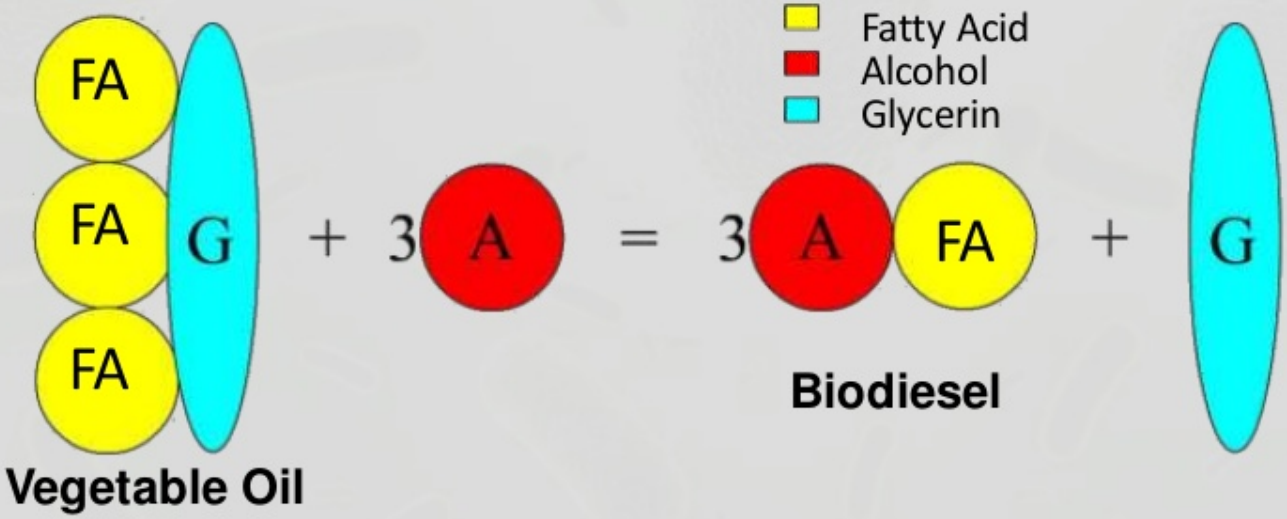
# Environmental Microbiology

Course 11: Biodiesel

Assoc. Prof. Dr. Emrah Şefik Abamor

# What is Biodiesel?

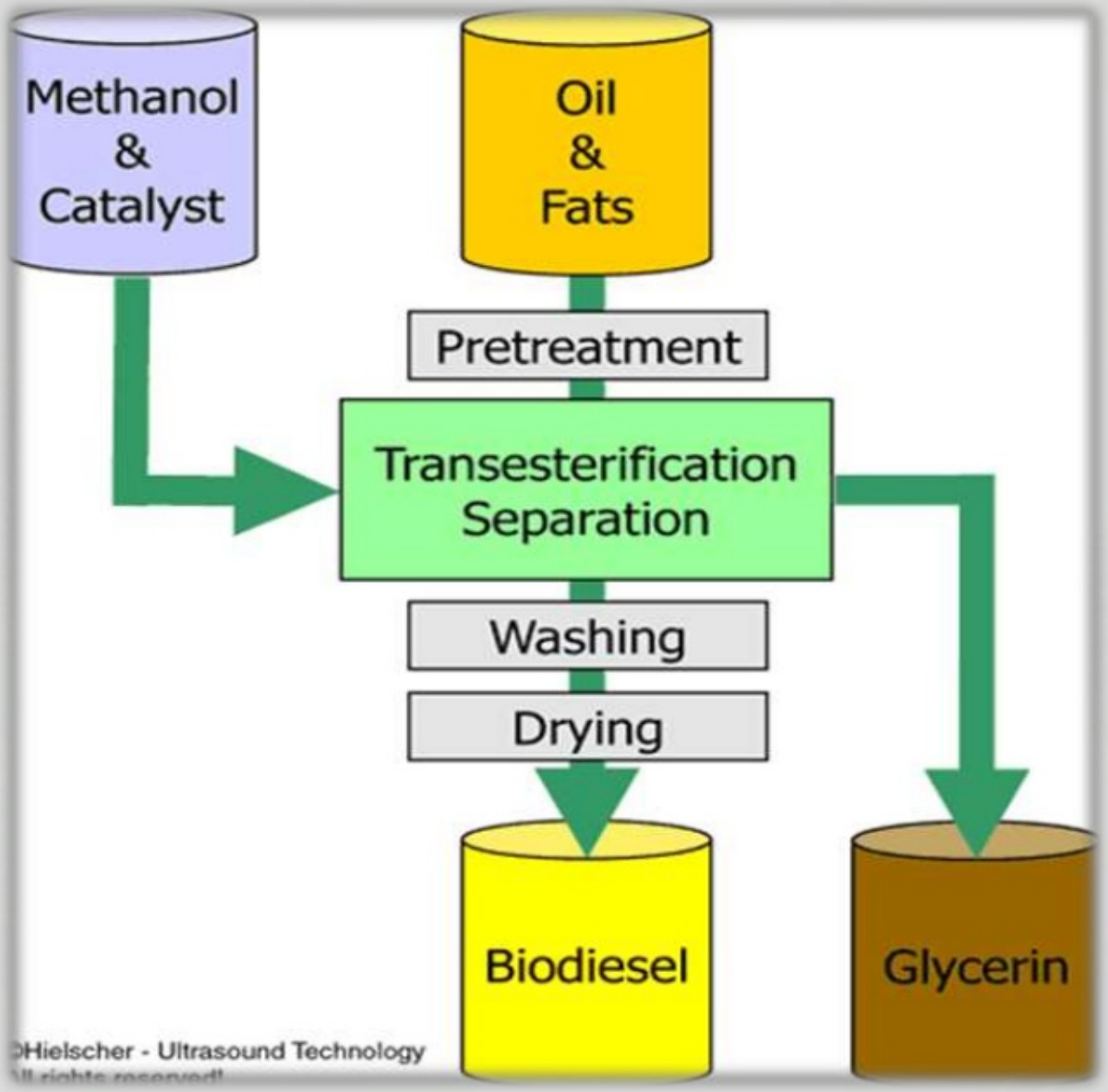
- Alternative fuel for diesel engines
- Made from vegetable oil, animal fat, microbes
- Lower emissions, High flash point (>300°F), Safer
- Biodegradable, Essentially non-toxic.



# Biodiesel is preferable to petrodiesel

- Environmental friendliness
- Renewability
- Reduced emissions
- Higher combustion
- Efficiency
- Improved lubricity
- Higher levels of safety

# Biodiesel Production Process




# Trans esterification

$$\begin{array}{lcccl} CH_2OOR1 & & \text{catalyst} & & CH_2OH \\ | & & \downarrow & & | \\ CHOOR2 & + \; 3CH_3OH & \Leftrightarrow & 3CH_3OORx & + \; CHOH \\ | & & & & | \\ CH_2OOR3 & & & & CH_2OH \end{array}$$

*Triglyceride* *3 Methanols* *Biodiesel* *Glycerin*

- R1, R2, and R3 are fatty acid alkyl groups.

# Microorganisms available for biodiesel production

- Alternative source of feedstock investigated for biodiesel production:
  - ❑ Microalgae
  - ❑ Bacillus
  - ❑ Fungi
  - ❑ Yeast

Lipids from all cannot be converted into biodiesel mainly due to ***less yield***

Oleaginous microorganisms are able to accumulate lipids above the 20% of their biomass, on dry basis.

- Biodiesel production using microbial lipids, which is named as single cell oils (SCO), has attracted great attention in the whole world.

# Single cell oil (SCO)

- Lipids obtained from microbes or single celled entity.

- Obtained from yeast, bacteria and microalgae.

- Lipid accumulation by micro-organisms depends on;
  - ❑ ***Genetic constituents***
  - ❑ ***Different culture conditions (pH, Temp, culture time, etc.)***

***"Single cell oil might be defined as the edible oils obtainable from microorganisms being similar in type and composition to those oils and fats from plants or animals"***

# What is Single Cell Oils?

- *Single Cell Proteins?*
- *Single Cell Oils (SCO):* triglyceride fats generated by microorganisms.
  - Lipid components are similar to those found in plant and animal.

→ *Applications*: animal feeds, aqua feeds, biodiesel.

# Oleaginous microorganism

- Microorganism that gathers >20-25% of their biomass as oil.

- Many yeast, fungi and several algae are maximum producers.
  - Algae → biodiesel
  - Yeast and fungi → edible oil
    - They are also capable of producing high levels of nutritionally important polyunsaturated fatty acids (PUFAs).

# Advantages of Oleaginous microorganism

- Growth on various substrates

→ utilizing by-product = reduce cost

- Ability to synthesize a divert

array of fatty products

→ many useful applications.

- Able to be genetic manipulation

→ selection for highest productivity.

# Condition for lipid accumulation

- Lipid accumulation is trigger when
  - Nutrients in the growth medium (usually Nitrogen source) is exhausted
  - Surplus of Carbon source (usually glucose)

→ Cells stop multiplying.

→ Cells convert Carbon source to Storage oils or fats.

# Condition for lipid accumulation

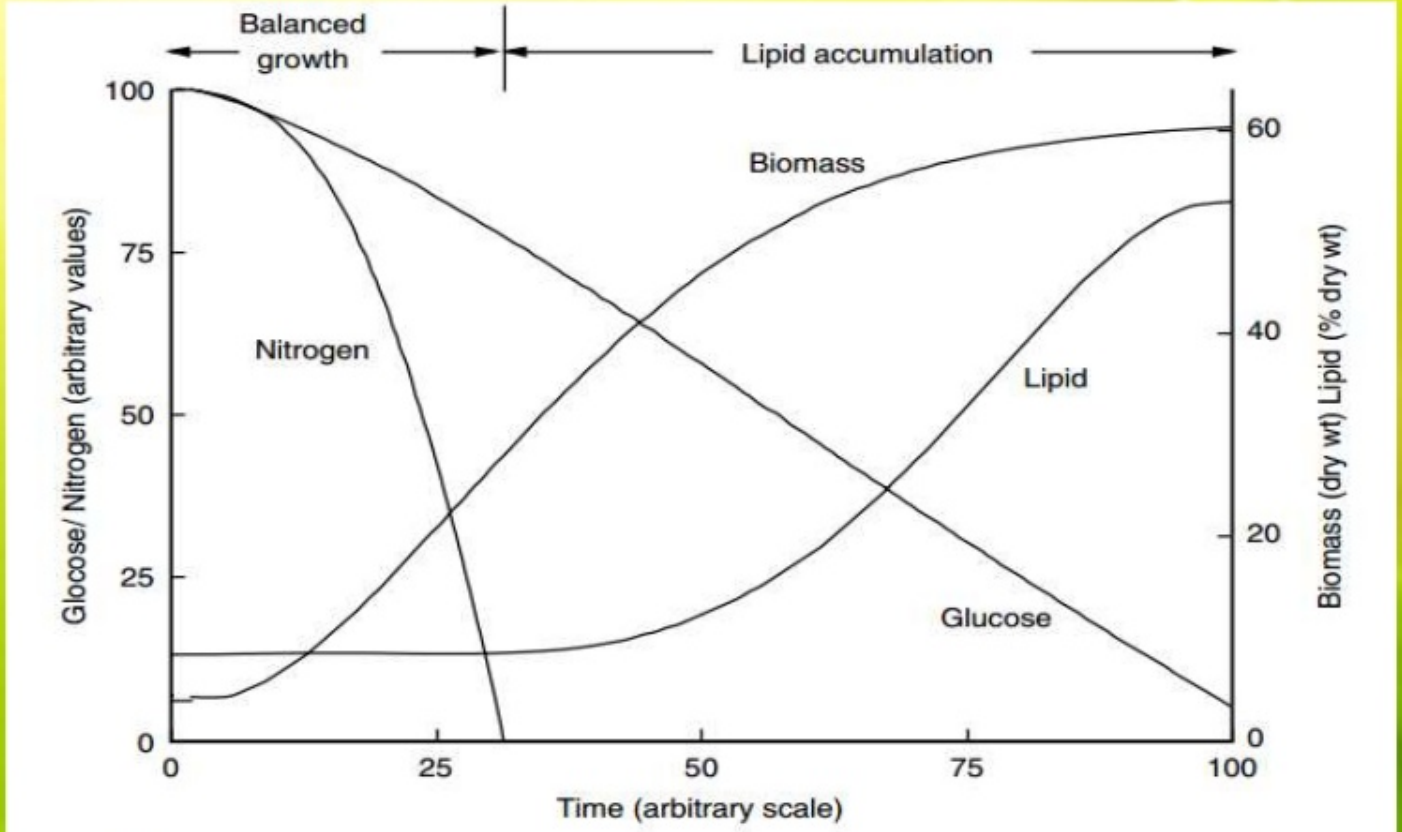



**Figure 17.1** The course of lipid accumulation by a typical oleaginous microorganism. The concentration of nitrogen (usually $NH_3$) in the medium is adjusted in the initial formulation so that it becomes exhausted after about the first 24 to 36 hours' growth.

# Microalgae

- Prokaryotic or eukaryotic photosynthetic microorganisms
- Can grow rapidly
- Live in harsh conditions due to their unicellular or simple multicellular structure
- Good candidates for biodiesel production,
  - ❑ higher photosynthetic efficiency
  - ❑ higher biomass production and
  - ❑ faster growth compared to other energy crops

# Continue..

- Can be induced to accumulate substantial quantities of lipids thus contributing to a high oil yield.

- Some of the common algae

  ***Chlorella***, *Crypthecodinium, Cylindrotheca, Dunaliella, Isochrysis, Nannochloris, Nannochloropsis, Neochloris, Nitzschia, Phaeodactylum, Porphyridium, Schizochytrium, Tetraselmis*

AVERAGE LIPID CONTENT- (1-70%)

MAXIMUM- 90%

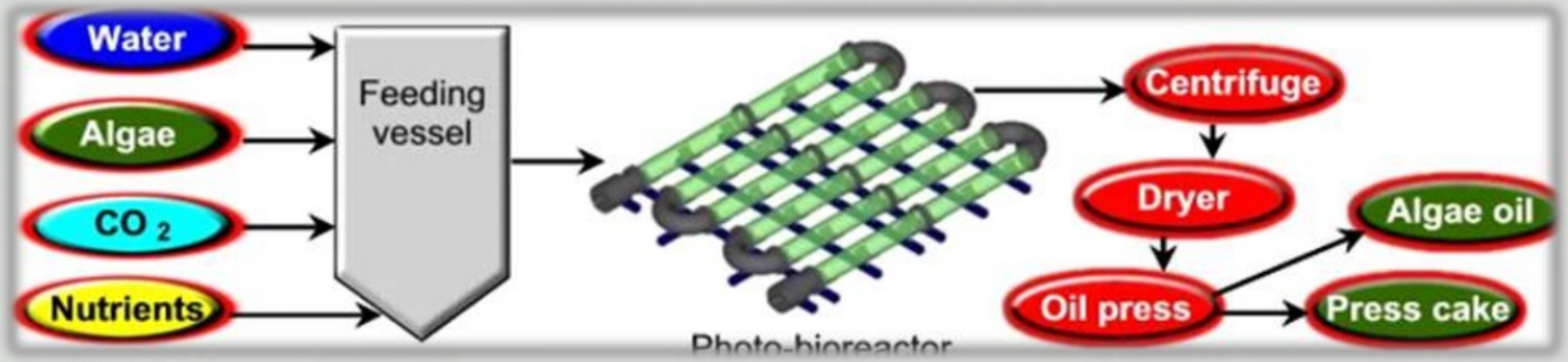
# How to obtain biodiesel from algae?

- Picking up the best algae
- Growing the algae
- Extraction
- Trans esterification
- Biodiesel

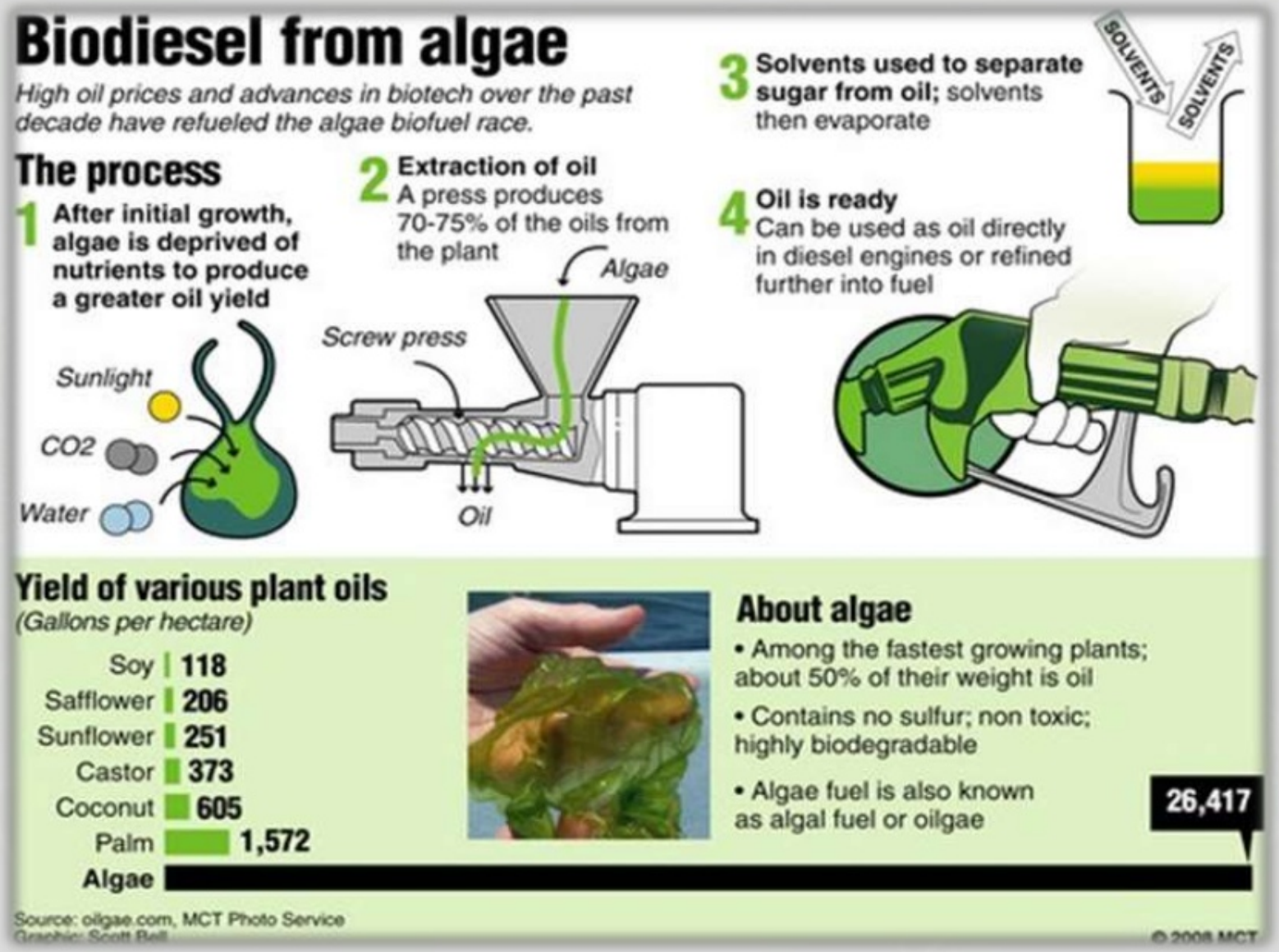
# Extraction

- ✓ Solvent extraction
- ✓ Mechanical extraction
- ✓ Super critical fluid extraction

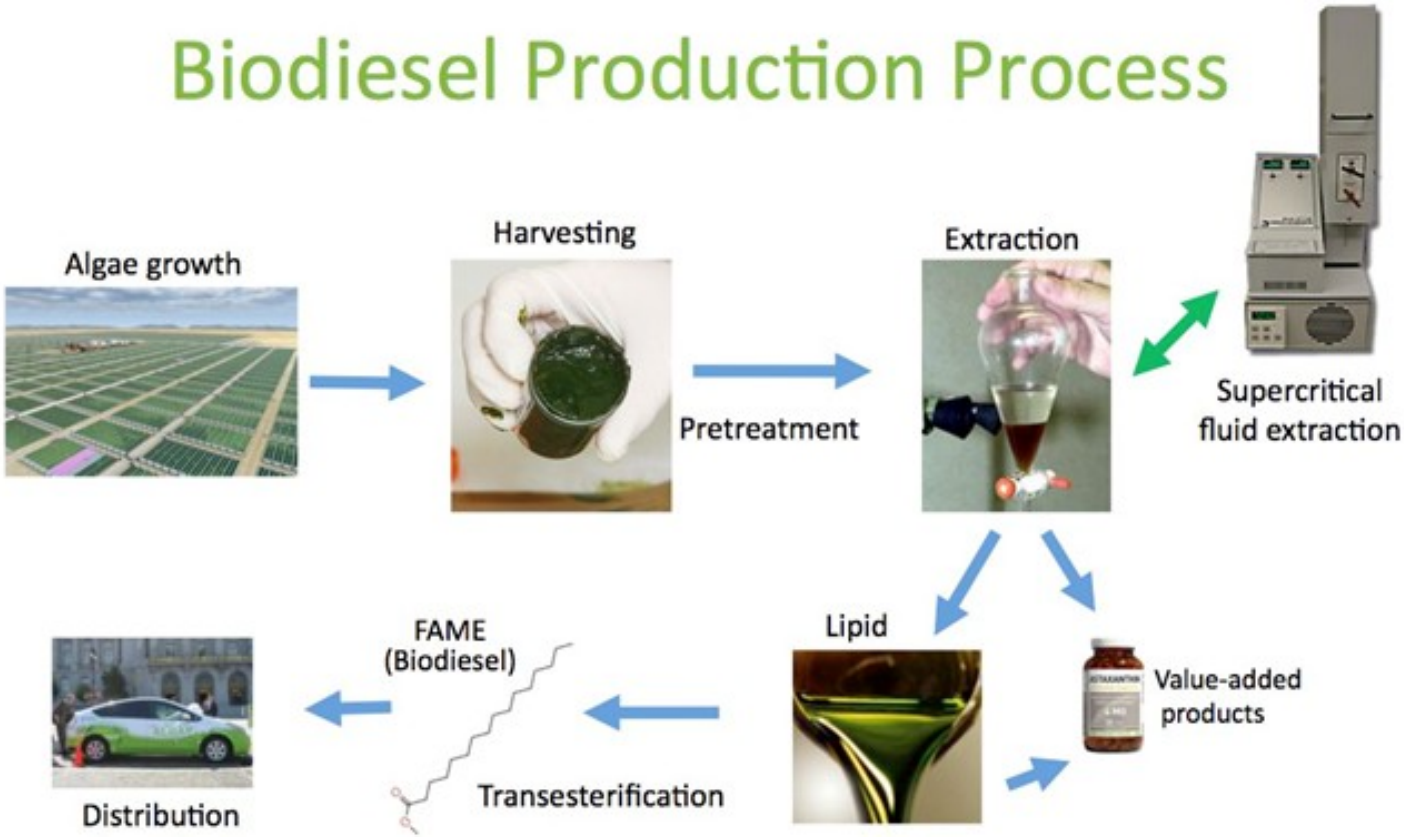
# Mechanical Extraction



# Solvent extraction



# Biodiesel Production Process



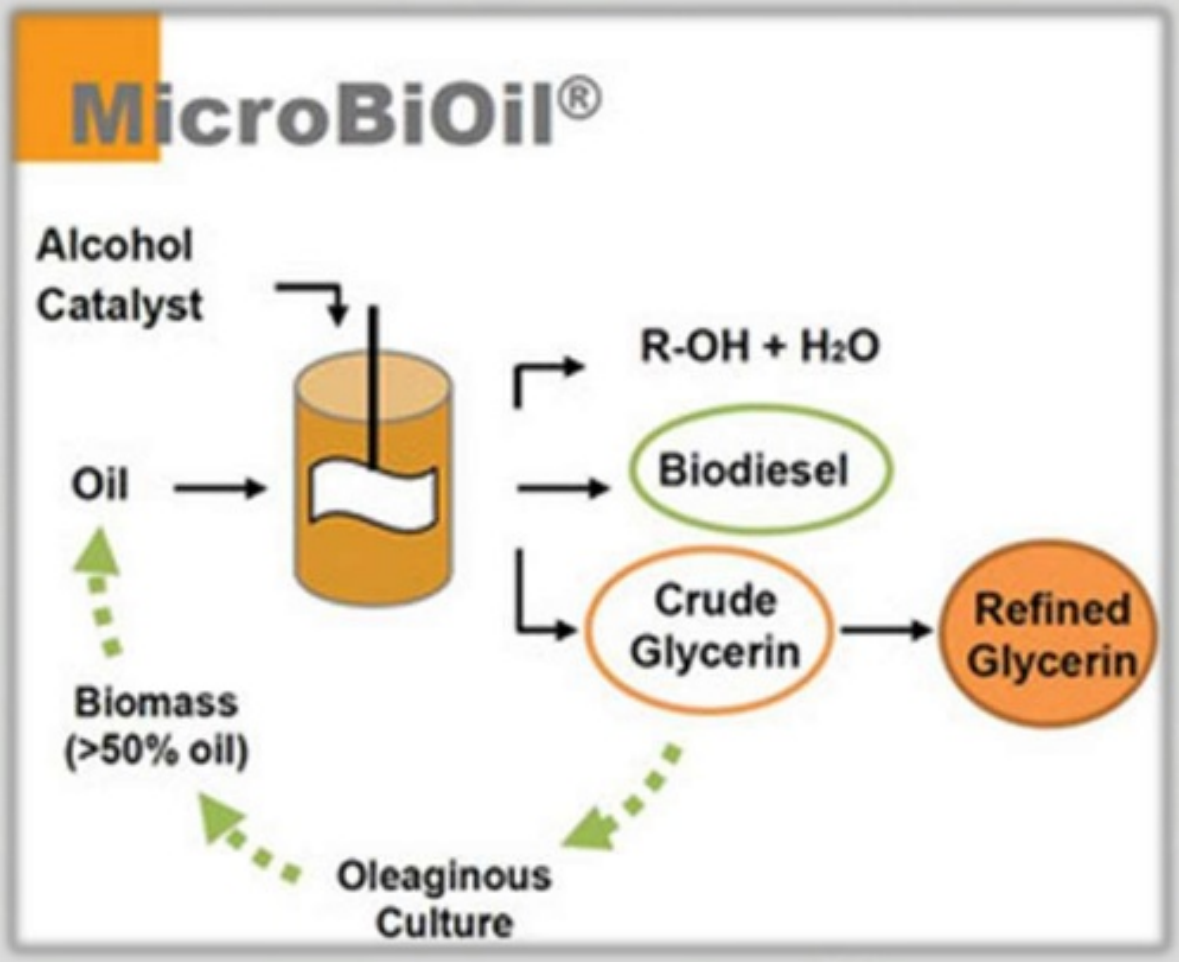
# Bacteria

- Bacteria can accumulate oil of about 20-40% of dry biomass
  - ❑ *Arthrobacter sp.* - 40%
  - ❑ *Acinetobacter calcoaceticus* - 38%

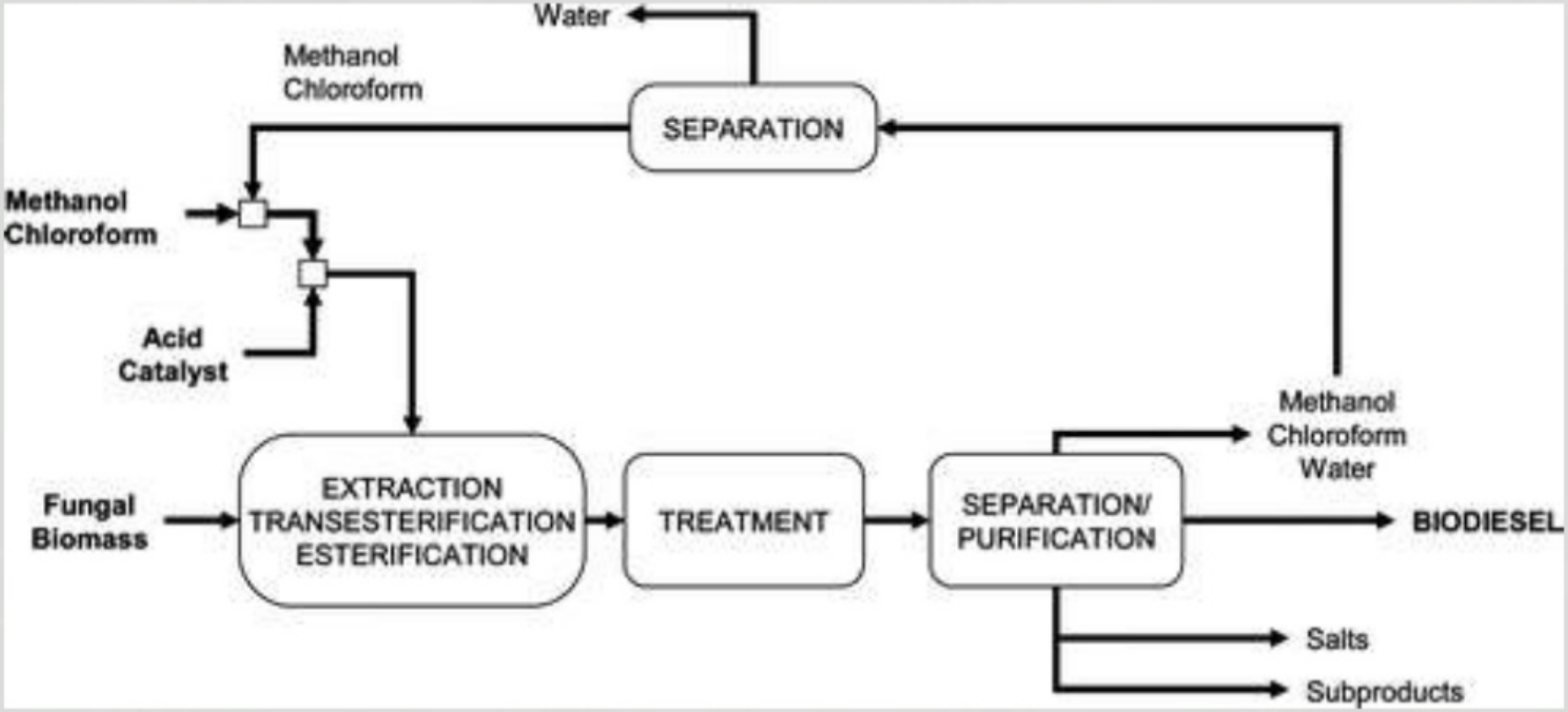
- Have a superiority in the production of biodiesel due to
  - ❑ Highest growth rate (reach huge biomass only need 12–24 h)
  - ❑ Easy culture method.

- Actinomycete group → high amount of fatty acids (up to 70% of the cellular dry weight)→ using **glucose** under growth-restricted condition.

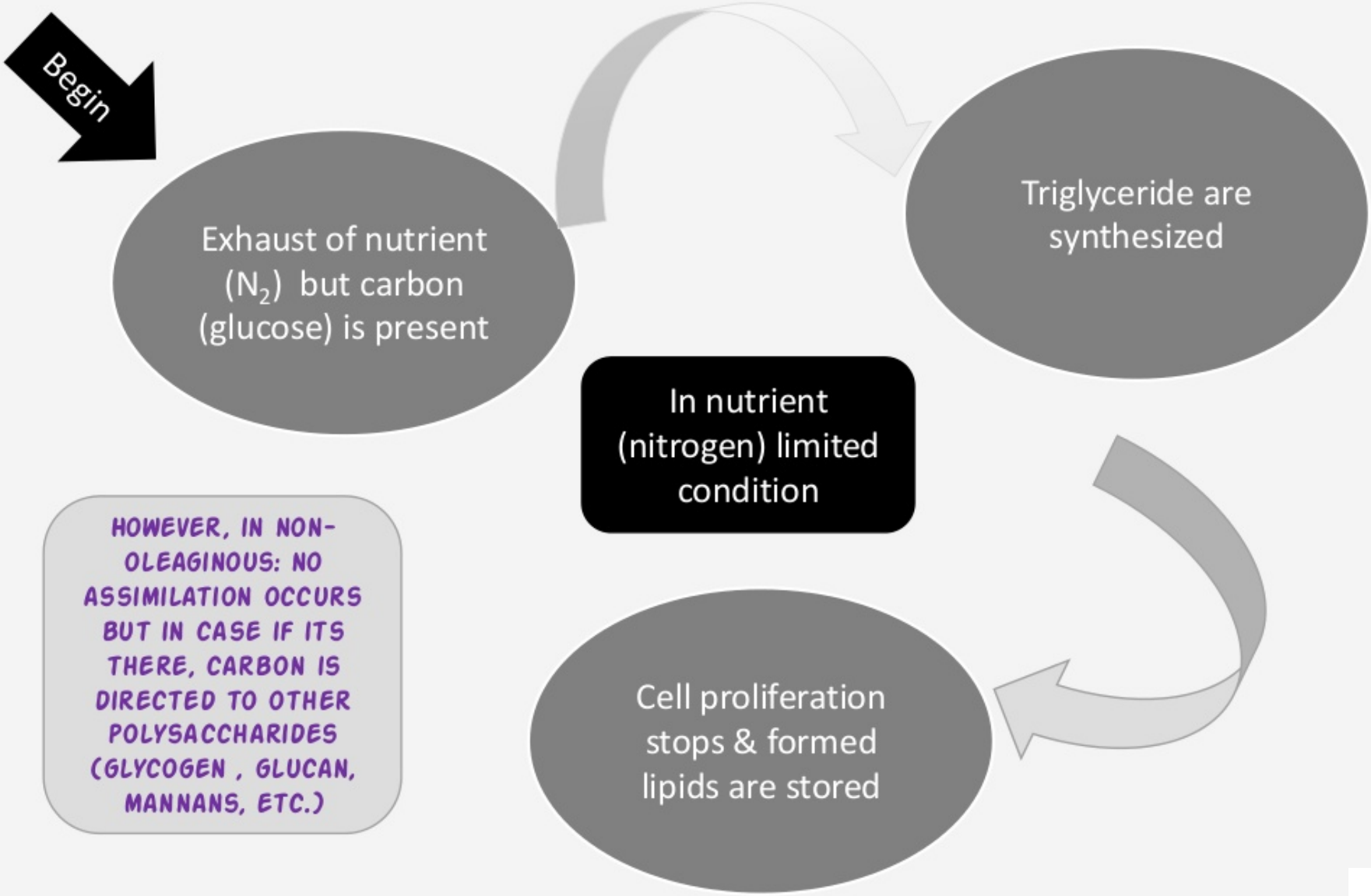
# Biodiesel from microorganism



# Biodiesel from Fungal Bio Mass



# How is lipid accumulated in oleaginous?




HOWEVER, IN NON-OLEAGINOUS: NO ASSIMILATION OCCURS BUT IN CASE IF ITS THERE, CARBON IS DIRECTED TO OTHER POLYSACCHARIDES (GLYCOGEN , GLUCAN, MANNANS, ETC.)

# Stages of Lipid accumulation in Batch culture

Prepare medium: high Carbon, low Nitrogen

↓

Microorganisms grow and multiply until a certain time

↓

Exhaustion of N, cells stop divide

↓

Cells convert C → storage lipid (intracellular)

↓

Lipid accumulation → cells expand

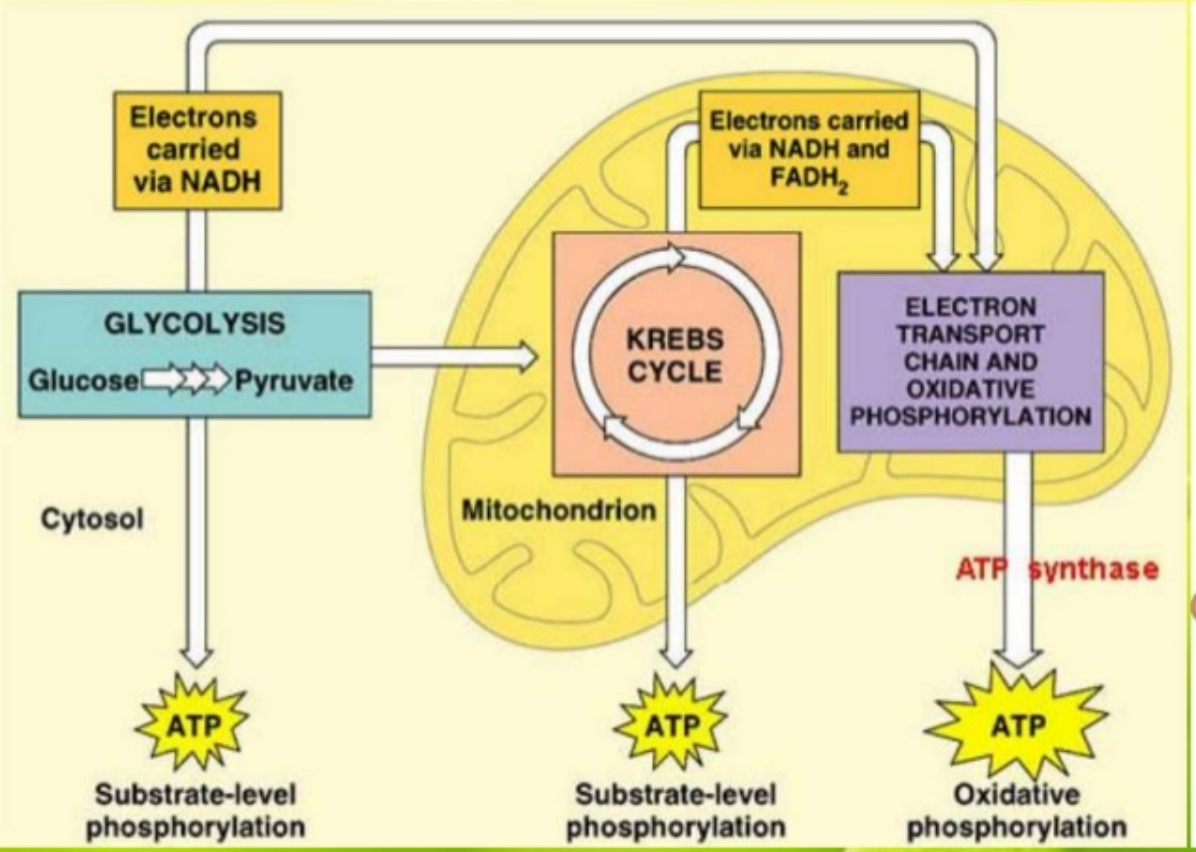
↓

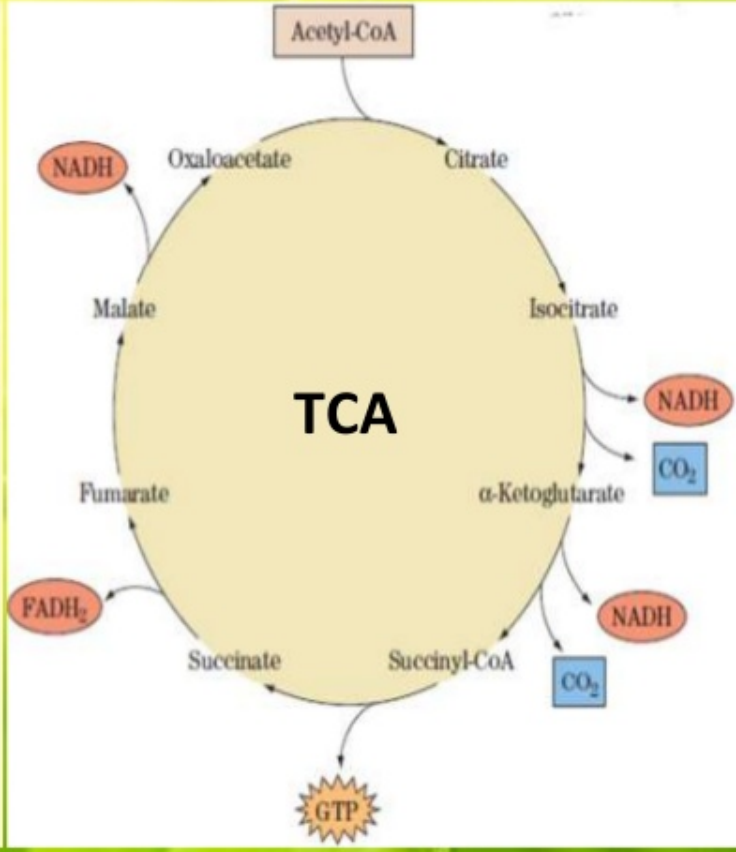
Cells reach limit of obesity → stop accumulating.

↓

...

# Biochemistry of Lipid accumulation




Cellular respiration of microorganism

# Biochemistry of Lipid accumulation

Exhaustion of N

↓

[AMP] is diminished;

Isocitrate dehydrogenase in mitochondrion stop working

↓

TCA is disrupted

↓

Citric acid in mitochondrion → cytosol

↓

**Enzyme ATP-citrate lyase:** Citric acid → acetyl-CoA
*(Oleaginous microorganisms only)*

↓

Acetyl-CoA →(fatty acid biosynthesis)→ triacylglycerols

# Biochemistry of Lipid accumulation

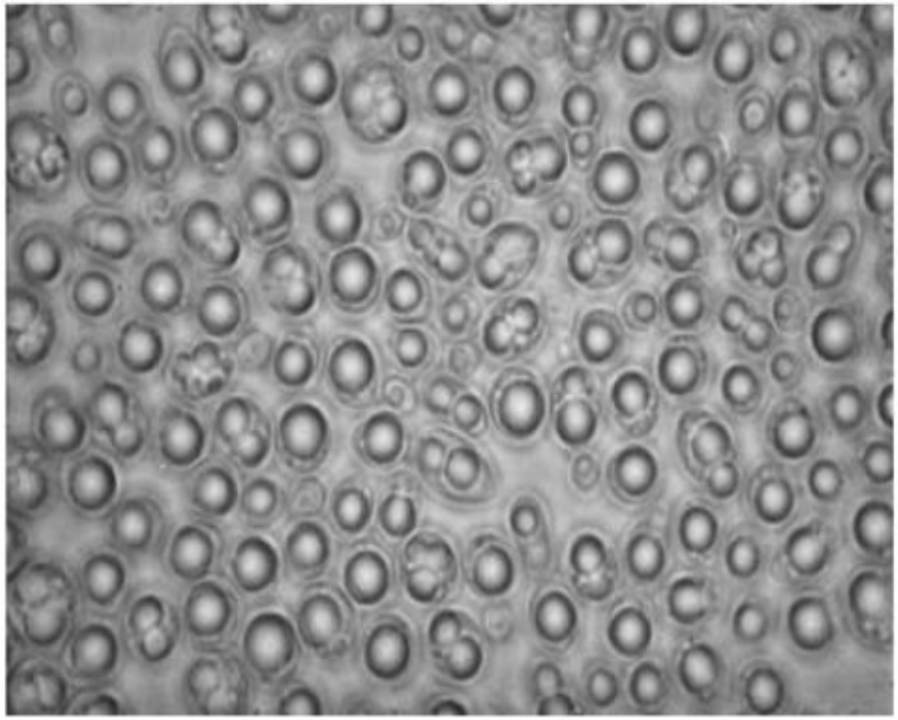



**Figure 17.2** Cells of an oleaginous yeast, *Cryptococcus curvatus* (formerly *Candida curvata* D), showing the accumulation of lipid in the form of discrete intracellular droplets.

# Activities that are unique to oleaginous organisms

Activity of ***Isocitrate dehydrogenase*** as a component of the TCA cycle is dependent on the presence of **AMP→** This allow **citric acid** accumulation during nitrogen limited condition

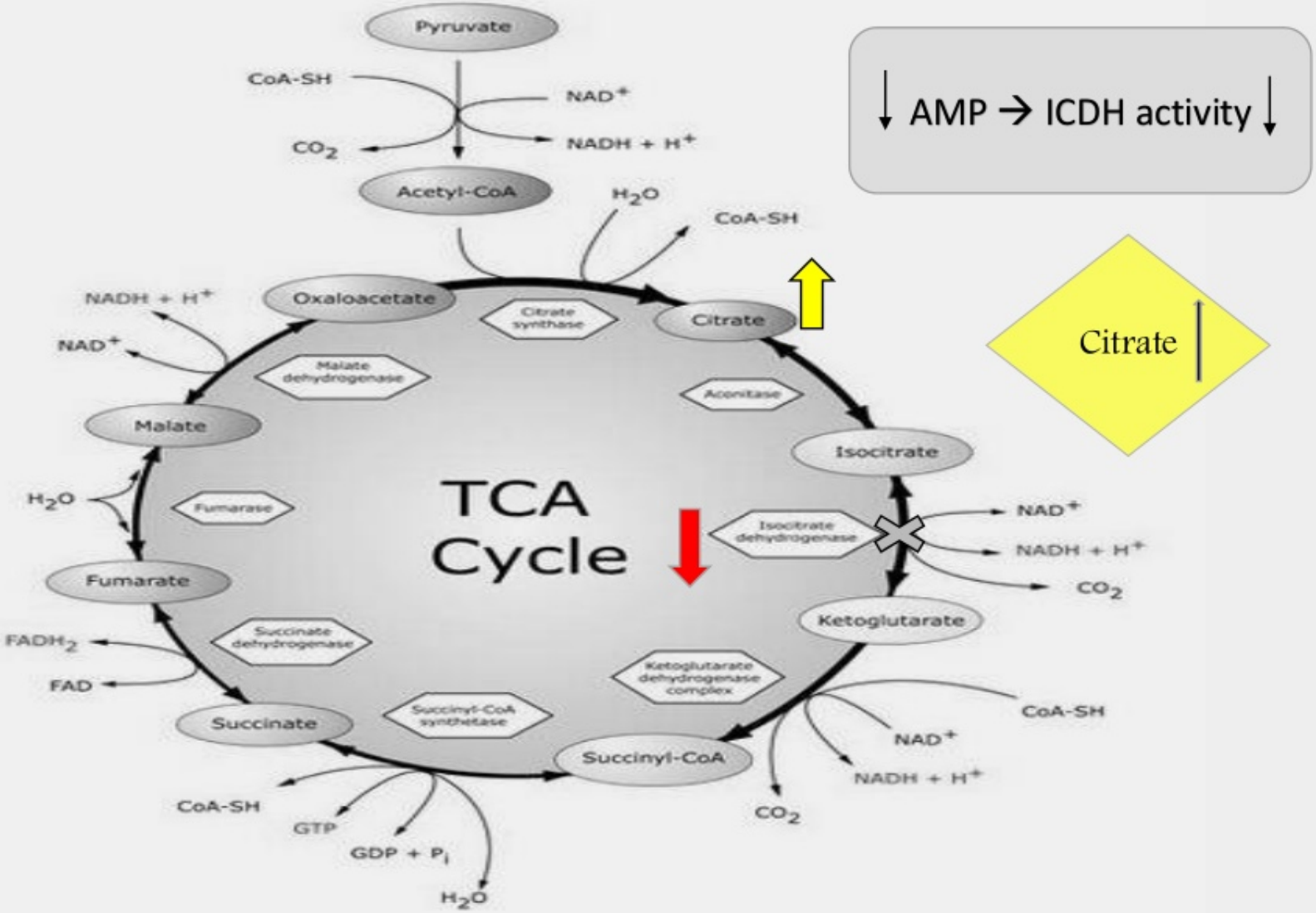
**AMP → inosine 5'- monophosphate + $NH_3$** Catalysed by: AMP deaminase

**Isocitrate ⟷ cis-aconitate ⟷ citrate** Catalysed by: aconitase

The formation of acetyl-CoA in oleaginous microorganisms has been attributed to the presence of ***ATP: citrate lyase*** (ACL, reaction no. 1) which does not appear to occur in the majority of non-oleaginous species.

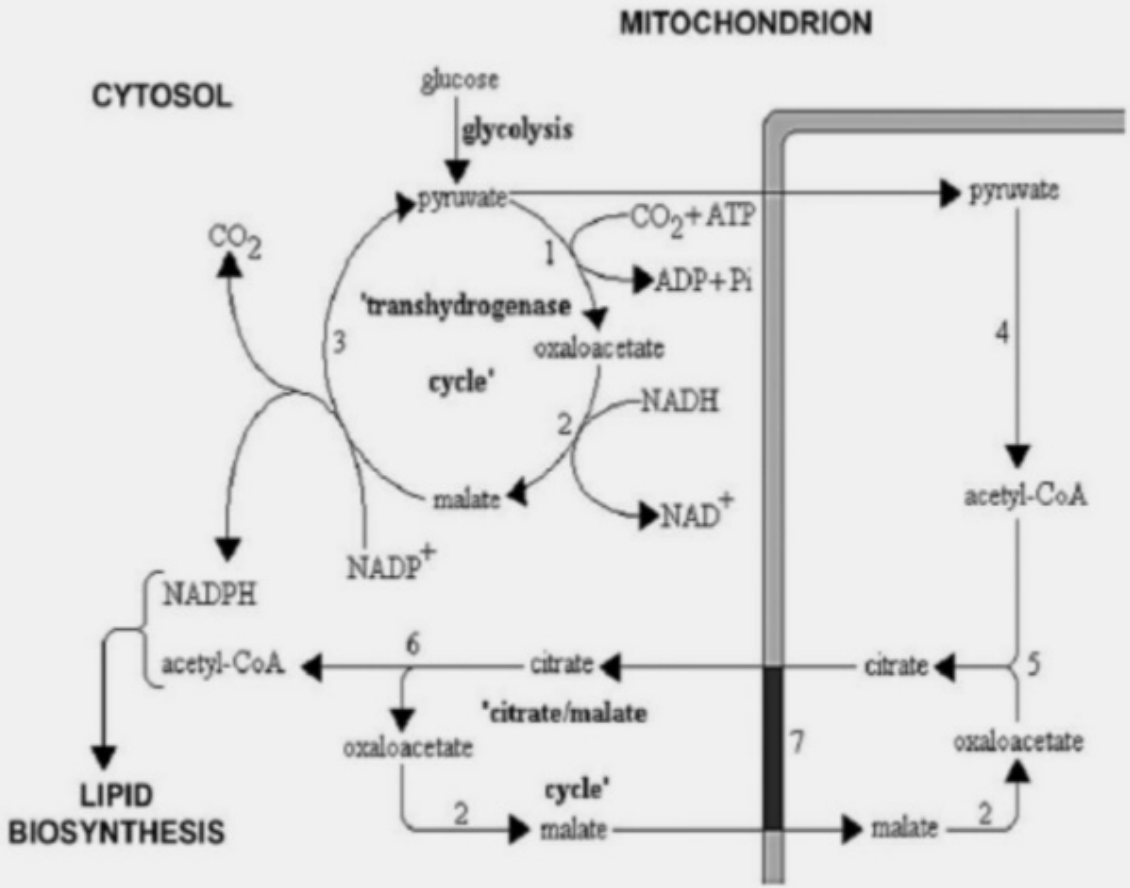
**Citrate+ CoA + ATP → acetyl- COA + Oxaloacetate + ADP + $P_i$**

# UNDER NITROGEN LIMITED CONDITION



# Biochemistry of Lipid Accumulation

Colin Ratledge (2004)




A scheme to show how the citrate/malate cycle and the cytosolic 'transhydrogenase' cycle could provide sufficient precursors of acetyl-CoA and NADPH for lipogenesis in oleaginous microorganisms (from Refs. [14,15]). Enzymes: 1, pyruvate decarboxylase; 2, malate dehydrogenase; 3, malic enzyme; 4, pyruvate dehydrogenase; 5, citrate synthase; 6, ATP:citrate lyase; 7, citrate/malate translocase. Net carbon balance: pyruvate → acetyl-CoA + $CO_2$. Net reaction for NADPH production: NADH + $NADP^+$ + ATP → $NAD^+$ + NADPH + $P_i$. The transhydrogenase cycle can operate independently of the carbon flux from citrate in the mitochondrion to acetyl-CoA in the cytosol and consequently can provide all the NADPH needed for both fatty acid biosynthesis and fatty acid desaturation and elongation reactions.

# Variation in the amount of lipid produced

- Two critical regulated enzymes, including **malic enzyme** and **ATP: citrate lyase (ACL),** have effect on lipid accumulation.

- Strong correlation between the presence of **ACL activity** and the **ability to accumulate lipid** in yeasts, fungi and other oleaginous microorganisms.

- Some yeast however have ACL activity but no high lipid accumulating ability.

"ACL ACTIVITY IS A PREREQUISITE BUT NOT THE SOLE FACTOR"

- Hence, other enzymes must also be responsible for controlling the extent of lipid biosynthesis in individual microorganisms.

## THE TOTAL MASS OF MICROBIAL LIPID IS ALSO REGULATED BY THE CONTENT OF FATTY ACID

- There are some different kinds of enzymes controlling fatty acid synthesis.

  Example:
  **Acetyl-CoA carboxylase (ACCase) → Rate limiting enzyme → catalyzes the first reaction of synthesis of fatty acid in microorganisms**

- Use of biotechnological tools to enhance the activity of ACCase could enhance the fatty acid production

# Improvement of microbial lipid production

- Research are aimed at **improving the economic competitiveness** of microbial lipids compared to plant and animal derived oils.

- Three main pathways are ongoing to improve economics of microorganism biodiesel.

1. Screening for potential oleaginous microorganism

1. Genetic and metabolic engineering

1. Making full use of byproducts

# I. Screening of potential oleaginous microorganisms

- Although several wild-type oleaginous microorganisms are able to synthesize rich oil, these strains have a limited ability to produce biomass.

- Making use of **mutation techniques** in microbial lipid production to filtrate better strain will get much more biomass than wild-type.

- Greece researcher reported in a ***Nitrogen limited condition,***

*Mortierella isabellina* → ↑ cell growth (up to 35.9 g/L) & enhance survival rate and glucose uptake rate even at a concentration of 100 g/L in media.

# II. Genetic and metabolic engineering

- Appropriate modification of genome of mo→ improve oil production

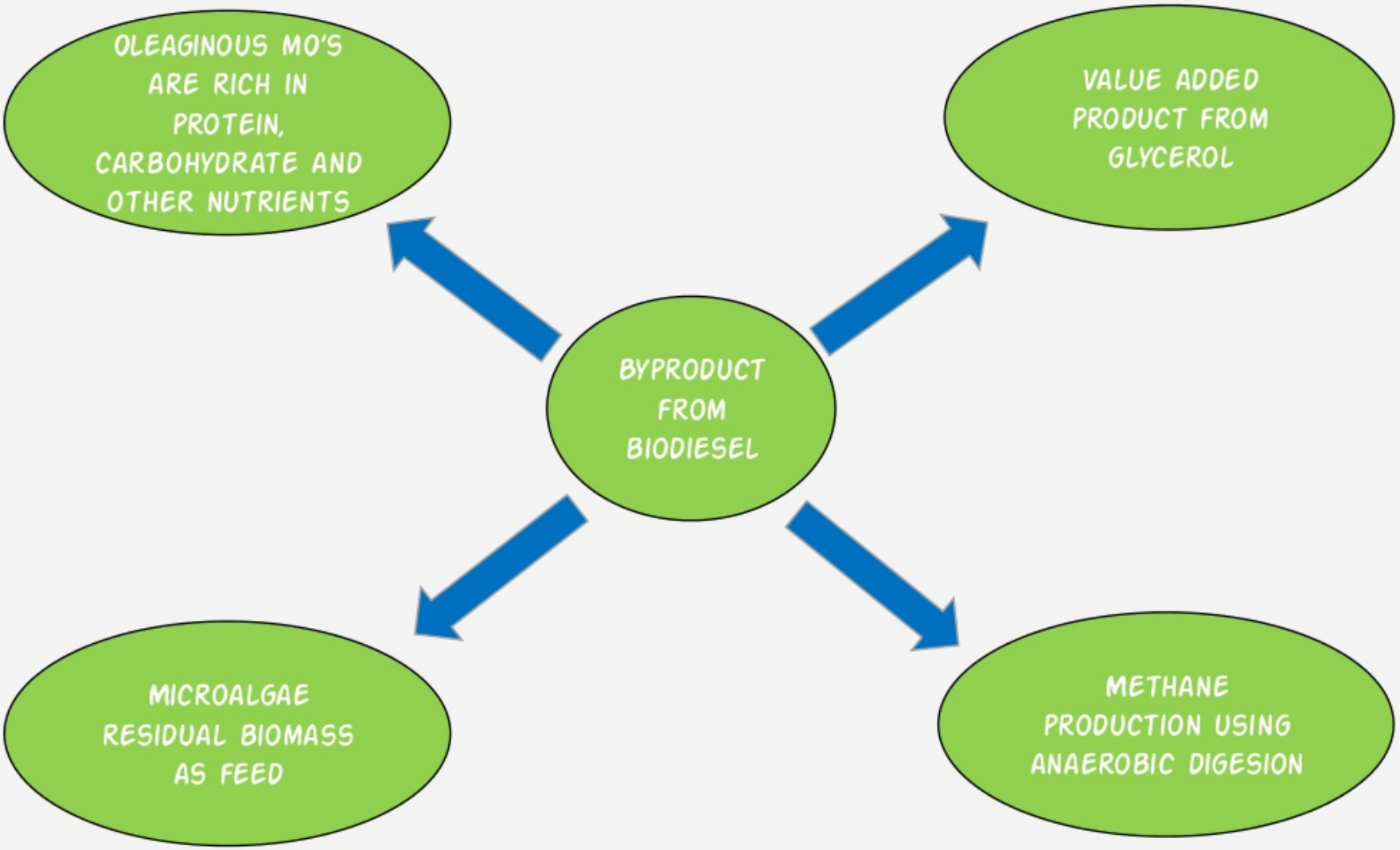
- However, production of stable engineered strains is an issue.

- Degree of unsaturation and length of carbon chain of fatty acid → regulated by enzymes→ however purification and study of their function is a major issue.

- Three genetic technologies are explored→ interdependent
  - ❑Cloning genes of critical enzymes
  - ❑Transgenic expression of these genes aimed to achieve a fine high-product microbial oil recombination strain
  - ❑Modification of cloned genes in order to engineer the expressed protein

- Genetically engineered *Pseudomonas citronellolis*, *E. coli* and *S. cerevisiae* for enhanced production of wax ester, fatty acid butyl ester and FAME respectively.

# III. Making full use of byproducts



# Environmental Microbiology

**Course 12: Water Pollution**

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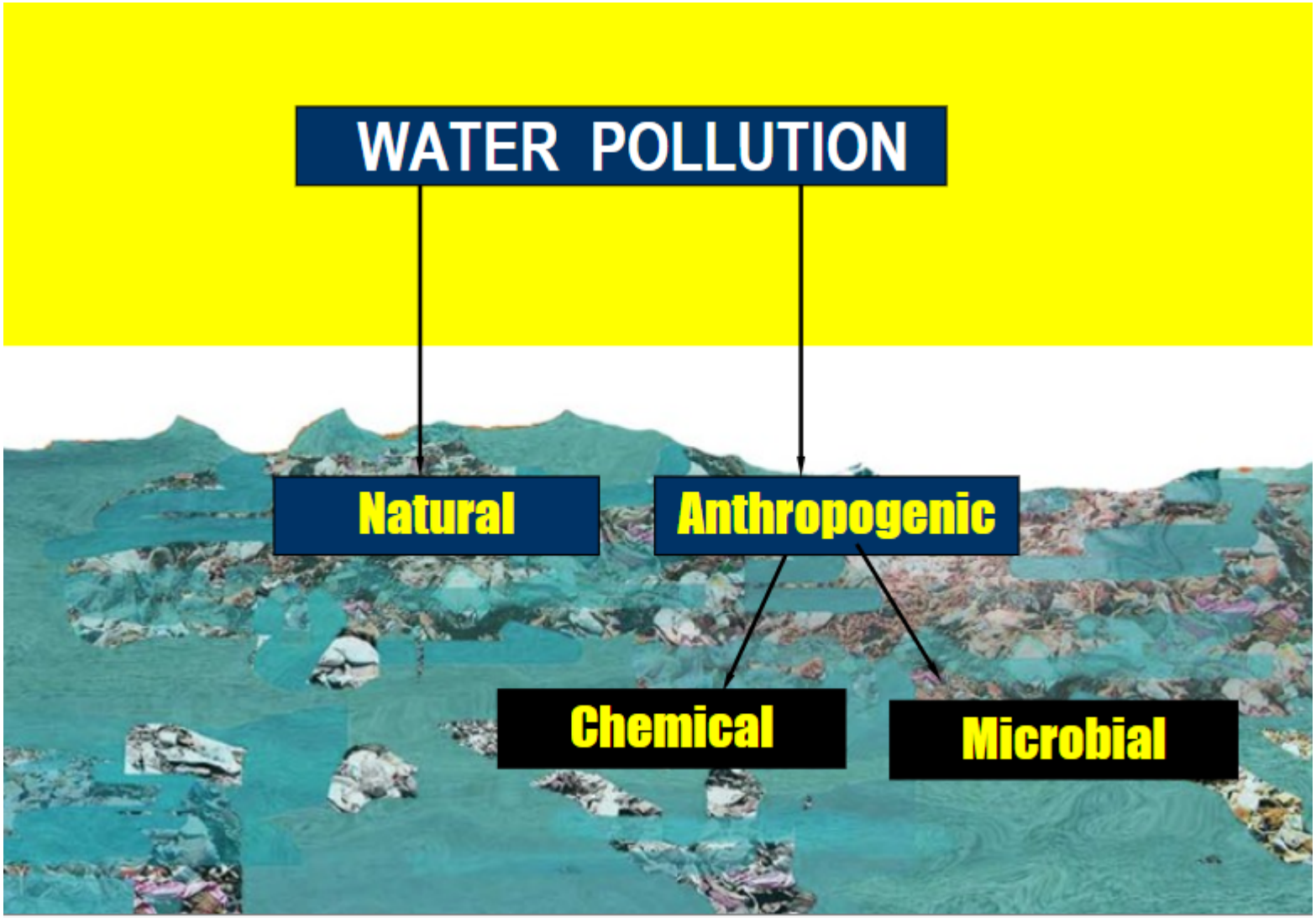
# Water Pollution

- Water pollution is any chemical, biological, or physical change in water quality that has a harmful effect on living organism or makes water unsuitable for desired uses.
- It has been suggested that it is the leading worldwide cause of deaths and diseases, and that it accounts for the deaths of more than 14,000 people daily.


WATER POLLUTION
Natural
Anthropogenic
Chemical
Microbial

- In addition, water-borne disease can be caused by the pollution of water with chemicals that have an adverse effect on health

## Water-borne disease caused by chemicals

- Arsenic
- Flouride
- Nitrates from fertilizers
- Carcinogenic pesticides (DDT)
- Lead (from pipes)
- Heavy Metals
- Chromium
- Nickel
- Cyanide

# Health effects

**Arsenic**

Cancer, vascular disease, liver disease, skin lesions, and neurological disorders, Arsenicosis (high levels of arsenic (GV =0.01mg/l)

Arsenicosis

**Fluoride**

Fluorosis (Severe skeletal problems)

Cause : high levels of fluorine (GV=1.5 mg/l)

**Chlorine**

Toxic and cause sufficient cell damage in the human body.

Fluorosis

**Iodine**

Enlargement of the thyroid gland and mental retardation.

**Nitrates**

High levels of nitrate in water can lead to blood poisoning and eventually death. Methaeglobinemia

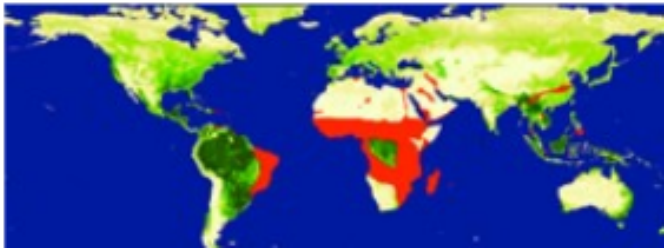
Main Cause : high levels of nitrates(GV=50mg/l)

# disease-causing agents (pathogens)

- bacteria (causing dysentery, enteritis)
  - coliform bacteria (*Escherischia coli* = *E. coli*)
    - normally live in intestinal tract of animals
    - indicators of fecal contamination if present in water
    - 0 bacteria/100ml water for drinking - WHO recommendation
    - 200 bacteria/100ml water for swimming - EPA recommendation
- viruses
  - infectious hepatitis
- parasites
  - protozoans (*Giardia*)
  - worms (*Schistosomiasis*)

*Giardia*

*Schistosomiasis occurrence*



# 4 Types of Water-Related Diseases

1. Water Borne: infections acquired by the ingestion of contaminated water
2. Water Washed: infections acquired due to insufficient water for bathing and hygiene
3. Water Based: infections transmitted by pathogens which have part of their life cycle in aquatic animals (snails, larvae, etc.)
4. Water Insect Vector: infections transmitted by vectors which live, breed or bite near water (often mosquitoes)

# Water and Sanitation-related Diseases

| Group Diseases | Group Diseases |
|---|---|
| Water-borne diseases (diseases transmitted by water) | Cholera; Typhoid; Bacillary dysentery Infectious hepatitis; Giardiasis |
| Water-washed diseases (caused by lack of water) | Scabies; Skin sepsis and ulcers; Yaws; Leprosy; Lice and thypus; Trachoma; Dysenteries; Ascariasis; Parathphoid |
| Water based diseases | Schistomiasis; Dracunuliasis; Bilharziosis; Filariasis; Threadworm |
| Water-related insect vector diseases | Yellow fever, **Dengue fever**, Bancroftian filariasis, Malaria Onchocerciasis |

# Water-borne Diseases

- Diseases caused by ingestion of water contaminated by human or animal excrement, which contain pathogenic microorganisms.
- Include cholera, typhoid, amoebic and bacillary dysentery and other diarrheal diseases.

**Diarrheal Diseases**

- Giardiasis (Protozoan)
- Cryptosporidiosis (Bacteria)
- Campylobacteriosis (Bacteria)
- Shigellosis (Bacteria)
- Viral Gastroenteritis (Virus)
- Cyclosporiasis (Parasite)

# Water-related Diseases

- Water-related diseases are caused by insect vectors, especially mosquitoes, that breed or feed near contaminated water.
- They are not typically associated with lack of access to clean drinking water or sanitation services
- Include dengue, filariasis, malaria, onchocerciasis, trypanosomiasis and yellow fever

# Water-washed Diseases

- Diseases caused by poor personal hygiene and skin and eye contact with contaminated water.
- These include scabies, trachoma, typhus, and other flea, lice and tick-borne diseases.

# Water-based Diseases

- Diseases caused by parasites found in intermediate organisms living in contaminated water.
- Includes Schistosomiasis and  Dracunculiasis

# Water related Disease

| Sl. No. | Transmission Route | Description | Disease Group | Examples |
|---|---|---|---|---|
| 1. | Water-borne | Transmission by consumption of contra minted water or person – to-person transmission due to lack of water and domestic cleanliness. | Feco-oral | •Diarrhoeal<br>•Dysentery<br>•typhoid |
| 2. | Water –washed | Person-to-person transmission due to lack of water and domestic cleanness | Skin and eye infections for personal | •Trachoma<br>•Scabies |
| 3. | Water-based | Transmission via an intermediate host which lives in water | Water based | •Schistosomiasis |
| 4. | Water-related | Transmission by insects which breed insect vector in water or bite near water | Water-related insect vector | •Malaria<br>•Filariasis |

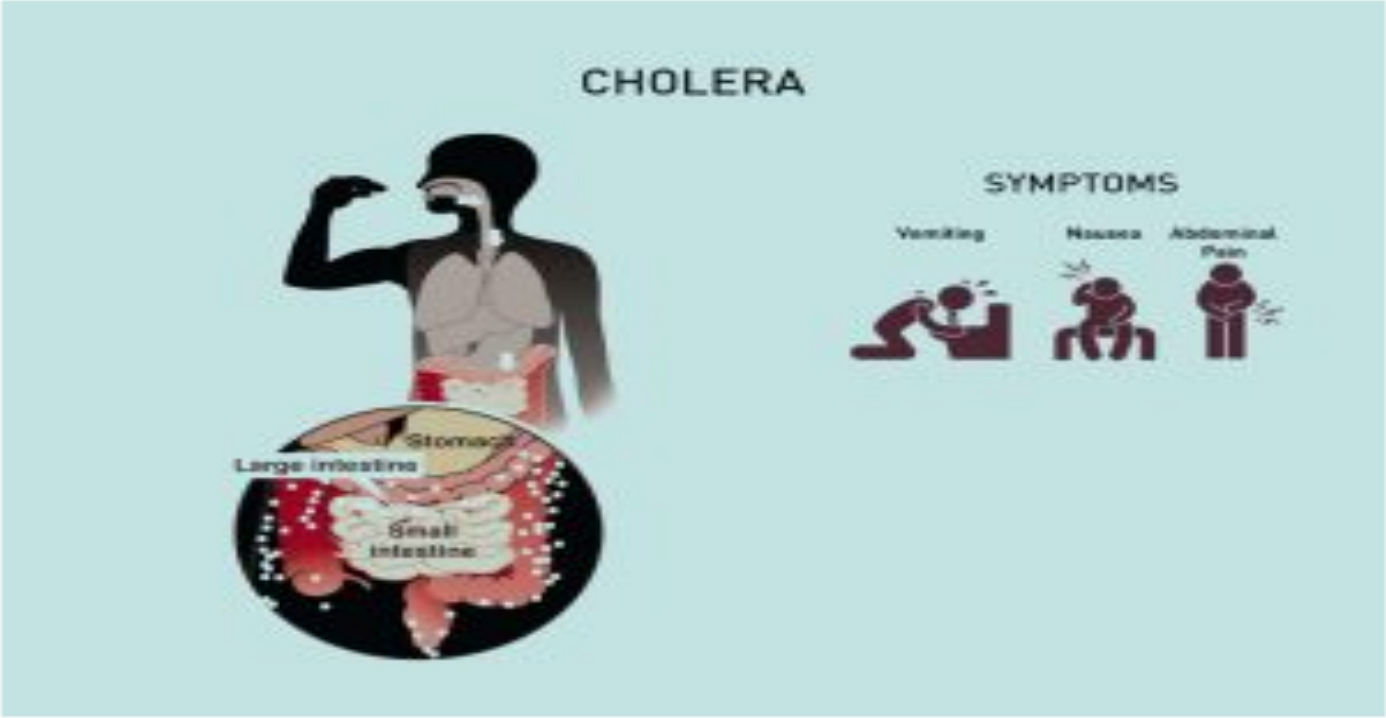
# Bacterial Contamination in Water

- **Campylobacter:** diarrhoea
- **E. coli:** Bloody diarrhoea, mild fever, anaemia
- **Pseudomonas:** dermatitis and infections in urinary tract, respiratory system, soft tissues, bones, joints, gastrointestinal tract
- **Shigella:** gastroenteritis, bacillary dysentery
- **Salmonella:** Nausea, vomiting, abdominal cramps, diarrhoea, fever, headache
- **Vibrio cholerae:** abdominal pains, mild fever, chills, and headache, watery diarrhoea followed by lethargy and dehydration.

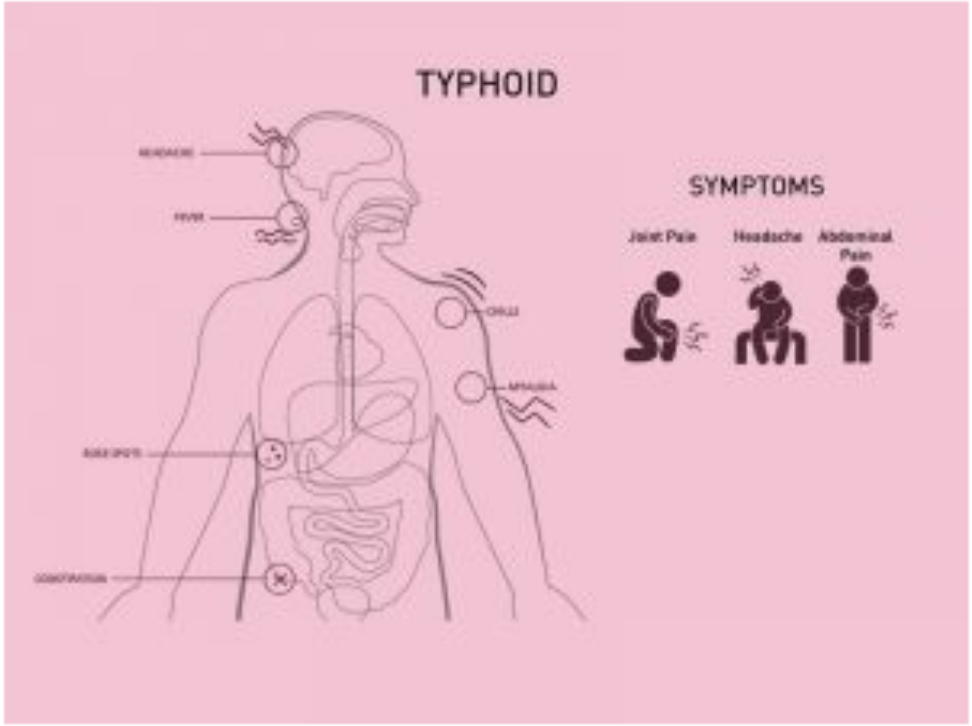
# Viruses Contamination in Water

- **Noroviruses** - Group of viruses that cause gastroenteritis, inflammation of lining of stomach and intestines
- **Hepatitis A –** Results in inflammation of liver
- Sources of infection:
  - ✓an infected person who didn't wash his or her hands after using washroom
  - ✓drinking untreated water or
  - ✓eating food washed in untreated water
- **Rotavirus:** Very common cause of severe diarrhoea among infants and children

- **Cholera:** Cholera is a waterborne disease and is diarrhoeal in nature. A person can get affected by Cholera by drinking water or eating food contaminated with the cholera bacterium. Thousands of people fall prey to this disease every year and many lose their lives as well. It can happen to both children and adults and some of the symptoms of cholera include vomiting, abdominal cramps, watery bowels, and fever.

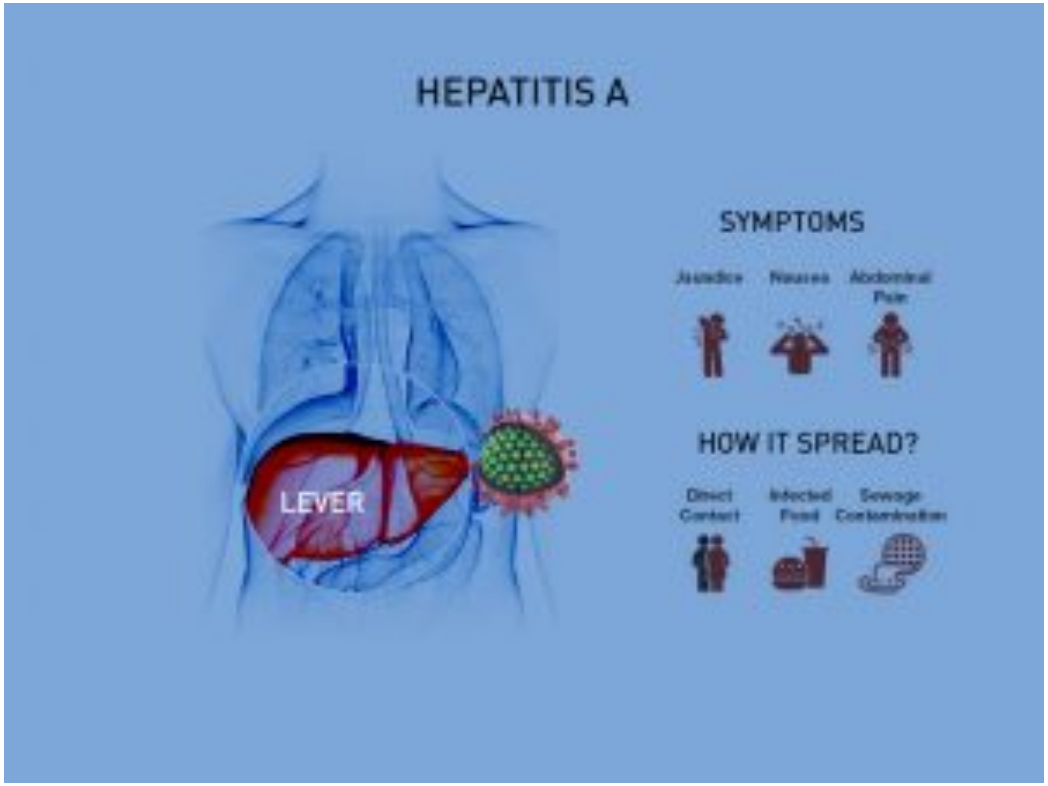


- **Typhoid:** It is another disease that gets transmitted by drinking contaminated water that carries 'Salmonellae Typhi bacteria'. Some of the symptoms through which you can understand that you are being affected by Typhoid are prolonged fever, loss of appetite, headache, constipation, exhaustion, sleepiness, and nausea. It can also be transmitted by close contact with the infected person.

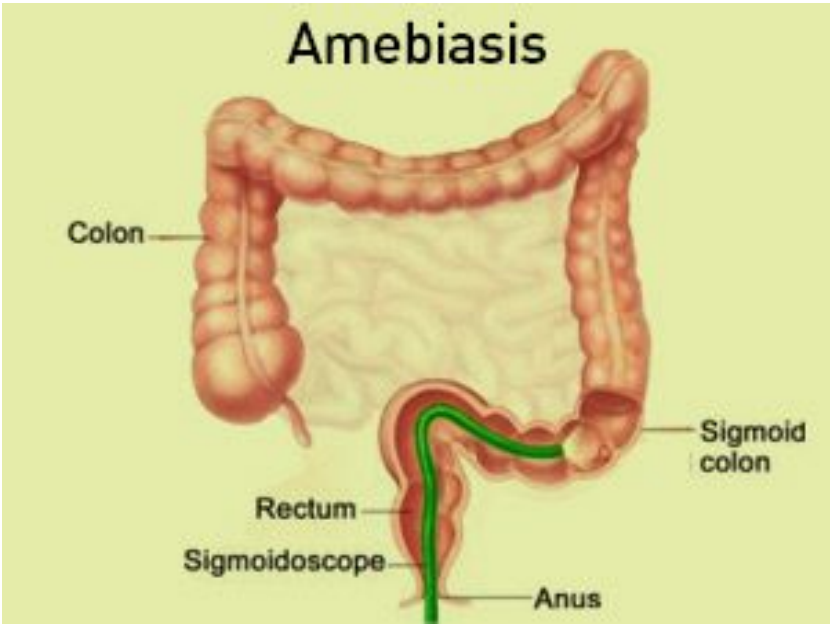


- **Diarrhoea:** Diarrhoea is one of the most common waterborne diseases that mostly affects children under the age of 5. The infection of diarrhea spreads through eating contaminated food and impure water. Some of the symptoms of diarrhea include dehydration, severe dizziness, loss of consciousness, pale skin and bloody stool, little or no urination. The attack of diarrhea can last up to 2 weeks by leaving the person dehydrated and if kept untreated the infected person can lose his/her life as well.

- **Hepatitis A:** Another type of waterborne diseases is Hepatitis A and it is caused by Hepatitis A virus, which affects the liver. It is normally spread by the fecal-oral route, by direct contact with the infected person or by ingestion of the contaminated food or water. Some of the symptoms that you can find in the infected person are nausea, vomiting, and fever.



- **Amebiasis:** Amebiasis is a kind of parasitic infection in the intestine that is caused by the protozoan Entamoeba histolytica, or E. histolytica. The single-celled protozoan usually enters the human body when the person swallows cysts through food or water. Cysts are an inactive form of parasite that stays alive for several months in the soil or environment, especially in the feces. So, you can say that the parasite can also enter the body through direct contact with fecal matter. Some of the symptoms of amebiasis are the loose stool, stomach pain, and abdominal cramping.



# What are indicator bacteria?

- The use of an organism that can serve as a surrogate for another is called **an indicator organism**.

- Trying to detect disease-causing bacteria and other pathogens in water is expensive and may pose potential health hazards.

- And it is not practical to test for pathogens in every water sample collected.

- Instead, the presence of pathogens is determined with indirect evidence by testing for an **"indicator"** organism such as coliform bacteria.

- Coliforms come from the same sources as pathogenic organisms.

## What are Coliforms?

- Coliforms are bacteria that are always present in the digestive tracts of animals, including humans, and are found in their wastes. They are also found in plant and soil material.

# Are Coliform Bacteria Harmful?

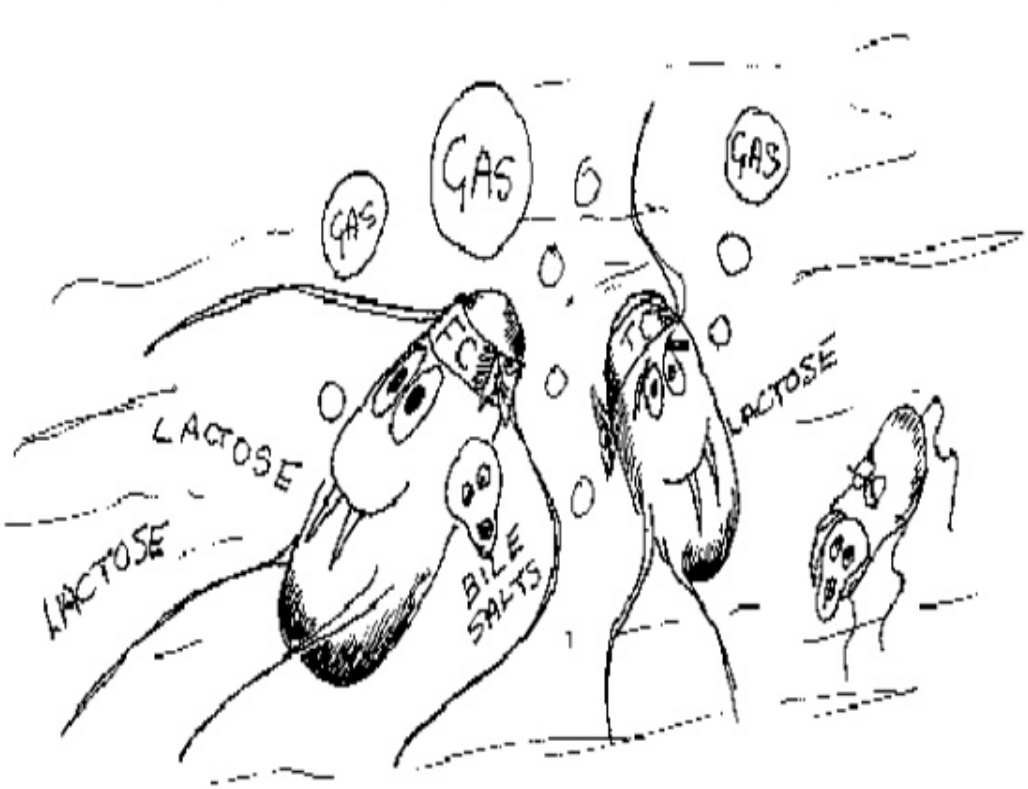
- Most coliform bacteria do not cause disease.
- However, some rare strains of E. coli, particularly the strain 0157:H7, can cause serious illness.
- Cases of E. coli 0157:H7 caused by contaminated drinking water supplies are rare.

# Characteristics of a Useful Indicator:

- Always present when pathogens are present
- Not present in the absence of the pathogen
- Correlated with degree of pollution
- More easily detectable than a pathogen
- Non-pathogenic in nature.

# Characteristics of coliforms:

- Aerobic or facultative,
- Gram-negative,
- Non-spore forming,
- Bacilli,
- Which ferments Lactose to form acid and/or gas with in 24 hours at 35°C



- Coliforms are relatively:

  -easy to identify.
  -are usually present in larger numbers than more dangerous pathogens.
  - and respond to the environment, wastewater treatment, and water treatment similarly to many pathogens.

- **As a result, testing for coliform bacteria can be a reasonable indication of whether other pathogenic bacteria are present.**

# What are Coliform Bacteria?

- Also commonly known as "indicator organisms", coliform refers to a wide variety of bacteria that can be found throughout the environment.
- This means that these organisms can be found in soil, water surfaces, vegetations as well as on the skin or intestinal tract of warm-blooded organisms such as humans.

# What are Coliform Bacteria?

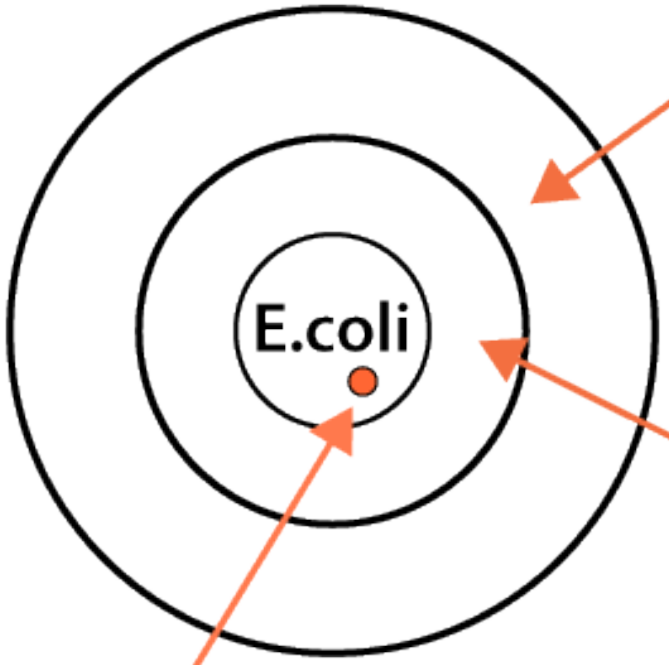
- Although some are pathogenic (capable of causing diseases - mild to life threatening diseases) most of them are harmless. **Regardless, detection of coliform (indicator organisms) indicates the presence of potential disease causing bacteria not only in water, but also in given foods and drinks (milk etc).**

# Types of Coliform Bacteria

- Divided into three main groups which include:

- Total coliform bacteria
- Fecal coliform bacteria
- E. coli

# Total Coliform, Fecal Coliform and E.coli




**Total Coliform**

Total Coliform =
Environmental Contamination

**Fecal Coliform**

Fecal Coliform + E.coli =
Fecal Contamination

**E.coli 0157:H7**

# Total coliform bacteria

- This group is largely composed of harmless, closely related bacteria. Apart from human and animal waste, total coliform bacteria can be found in such environments as water, vegetation and soil where they live freely.
- While they are generally harmless, the presence or detection of this group of bacteria in drinking water or water source that supply drinking water is important because they are indicative of possible contamination.

# Total coliform bacteria

- If detected in a water sample, **this suggests that disease causing coliform may also be present and thus the need to treat the water source or determine the source of contamination** (environmental contamination etc). **Thermotolerant coliforms** are good examples of total coliform bacteria. These are coliform that are capable of fermenting lactose at 45 degrees.

# Total coliform bacteria

- Detection of total coliform does not necessarily mean that disease causing bacteria are present in water
- Testing the presence of total coliform bacteria basically involves growing them in lactose media at about 35 degrees

# Fecal Coliform Bacteria

- Fecal coliform bacteria (FC) are a subgroup of the total coliform bacteria that can be found in the intestines and feces of warm blooded animals (human beings, pigs, cows, dogs, pigs etc). E. coli is an example that typically resides in the intestinal tract of warm-blooded animals and thus the animal's fecal matter.

# Fecal Coliform Bacteria

- Compared to total coliform bacteria, which are largely harmless, the fecal are composed of both pathogenic and non-pathogenic bacteria. As such, **their detection in a sample of drinking water is an indication that the water is contaminated by sewage.**

# Fecal Coliform Bacteria

- The presence of these bacteria is also very important because the source of the bacteria is well known compared to the source of total coliform bacteria (TC). Here, therefore, it becomes easier to locate and fix the source of the problem and treat the water more effectively in order to prevent possible diseases associated.

# E. Coli (Coliform)

- E. coli is a sub-group of fecal coliform bacteria and is largely composed of E. coli (Escherichia coli). Compared to others, E. coli are almost exclusively found in the intestines of warm-blooded animals where they are able to live and reproduce.

# E. Coli (Coliform)

- Although they are mostly harmless in the host's intestines, there are strains of E. coli (e.g. E.coli 0157:H7) that can cause serious illnesses. **Detection of these organisms in water is indicative of fecal contamination (recent contamination in most cases) as well as possible presence of other pathogenic organisms that may include viruses.** In such cases, water is contaminated by sewage or animal feces.

# What happens if total coliform bacteria are confirmed in my water?

- If total coliform bacteria are confirmed (at least 2 samples with coliform bacteria present) in your drinking water, **your water system should be inspected to find and eliminate any possible sources of contamination.** Once the source is identified, it can usually be resolved by making system repairs, flushing, and adding chlorine for a short period of time. The state Health Department works with water systems and utility managers to help resolve such problems.

# What happens if fecal coliform bacteria or E. coli are confirmed in my water?

- Confirmation of fecal coliform bacteria or E. coli in a water system indicates recent fecal contamination, which may pose an immediate health risk to anyone consuming the water. It **will be issued within 24 hours to alert all water users that there is a health risk associated with the water supply.** In most cases, the use of boiled or bottled water will be recommended for drinking and cooking. The notice will inform customers of actions being taken to correct the problem, and when the problem will likely be resolved.

# Most Probable Number & Membrane Filter methods

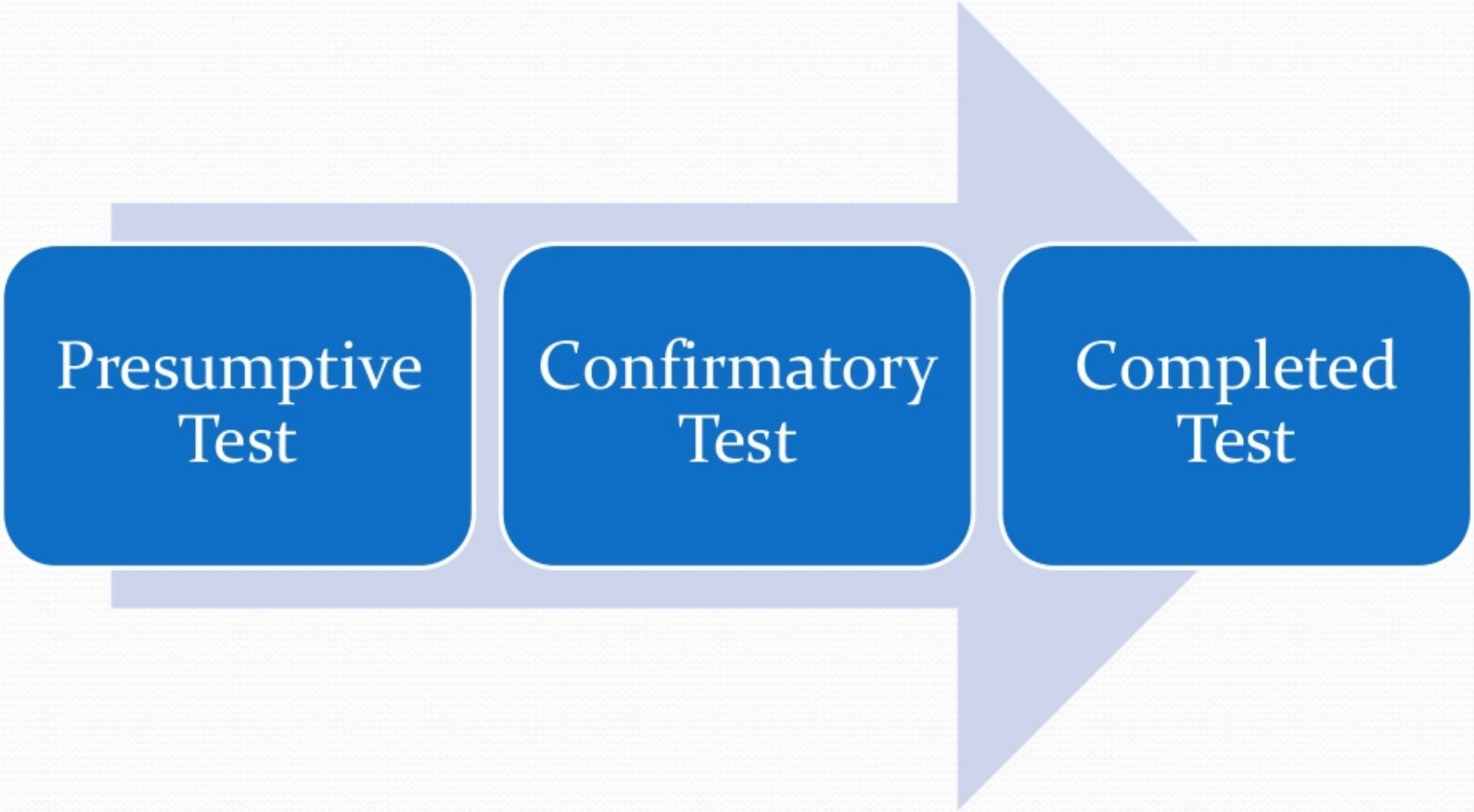
# Most Probable Number OR Multiple tubes method:

- The **Most Probable Number** method is used to check **potability** (if water is safe enough to be drinking water) of water.
- The MPN method looks for the presence of potential pathogenic bacteria that may be in the water due to **contamination** of the water supply.
- Water supplies are generally derived from ground sources and have to be checked for safety levels of bacterial-contamination.

- **MPN** method enumerates the enteric bacteria called coliforms, specifically **fecal coliforms** (*E. coli*)

- Coliforms are **Gram negative bacilli that have the ability to ferment lactose with the production of acid and gas**.

## ➢MPN test includes 3 levels of testing:



# 1-Presumptive Test:

- The **presumptive** test looks for presence of coliforms in the water sample by inoculating lactose broths with the water sample.

## Composition of lactose broth:

| Ingredients per liter of demineralized water: | |
|---|---|
| Peptone 190 (Pancreatic Digest of Gelatin) | 5.0 g |
| Beef Extract | 3.0 g |
| Lactose | 5.0 g |
| pH 6.7 ± 0.2 at 25C | |

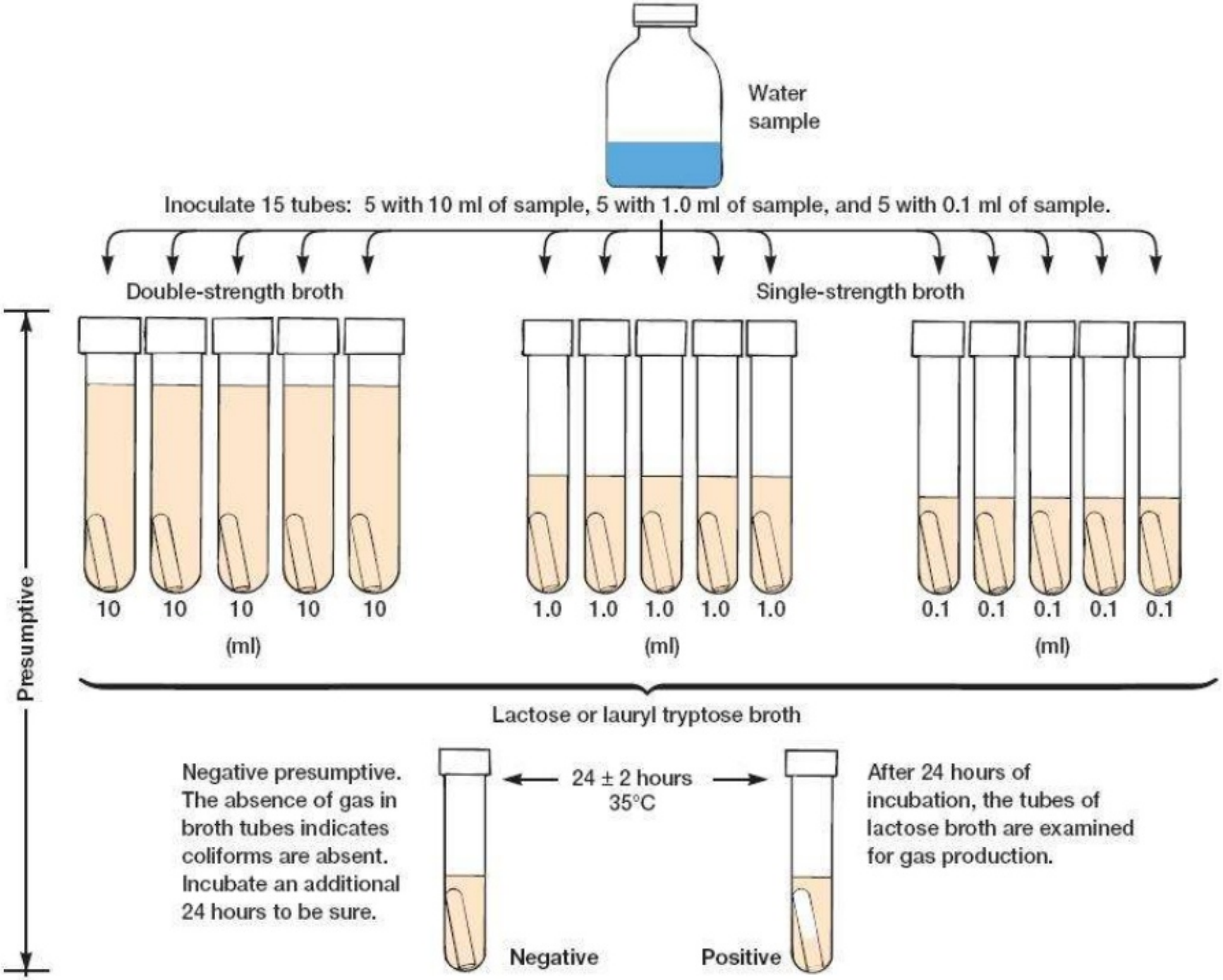
## Procedure:

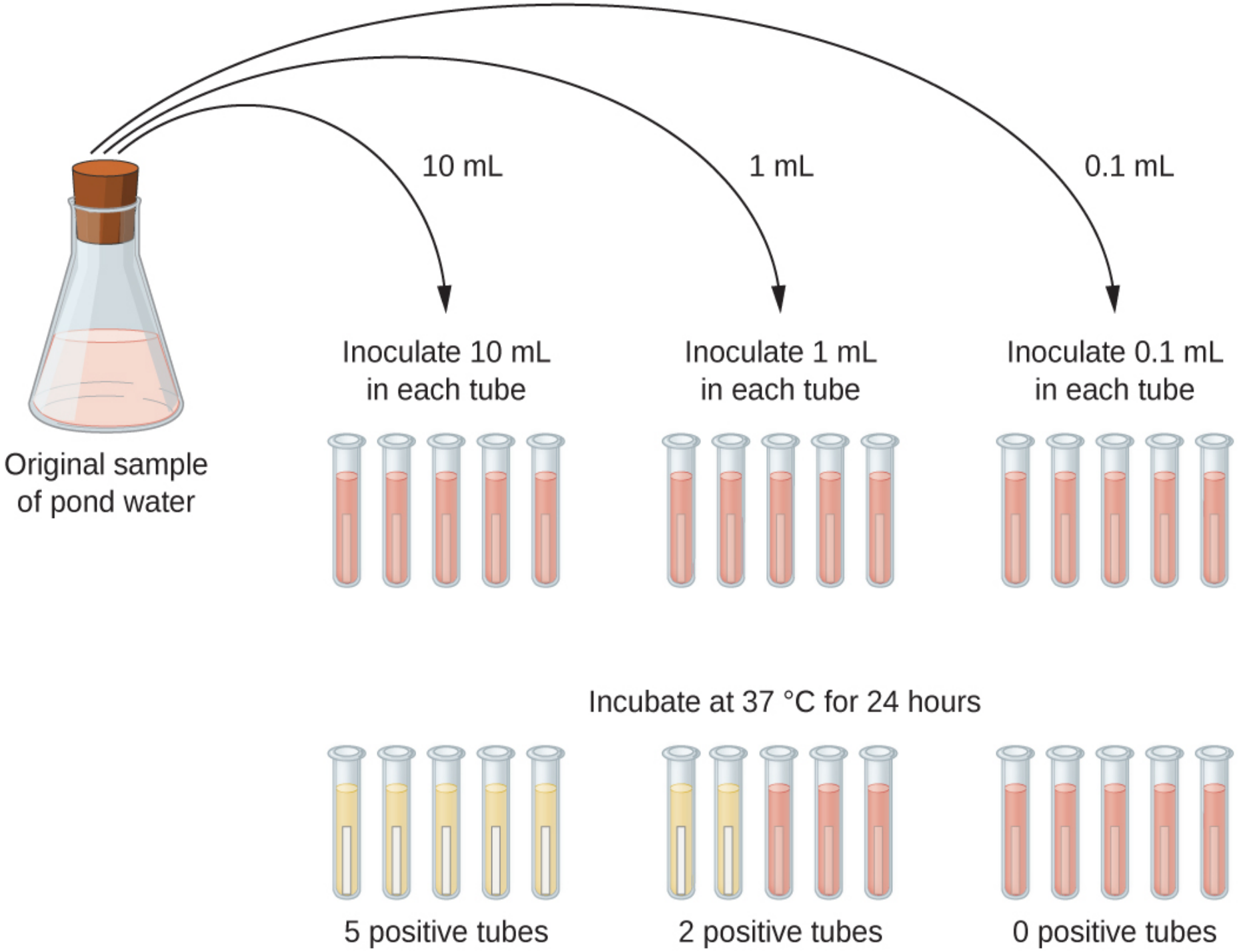
- Took three sets of test tubes (with Durham tube) containing **lactose broths(2ml)** are inoculated with varying dilution of the sample:-

- first set of 5 tubes inoculated with 10ml of sample;
- second set of 5 tubes inoculated with 1ml of sample;
- third set of 5 tubes inoculated with 0.1ml of sample

- Then all test tubes incubate at 35℃ for 24 hours.

- Those tubes that show presence of **acid and gas** are marked as **+ve** and those with **no acid/gas as -ve.**
- Then count the +ve marked tubes from all sets.
- The combination of positives in the 3 sets is used to figure out the MPN /100ml of water using the table provided.


Water sample
Inoculate 15 tubes: 5 with 10 ml of sample, 5 with 1.0 ml of sample, and 5 with 0.1 ml of sample.
Double-strength broth
Single-strength broth
10 10 10 10 10
(ml)
1.0 1.0 1.0 1.0 1.0
(ml)
0.1 0.1 0.1 0.1 0.1
(ml)
Presumptive
Lactose or lauryl tryptose broth
Negative presumptive. The absence of gas in broth tubes indicates coliforms are absent. Incubate an additional 24 hours to be sure.
24 ± 2 hours
35°C
Negative
Positive
After 24 hours of incubation, the tubes of lactose broth are examined for gas production.


10 mL
1 mL
0.1 mL
Original sample of pond water
Inoculate 10 mL in each tube
Inoculate 1 mL in each tube
Inoculate 0.1 mL in each tube
Incubate at 37 °C for 24 hours
5 positive tubes
2 positive tubes
0 positive tubes

***MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when five 10-ml, five 1-ml and five 0.1 ml test portions are used)***

| No. of tubes giving a positive reaction : | | | MPN (per 100 ml) | 95% confidence limits | |
|---|---|---|---|---|---|
| 5 of 10 ml | 5 of 1 ml | 5 of 0.1 ml | | Lower | Upper |
| 0 | 0 | 0 | <2 | <1 | 7 |
| 0 | 1 | 0 | 2 | <1 | 7 |
| 0 | 2 | 0 | 4 | <1 | 11 |
| 1 | 0 | 0 | 2 | <1 | 7 |
| 1 | 0 | 1 | 4 | <1 | 11 |
| 1 | 1 | 0 | 4 | <1 | 11 |
| 1 | 1 | 1 | 6 | <1 | 15 |
| 2 | 0 | 0 | 5 | <1 | 13 |
| 2 | 0 | 1 | 7 | 1 | 17 |
| 2 | 1 | 0 | 7 | 1 | 17 |
| 2 | 1 | 1 | 9 | 2 | 21 |
| 2 | 2 | 0 | 9 | 2 | 21 |
| 2 | 3 | 0 | 12 | 3 | 28 |
| 3 | 0 | 0 | 8 | 1 | 19 |
| 3 | 0 | 1 | 11 | 2 | 25 |
| 3 | 1 | 0 | 11 | 2 | 25 |
| 3 | 1 | 1 | 14 | 4 | 34 |
| 3 | 2 | 0 | 14 | 4 | 34 |
| 3 | 2 | 1 | 17 | 5 | 46 |

Standard :

If MPN/100 ml is 3 or less than 3 than water is suitable for drinking

## 2. Confirmed test:

Some microrganisms other than coliforms also produce acid and gas from lactose fermentation. In order to confirm the presence of coliform, confirmatory test is done.

From each of the fermentation tubes with positive results transfer one loopful of medium to:

1. 3 ml  lactose-broth or brilliant green lactose fermentation tube,
2. to an agar slant and
3. 3 ml tryptone water.

Incubate the inoculated lactose-broth fermentation tubes at 37°C and inspect  gas formation after 24 ± 2 hours. If no gas production is seen,further incubate up to  maximum of  48 ±3 hours to check gas production.

The agar slants should be incubated at 37°C for 24± 2 hours and Gram-stained preparations made from the slants should be examined microscopically.

The formation of gas in lactose broth and the demonstration of Gram negative, non-spore-forming bacilli in the corresponding agar indicates the presence of a member of the coliform group in the sample examined.

The absence of gas formation in lactose broth or the failure to demonstrate Gram-negative, non-spore-forming bacilli in the corresponding agar slant constitutes a negative test *(absence of coliforms in the tested sample)*.

**Tryptone water Test**

1. Incubate the tryptone water at (44.5 ±0.2°C) for 18-24 hours
2. Following incubation, add approximately 0.1ml of Kovacs reagent and mix gently.
3. The presence of indole is indicated by a red colour in the Kovacs reagent, forming a film over the aqueous phase of the medium.

a. Confirmatory tests positive for indole, growth, and gas production show the presence of thermotolerant *E. coli*.

b. Growth and gas production in the absence of indole confirm thermotolerant coliforms.

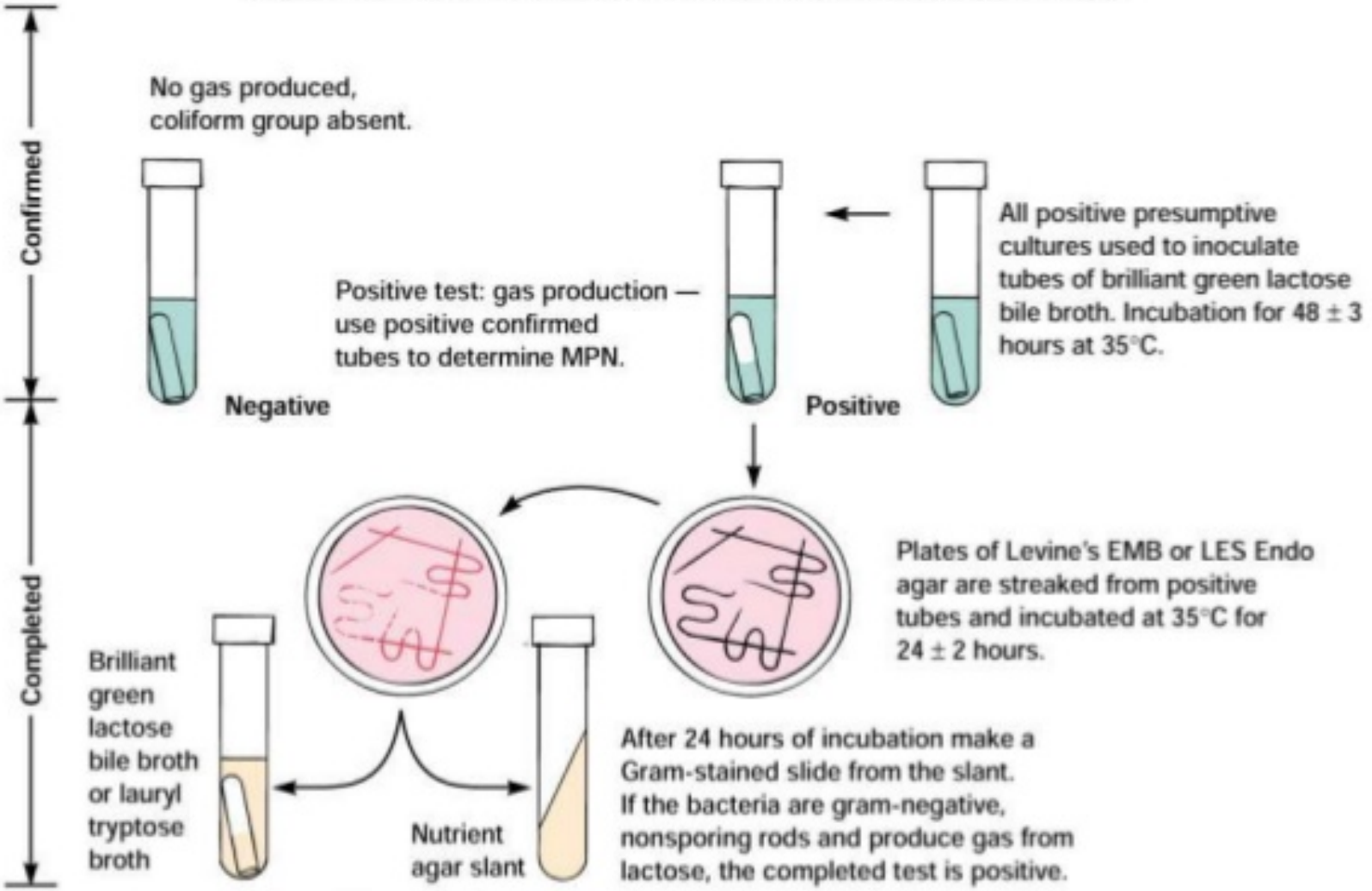
## 3. Completed test:

Since some of the positive results from the confirmatory test may be false, it is desirable to do completed tests. For this inoculum from each positive tube of the confirmatory test is streaked on a plate of EMB or Endo agar.

In this process, a loopful of sample from each positive BGLB tubes is streaked onto selective medium like Eosin Methylene Blue agar or Endo's medium. One plate each is incubated at 37°C and another at 44.5± 0.2°C for 24 hours.

# Flow chart of Confirmed and Completed MPN





***Negative for Lactose Fermentation / gas production***

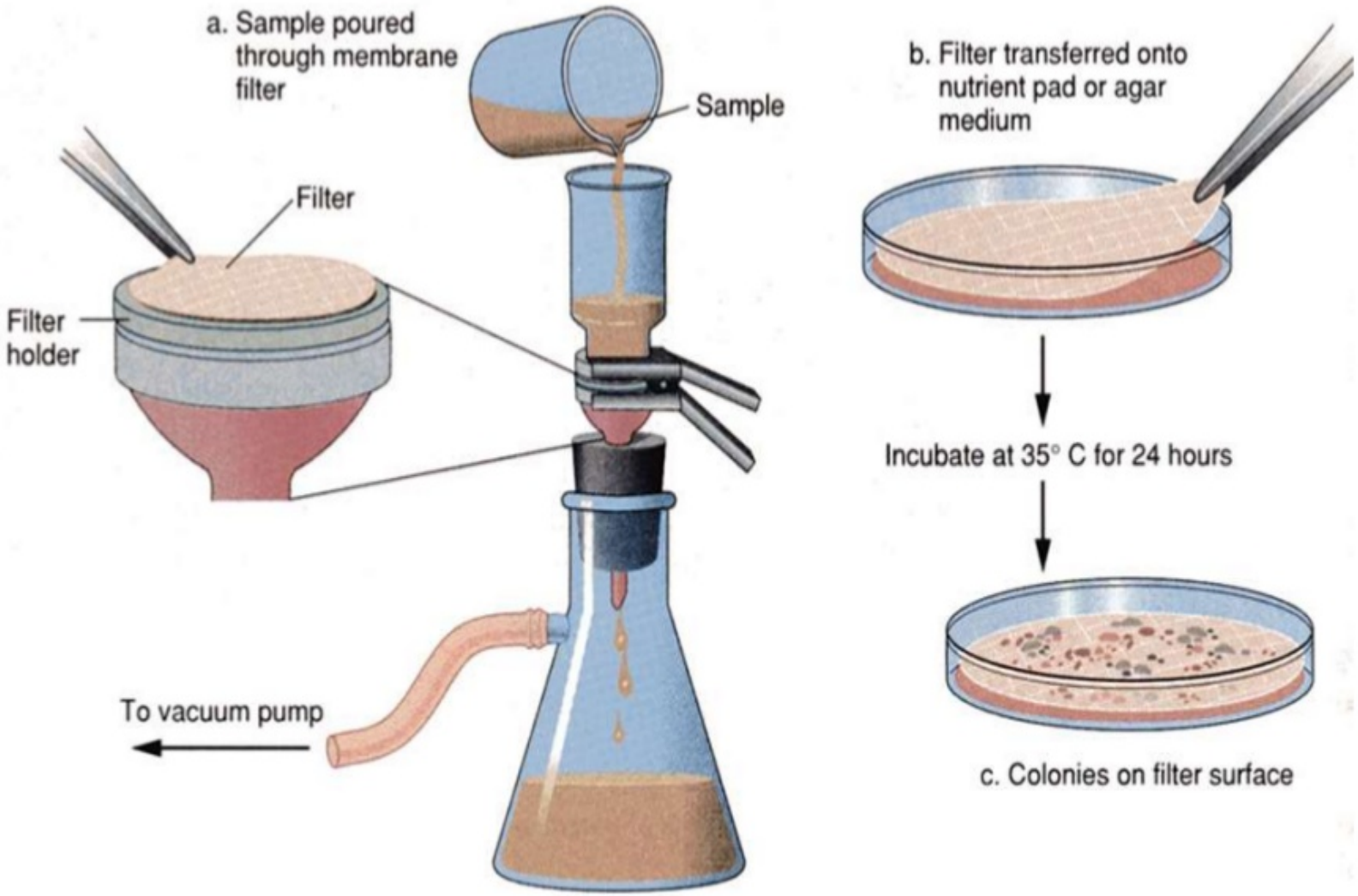
***Positive for Lactose Fermentation / gas production***

# Completed


Typical coliform colonies confirmed on EMB agar.
Non-*E. coli* coliforms (left) and *E. coli* (right).

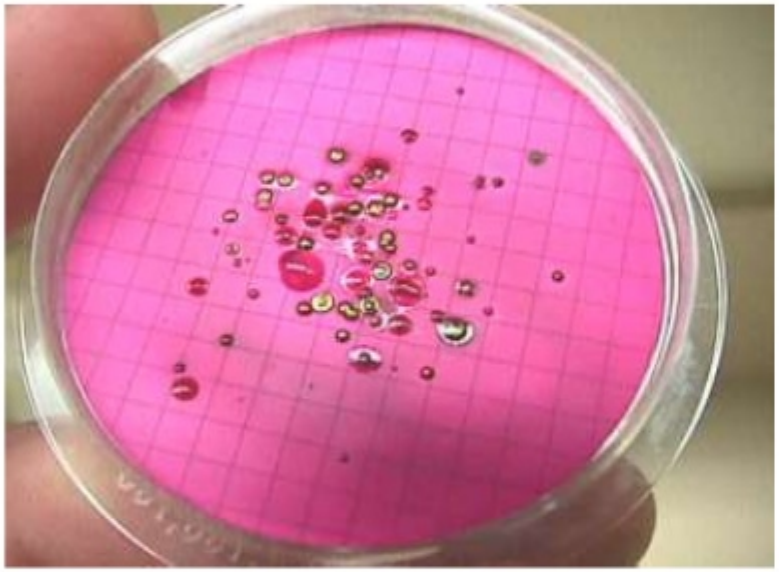
# Membrane Filter Methods

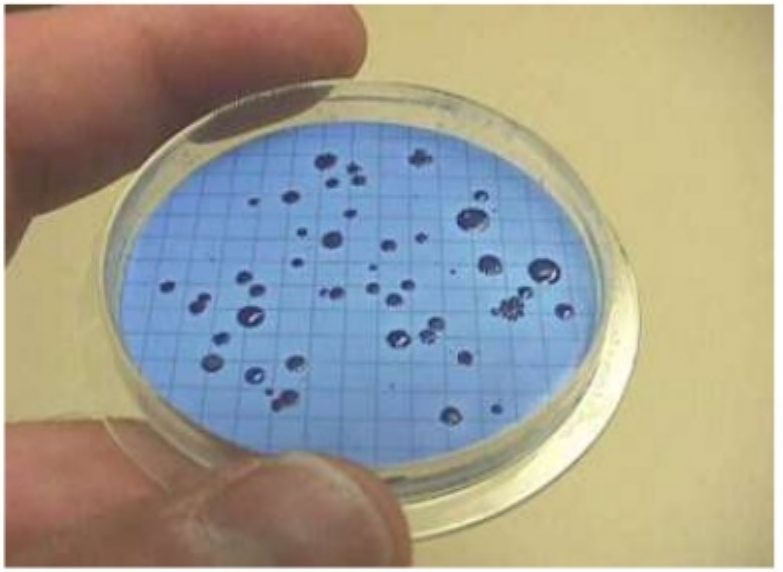
* Filter water through a membrane filter(Bacterial cells can not pass through)
* Place membrane on selective media(EMB)
* Incubate
  * 35°C
* Count colonies


a. Sample poured through membrane filter
Sample
Filter
Filter holder
To vacuum pump
b. Filter transferred onto nutrient pad or agar medium
Incubate at 35° C for 24 hours
c. Colonies on filter surface

# Membrane filtration method

Results:





Coliform bacteria produce colonies with a characteristic **"metallic green sheen"**