophotometer tube then determine the OD at 600 nm. Record and discuss the results which give higher growth.

**b. <u>Biomass measurement:</u>**

After incubation period ,the culture transfer to centrifuge tube ,and the centrifuging at 10000rpm for 15 Min. for precipitation of bacterial biomass ,the supernatant removed ,and precipitate (biomass) transferring to pre weighted filter paper ,and then dried in oven at 70 C° over night ,weighting again to determine the dry weigh as follow.

$$g\backslash L = \frac{W2-W1}{\text{Sample volume(ml)}} \times 10000$$

W1=weight of filter paper empty
W2= weight of filter paper of drieing

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## Biodegradation of aromatic hydrocarbon compounds

The biodegradation of aromatic compounds was considered to be more difficult compare with the degradation of aliphatic hydrocarbons and the enzymes in this process different from those which be involved in the oxidation of aliphatic compounds. In oxidation, aromatic compounds were degrading to intermediate compounds such as catechol and protocatechuate. See figure (1).

Poly aromatic hydrocarbons considered as one of pollutants in soil and water. These persist in the environment for long time and seem to be non degradable under natural conditions. The removal of these pollutants from contaminated area very important because most of these pollutants were carcinogenic or mutagenic for organisms.

The stability of these pollutants in environmental system and its resistance to biodegradation due to: their low solubility in water and its adsorption on soil molecules that made its bioavailability for soil microorganisms very low. The solubility and availability of these compounds decrease with increase molecular weight of these compounds.

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds having two or more fused aromatic rings. Generally, two- to three-ringed PAH compounds degrade at a relatively faster rate than PAH compounds having more than three rings. In some instances, the degradation rate of higher ringed PAHs is facilitated by the presence of other structurally related two- or three-ringed compounds. Also, higher molecular weight PAHs may be co-metabolized (in mixed culture) to simpler intermediates in the presence of supplementary carbon sources.

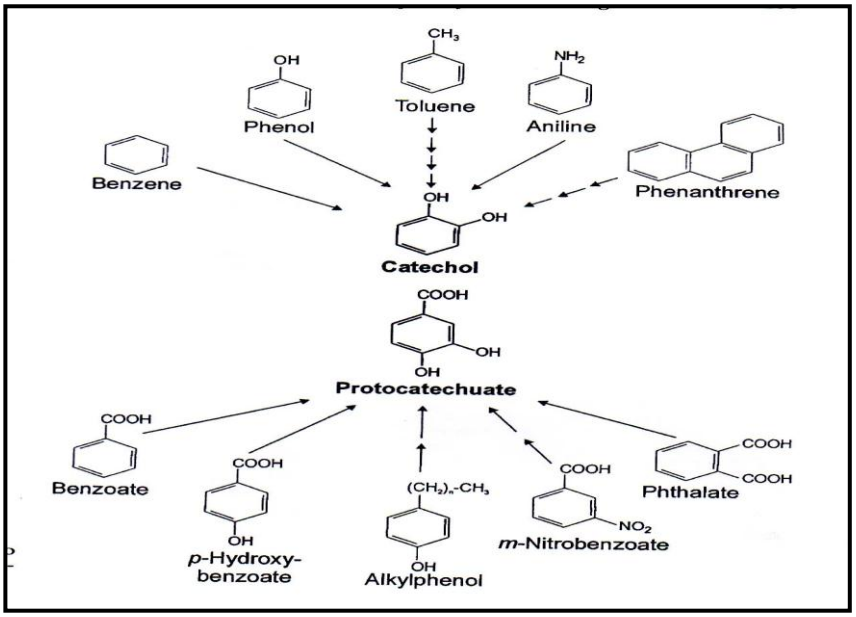


**Figure 1** : Degradation of poly aromatics to Catechol and Protocatechuate

## Procedure:

### A. Testing the capability of bacteria to degrade poly aromatic compounds in solid media

1. Prepare the mineral salt medium with same constituents as in lab 4 in (g/l).
2. Add 15 g of nutrient agar to 1 L mineral salt medium to solidify the plates.
3. The plates were poured and dried overnight in the incubator at 30°C then, 0.2 ml of a solution containing 1.0 g of naphthalene and 0.5 g of anthracine per 100 ml of 95% ethanol were uniformly spread on the surface of the agar plates (while gently turning around).
4. Then plates were further dried overnight at room temperature to allow the ethanol to evaporate before inoculation. The ethanol immediately vaporized from the surface of the plates at room temperature, and a white thin layer of anthracine and thin layer of naphthalene remained on the surface of the plates.
5. Then inoculum's of bacteria (OD of 0.6 at 600 nm) grown over night in 10 ml nutrient broth added as spots to surface of the plates, and incubated at 30°C for seven days.
6. Results: Naphthalene -degrading ($Nah^+$) and anthracine – degrading ($Anth^+$) bacteria appear as colonies surrounded by zones of clearing on the surface of plates.

### B. Testing the capability of bacteria to degrade the poly aromatic compounds in liquid media:

## Procedure:

1. Add to elementary 250 ml conical flasks 50 ml of mineral salt medium as prepared in lab. 4 then adjust the pH of solution to 7 and autoclave the flasks.
2. After sterilization of flasks 0.01% of naphthaline and anthracin was add to each flask.
3. Then overnight growth culture in nutrient broth for 16-18 h at the level 1 %( v/v) were ads to each flask as inoculums.
4. Incubate the flasks in shaker incubator at 30 ˚C and 120 rpm, for 7 days.
5. After incubation period determine the followings:

**a. Measurement the optical density (OD) of the culture:**

1. Transfer 2 ml of culture medium into the spectrophotometer tube then determine the OD at 600 nm. Record and discuss the results which give higher growth.

**b. Biodegradation test:**

1. Transfer 30 ml from culture medium into 50 ml centrifuge tubes, and then centrifuge the cultures at (10.000 g, for 15 min).
2. The precipitate cells transferred to weighted container and dried overnight at 105˚C in oven and reweighed, to determine the dry weight of bacteria.
3. Take 2 ml from the supernatant after centrifugation and determine the absorbance or the concentration of solution in UV- visible spectrophotometer at 270 nm.
4. Determine the absorbance of control sample (all the amendments with out inoculums) as the same condition above.
5. Calculate the biodegradation percent as:

**Biodegradation %** = Absorbance of sample / absorbance of control × 100.

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## Bioremedation of pollutants

**-Bioremedation** is a waste management technique that involves the use of microorganisms to remove or neutralize pollutants from a contaminated site. According to the United States( EPA), bioremediation is a "treatment that uses naturally occurring microorganisms to break down hazardous substances into less toxic or non toxic substances".

-The population of microbes may use the soil matrix, groundwater or both as a substrate. The soil is amended with nutrients, while moisture and oxygen are maintained at near optimum levels for degradation to occur. The aerobic microbes utilize the organic carbon in the contaminant as an energy and organic carbon source for growth. They typically utilize oxygen and produce carbon dioxide and water in this process.

-Bioremedation technologies can be generally classified as in situ or ex situ. Selection of the appropriate technology depends on the solubility, volatility and adsorptive ability of the contaminant, as well as the location and the extent of contamination.

- ***In-situ*** **bioremediations :** involves treating the contaminated material at the same site.

**-** ***Ex-situ*** **bioremediations :** treat the soils or groundwater after removal from its original environment to another location.

-Some examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation.

## Factors affecting microbial bioremediation:

The rate of microbial treatment of pollutants is dependent upon many biological, chemical and physical parameters. Environmental enhancements, such as adjustment of pH, temperature, nutrient level, and aeration, are usually necessary to facilitate the bioremediation process.

## Biostimulation

Usually involves the addition of nutrient and oxygen to help indigenous microorganism for biodegradation. These nutrients are allowing microbes to create the necessary enzyme to break down the contaminants. The important nutrients are nitrogen, phosphorous and carbon sources.

### 1- Nitrogen:

Nitrogen is an essential macronutrient for microbial growth and reproduction. A ratio of carbon to nitrogen in the soil for effective degradation has been estimated to be 10:1. Many soils contaminated with hydrocarbons are nutrient limited .This phenomenon is caused by excessive carbon loading from the fuel without any significant nitrogen inputs.

Consequently, it may be necessary to amend the soil with nitrogen, in order to initiate bioremediation .Some of the different forms of nitrogen that have been used by researchers for bioremediation are NH4Cl, $KNO_3$ $NaNO_3$, NH4$NO_3$ , urea , glutamic acid , oleophilic nitrogen, etc .

### 2- Phosphorus

Microorganisms require phosphorus in the biosynthesis of nucleic acids and cell membranes. Many studies have indicated that addition of phosphorus increase the rate of mineralization of many hydrocarbons, such as diesel fuel in the soil. The optimal carbon to phosphorus ratio for petroleum degradation by a mixed microbial population in soil has been estimated to be 20:1. These observed differences in required C: P ratio may be due to the low bioavailability of phosphorus when added to soils as a result of sorption and precipitation.

## Procedure

1- Prepare mineral salt medium as previous experiments
2-Add for each 250 ml conical flasks in duplicate the following:
- 50 ml mineral salt medium.
- 1% hydrocarbon source (waste oil).
- 0.4% one of the nitrogen source ($NH_4Cl$, $KNO_3$, $NaNO_3$, $NH_4NO_3$, urea, glutamic acid).

3- Adjust the pH of flasks to 7, then autoclave the flasks.
4- After sterilization, add 1% of overnight inoculums of bacterial culture for each flask.

5- Incubate the flasks at 30$^{o}$C in shaker incubator for 72h and 150 rpm.
6- Estimate the optical density of the cultures, dry weigh of the cells and emulsification activity.
7- Then record the results of the experiments, and determine the best nitrogen source for biodegradation and emulsification activity.
8- Draw a figure to illustrate the results above, and discus the result.

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## Biosurfactant Production by bacteria

**Biosurfactants** are surface-active substances derived from living organisms, especially microorganisms.

**Biosurfactants** are amphiphilic compounds, containing hydrophobic and hydrophilic moieties. The hydrophilic moiety can be carbohydrate, amino acid, phosphate group or some other compounds, whereas the hydrophobic moiety usually is a long chain fatty acid.

**Biosurfactants** are being investigated as replacements for synthetic surfactants **because** they are biodegradable, less sensitive to extreme environments and can be produced on renewable substrates.

**The potential applications** of biosurfactants in industrial include emulsification and foaming for food processing, wetting and phase dispersion for cosmetics and textiles, or solubilization for agrochemicals and in pharmaceutical products. In addition, biosurfactants can be used in environmental applications such as bioremediation and dispersion of oil spills.

**Biosurfactants** can be divided into **4 groups** based on their overall structures. They are glycolipids, phospholipids, lipoproteins or lipopeptides.

**Biosurfactants** can be commercially produced at levels of up to 100 g/L, as reported for rhamnolipids from *Pseudomonas sp.* This production level, combined with the use of cheap renewable substrates as organic wastes, makes the cost of biosurfactants competitive with the cost of synthetic surfactants.

**Alternative substrates** have been suggested for biosurfactant production, especially water-miscible agro-industrial wastes: molasses, whey, distillery wastes. However, there are few examples of the use of hydrophobic wastes as cheap substrates, for example, waste frying oils, used lubricant oils and oily sludge from petroleum refineries.

**It is reported** in the literature that the genus ***Pseudomonas*** and ***Serratia*** are capable of using different substrate, such as hydrocarbons, vegetable oils, olive oils, n- paraffin's to produce biosurfactants.

## Procedure:

### 1. preparation of inoculums:

The isolates of ***Pseudomonas aeruginosa*** or ***Serratia marcescens*** were grown in nutrient broth for 16–18 h 30 ˚C. This culture was used as stock culture inoculums at the 1% (v/v) level.

### 2. Preparation of mineral salt medium

A mineral salt medium with the following composition (g/L) was used for biosurfactant production: K2HPO4 (1), KH2 PO4 (1), Mg SO4 · 7H2O (0.6), Fe SO4 · 7H2O (0.01), NaCl (0.05), CaCl2 (0.02), yeast extract (0.5). The pH of the medium was adjusted to 7.0.

**Media and cultivation conditions for biosurfactant production:**

1. Add 50 ml of mineral salt medium into 250 ml conical flask. Then add 1% of olive oil as source of carbon to each flask, and then sterilize the medium.
2. Add 1% of grown bacterial culture in nutrient broth to each conical flask, and then incubate the flasks in a rotary shaker incubator at 150 rpm for 72 h at 30 °C.
3. After the incubation ending, the flasks removed from the shaker incubator. Record your notes from visual observation in the flasks.
4. Extraction and estimation of Crude biosurfactant:
    - **a.** The content of the culture transfers into 50 ml centrifuge tubes. The culture medium was centrifuged at 10.000 rpm at 4°C for 15 min.
    - **b.** The culture inside the centrifuge tube separated into three layers: the oil layer located at the surface of tubes, then tha supernatant and the biomass at the bottom.
    - **c.** The supernatant extract by using the organic solvent system chloroform: methanol (2:1, v/v); or diethyl either. The extract transfer into weighted container.
    - **d.** The extract concentrated and dried at room temperature and reweighed. The results calculated as:

**Biosurfactant** (g/l) = **weight of biosurfactant / sample volume × 1000**

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## Bioabsorbtion of heavy metal by microorganism

The discharge of heavy metals into aquatic ecosystems has become a matter of concern in many countries over the last few decades. These pollutants are introduced into the aquatic systems significantly as a result of various industrial operations. The pollutants of concern include lead, chromium, mercury, uranium, selenium, zinc, arsenic, cadmium, gold, silver, copper and nickel. These toxic materials may be derived from mining operations, refining ores, sludge disposal, fly ash from incinerators, the processing of radioactive materials, metal plating, or the manufacture of electrical equipment, paints, alloys, batteries, pesticides or preservatives. Heavy metals such as zinc, lead and chromium have a number of applications in basic engineering works, paper and pulp industries, leather tanning, organochemicals, petrochemicals fertlisers, etc. Major lead pollution is through automobiles and battery manufacturers. For zinc and chromium the major application is in fertliser and leather tanning respectively. Over the few decades, several methods have been devised for the treatment and removal of heavy metals.

Measurements were made of the adsorption of lead, cadmium, zinc, nickel and uranium per unit surface area of microorganisms, two clays (reference smectite, kaolinite), and two soils (untreated Vertisol and treated Vertisol) to quantify the selective affinity. The adsorption of heavy metals and uranium by the samples was influenced by soil constituents, and increased with increasing Pb, Ni, Zn, Cd and U concentrations. Microorganisms were also compared with these abiotic soil constituents for their ability to adsorb these metals. Dead cells adsorbed the largest quantity of all heavy metals than live cells and other soil components. At pH 6.0, the uptake percentage of U(VI) by dead cells was higher than that of any of the other metal ions. On the basis of amounts of adsorption on adsorbents, the selectivity sequences were varied. The most common sequences were $U \gg Pb > Cd \approx Zn > Ni$. The results verified the importance of geochemical parameters of soils such as type of clay mineral, oxide mineral content, and organic content, for controlling metal uptake. The results also suggest that bacterial membrane cells can be successfully used in the treatment of mixed metal-contaminated wastes. .

An investigation of the removal and recovery of uranium from aqueous systems using microbial biomass has been described previously. To establish which microorganisms accumulate the most uranium, we

extended our investigation of uranium uptake to 83 species of microorganisms, 32 bacteria, 15 yeasts, 16 fungi and 20 actinomycetes. Of these 83 species of microorganisms tested, extremely high uranium-absorbing ability was found in ***Pseudomonas stutzeri***, ***Neurospora sitophila***, ***Streptomyces albus*** and ***Streptomyces viridochromogenes***.

The selective accumulation of heavy metal ions by various microorganisms has also been examined. Uranyl, mercury and lead ions were readily accumulated by almost all the species of microorganisms tested. Actinomycetes and fungi differ from many bacteria and most yeasts in their selective accumulation of uranium and mercury.

In addition to this fundamental research, uranium recovery was investigated in immobilized ***Streptomyces albus***, a microorganism with high uranium-uptake ability. These immobilized cells adsorbed uranium readily and selectively. The immobilized cells recovered uranium almost quantitatively and almost all uranium absorbed was desorbed with 0.1 M $Na_2CO_3$. The dry weight of the free cells decreased by 50% during 5 adsorption-desorption cycles. However, the dry weight of the immobilized cells decreased by only 2% during 5 cycles. These results showed that microbial cells are more stable after immobilization and can be used repeatedly for the process of uranium adsorption-desorption.

## Factors affecting Biosorption :

The investigation of the efficacy of the metal uptake by the microbial biomass is essential for the industrial application of biosorption, as it gives information about the equilibrium of the process which is necessary for the design of the equipment. The metal uptake is usually measured by the parameter 'q' which indicates the milligrams of metal accumulated per gram of biosorbent material and 'qH' is reported as a function of metal accumulated, sorbent material used and operating conditions.

The following factors affect the biosorption process:

1. Temperature seems not to influence the biosorption performances in the range of 20-35 $^0$C.

2. pH seems to be the most important parameter in the biosorptive process: it affects the solution chemistry of the metals, the activity of the functional groups in the biomass and the competition of metallic ions .

3. Biomass concentration in solution seems to influence the specific uptake: for lower values of biomass concentrations there is an increase in the specific uptake. Gadd et al. 1988 suggested that an increase in

biomass concentration leads to interference between the binding sites. Fourest and Roux, 1992 invalidated this hypothesis attributing the responsibility of the specific uptake decrease to metal concentration shortage in solution. Hence this factor needs to be taken into consideration in any application of microbial biomass as biosorbent.

4. Biosorption is mainly used to treat wastewater where more than one type of metal ions would be present; the removal of one metal ion may be influenced by the presence of other metal ions. For example: Uranium uptake by biomass of bacteria, fungi and yeasts was not affected by the presence of manganese, cobalt, copper, cadmium, mercury and lead in solution. In contrast, the presence of $Fe^{2+}$ and $Zn^{2+}$ was found to influence uranium uptake **by *Rhizopus arrhizus***. And cobalt uptake by different microorganisms seemed to be completely inhibited by the presence of uranium, lead, mercury and copper.

## Use of Recombinant bacteria for metal removal:

Metal removal by adsorbents from water and wastewater is strongly influenced by physico-chemical parameters such as ionic strength, pH and the concentration of competing organic and inorganic compounds. Recombinant bacteria are being investigated for removing specific metals from contaminated water. For example a genetically engineered ***E.coli***, which expresses $Hg^{2+}$ transport system and metallothionin (a metal binding protein), was able to selectively accumulate 8 μmole $Hg^{2+}$***/g*** cell dry weight. The presence of chelating agents $Na^{+}$, $Mg^{2+}$ and $Ca^{2+}$ did not affect bioaccumulation.

## Choice of metal for biosorption process:

The appropriate selection of metals for biosorption studies is dependent on the angle of interest and the impact of different metals, on the basis of which they would be divided into four major categories: (i) toxic heavy metals (ii) strategic metals (iii) precious metals and (iv) radio nuclides. In terms of environmental threats, it is mainly categories (i) and (iv) that are of interest for removal from the environment and/or from point source effluent discharges.

Apart from toxicological criteria, the interest in specific metals may also be based on how representative their behaviour may be in terms of eventual generalization of results of studying their biosorbent uptake. The toxicity and interesting solution chemistry of elements such as chromium, arsenic and selenium make them interesting to study. Strategic and

precious metals though not environmentally threatening are important from their recovery point of view.

## Procedure(1):

A method to remove heavy metals concentrations in water down to very low levels is described. The method calls for the addition of a soluble sulfide to the water. This is followed adding a soluble iron reagent such as ferrous sulfate or ferrous chloride. The water is aerated. As an alternative to aeration, the pH of the water can be increased. Finally, the solids generated from the above steps are separated from the water. This method has been shown to remove heavy metals, particularly copper and zinc, from actual industrial wastewater to very low concentrations, i.e., below about 100 ppb. Furthermore, the treated water is free of sulfide. The byproduct sludge comprises iron sulfide, iron oxides, iron hydroxides, and the heavy metal sulfides.

## Procedure(2):

The jar test was used to determine the optimum treatment conditions for removal of heavy metals in four synthetic wastewaters. Each wastewater contained a single heavy metal, Hg, Ag, Cr or Fe, at the concentrations of 153, 214, 37 and 135 mg/l, respectively. The synthetic wastewaters were prepared from $HgSO_4$, $Cr_2(SO_4)3.H_2O$, $Ag_2SO_4$ and $Fe(NO_3)3.9H_2O$, respectively. The organic precipitant used in this research was non-toxic dithiocarbamate complexes with a chelating group of $–NH\text{-}CS_2Na$. The organic precipitant solution used for experimentation was prepared by diluting the stock solution by 50 times. The pH and ORP values of the solution were 11.5 and -500 to -520 mV, respectively. Firstly, the pH of the synthetic wastewaters was adjusted by adding 50% (w/v) NaOH solution, to attain pH values ranging from 0.4 to 10. A fixed amount of dithiocarbamates was then added. The jar test conditions were 30-min rapid mix and 20- min slow mix. Dissolved and total concentrations in the effluents after 30-min settling were measured to determine the optimum initial pH condition. Then, the optimum dosage of dithiocarbamates was determined by adding various dosages according to the stoichiometric values for the reactions with Hg and Ag, as shown in Equations (1) and (2). $R–NH\ CS_2Na\ .\ 2\ R–NH\text{-}CS_2Hg + Na_2SO_4$ (1) Stoichiometric value for Hg: –NH-CS2Na is 1: 0.82 (by weight) $R–NH\text{-}CS_2Na\ .\ 2\ R–NH\text{-}CS_2\ Ag + Na_2SO_4$ (2) Stoichiometric value for Ag : –NH-CS2Na is 1: 1.53 (by weight) Consequently, experiments were conducted with real wastewater samples obtained from open-reflux COD

analysis of the environmental laboratory at Chulalongkorn University, Thailand. The treatment conditions determined from the synthetic wastewater experiments were adopted. The real wastewater was diluted with tap water by 10 folds prior to experimentation. This sufficient dilution helped to prevent danger from pH adjustment of the extremely strong acid wastewater with NaOH. In reality, dilution of this COD analysis wastewater with basic (alkaline) wastewater is also expected in waste treatment facilities. The final concentrations of the diluted wastewater were 201, 182, 46.4 and 138 for Hg, Ag, Cr and Fe, respectively. Measurements of pH, ORP and concentrations of dissolved and total heavy metals were conducted with untreated wastewater, pH-adjusted wastewater and treated supernatant effluent after 30-minute settling. An atomic absorption spectrometer (Model: Analyst 800, Perkin Elmer Co.) with a hydride generation method was used for Hg analysis, while an atomic absorption spectrometer with a direct air-acetylene flame method was adopted for analysis of Cr, Ag and Fe.