Laboratory Manual

Microbial Biotechnology (BT512P)

Contents

Practical No: 1

Isolation and Screening of potential microbes from different environmental sources

Introduction

There are approximately 10,000 known species of microbes. It is estimated that approximately 10,000 to 100,000 unidentified species are yet to be studied. A single spoonful of soil can have 100 million bacteria. A scraping of your gums can yield 1 million bacteria per cm2 (a cm2 is about the size of your little fingernail). The bacteria in and on human bodies make up about 10% of the dry body weight.

Most of the currently known species of bacteria have been identified using traditional microbiological techniques such as the gram staining, morphology, and metabolic reactions. Bacteria rarely live alone and tend to form communities with other bacteria. This is true for both the environmental bacteria and the ones colonizing human bodies.

The first requirement for physically isolating a bacterium is that it should be culturable in the laboratory. This requires prior knowledge of optimum temperature for bacterial growth, optimal oxygen requirements, and important nutritional needs.

Two standard methods are frequently used to isolate microorganisms.

1. Streaking on an agar plate
2. The Pour Plate method

Streaking on an agar plate involves the successive dilutions of desired organisms until the cells are at a density low enough to give rise to recognizable individual colonies spatially separated from each other. In the pour plate method, samples are diluted sufficiently before adding cool molten agar. The inoculated mixture is then carefully poured into a petri plate. The isolated cells give rise to individual colonies growing onto the agar plate. This technique can be a little tricky because if the melted agar will be too hot, it kills all the bacteria. However, if the melted agar is too cold, a big lump will form in the Petri dish. The streaking method yields individual colonies on the surface of the agar. This technique is much faster and easier to master.

Overview

E. coli readily grows aerobically on tryptic soy agar (TSA). However, E. coli has a tan appearance at all temperatures and is a rapidly dividing microbe that forms large colonies. The ability to isolate and visualize the individual colonies on the plate is highly dependent upon appropriate incubation conditions. The plate should be incubated at room temperature for 48 hrs. Escherichia coli can then be identified based on colony size and pigment.

Materials

- 1 mixed-culture in tryptic soy broth (TSB) tube containing Escherichia coli
- 1TSA plates

Streaking Procedure

There are several ways of streaking in order to achieve desired isolation. Quadrant method of streaking is described below.

1. Label the petri plate with the date, section, and name of the organism to be cultured.

Use Aseptic technique to obtain a loop-full of organisms from tryptic soy broth (TSB) tube. Make sure that the broth is mixed properly so that the organisms are uniformly suspended in the broth.

2. Close the TSB tube after acquiring the sample.

3. The next step can be performed by keeping the plate on the lab bench or holding it in hand. Lightly drag the loop back and forth across the solid surface of the agar. (Refer to Figure 1).

- Drag the loop with light hands as strong exertion will result in greater bacterial deposit.
- The general idea is to decrease the bacterial concentration with each swipe.
- Four to five zigzags seem to work well.

4. Pass the metal loop through the flame of a Bunsen burner in order to sterilize it. In case of a plastic loop, properly discard it in a cavicide container.

5. Do not go back into the original broth tube.

6. Touch the loop on the agar surface against the far end of the first streak. Repeat by dragging back and forth.

 Do not drag into the center of the plate.

- Faint indentations of the streaking line on the agar surface will be clearly visible.

7. Using a sterile loop, repeat the procedure on the second streak.

8. Using a sterile loop, repeat the procedure on the third streak. Slowly drag the loop in a zigzag manner from the last streak towards the center of the plate.

- Individual isolated colonies will be visible in the last streak.

9. Sterilize the metal loop again with the help of a Bunsen burner. Cover the plate again.

10. Place the streaked petri plates in the incubator at 37 Degree Celsius for about 24 hours.

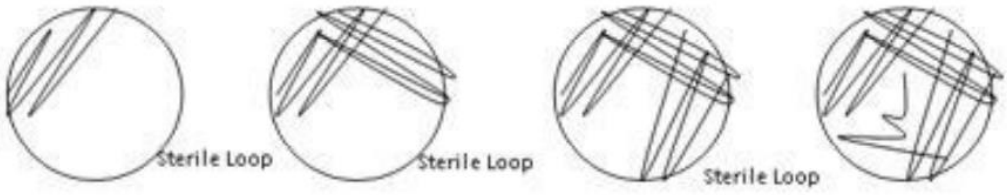


Figure 1: Quadrant method of streaking for isolation.

Note

- It is essential that the loop is sterilized every time before streaking, by passing it through the flame of a Bunsen burner
- The plate should not be uncovered for a long time as it greatly increases the chances of contamination.

Screening

The process involving isolation, detection and separation of microorganisms of interest from a mixed population by using highly selective procedures is called Screening. Important things to be considered while screening are as follows:

Choice of source: - Samples for screening are taken from soil, water, and milk.

Choice of substrate: - Relevant nutrients and growth factors should be supplied for growth of desired microorganisms.

Choice of Detection: - Proper isolation and detection of desired microorganisms is important.

Primary Screening

It’s a process for detection and isolation of microorganisms of interest. It determines which microbe is able to produce a particular compound of interest but it doesn’t provide any idea about the production or yield potential of that microorganisms.

1. Primary Screening of Organic acid producing microorganisms

The dyes sensitive to pH alterations can be used for detecting microorganisms that are capable of producing organic acids. These dyes undergo color changes with the alteration in pH. Dyes such as Neutral red, Bromothymol blue are added to poorly buffered nutrient agar media. Colonies are sub-cultured to make stock culture. Incorporation of $CaCO_3$ in nutrient media is also used to screen organic acid producing microbes due to the formation of clear zone of dissolved $CaCO_3$ around the colony.

2. Primary screening of Antibiotic producing microorganisms

Crowded plate technique is used for screening antibiotic producing microorganisms. It does not give information about sensitivity of antibiotics against other microorganisms. Dilutions of the soil sample are prepared and then introduced into the culture plates so that they can give rise to at least 300 to 400 colonies per plate. Colonies showing antibiotic activity are indicated by zone of inhibition around the colony. Such colonies are sub cultured and purified by streaking before making stock cultures. The purified cultures are then tested to find the Microbial Inhibition Spectrum.

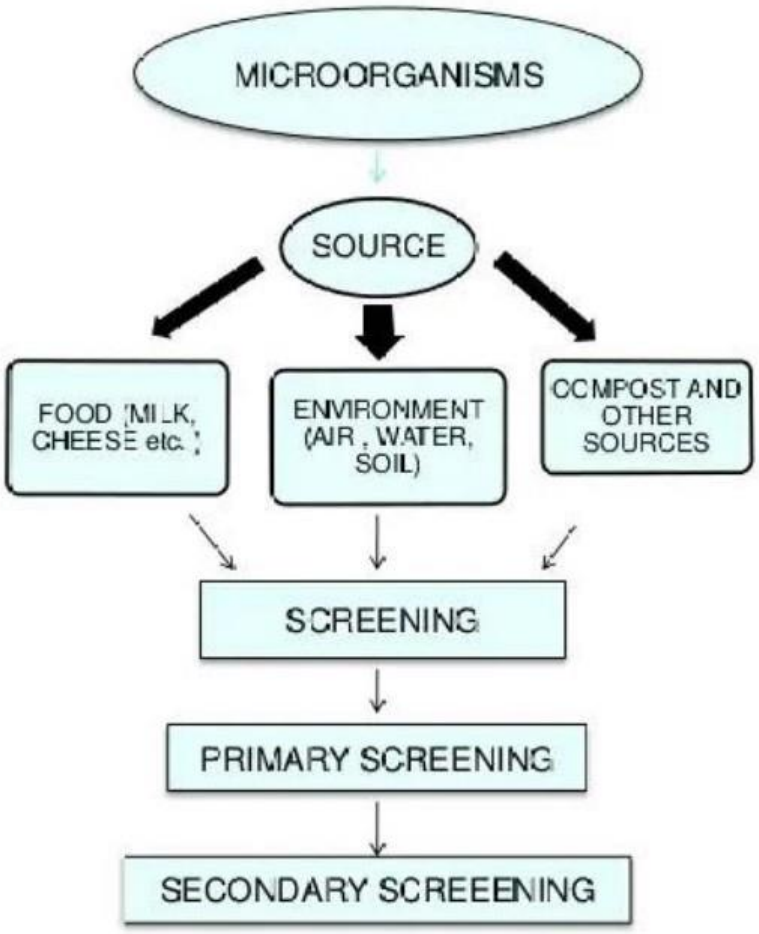


Figure: A flow sheet showing steps in screening of microorganisms

Secondary Screening

It is a systematic screening program intended to isolate industrially important or useful microorganisms. This screening methodology is useful in sorting or separating microorganisms that have real commercial value. The microorganism having poor applicability in fermentation processes are discarded. This screening provides information about whether the product formed by microorganism is novel or not which can be accomplished by paper or thin layer chromatographic techniques. Secondary screening also shows whether the product possesses physical properties such as UV light absorption or fluorescence or unique chemical properties that can be exploited to detect the compound during paper chromatography.

Example of Secondary Screening-Antibiotic producing Streptomyces species

Streptomyces isolates are streaked as narrow bands on nutrient agar plates which are

incubated later on. Test organisms are then streaked from the edge of the plates without touching streptomyceal isolate and are then incubated. At the end of incubation, growth inhibitory zones for each organism are measured in mm. Such organisms are passed through further testing by growing the culture in sterilized liquid media and then incubating it at a constant temperature in a mechanical shaker.

Isolation and Screening of alpha amylase producing bacteria

1. Take one gram of sample (feed, soil, flour, and compost).

2. Take 3 test tubes and add 9 ml of distilled water in each test tube. Cotton plug the test tubes and sterilize in an autoclave.

3. Add one gram of sample in first test tube, mix well and then transfer one ml from the first test tube to the second test tube to prepare 10^{-2} dilution. Then take one ml from the second test tube and add the third test tube to prepare 10^{-3} dilution.

4. Take 200 µL from 10-3 dilution and spread it on starch agar plates (Nutrient agar medium containing 1% starch, pH 7.0).

5. Incubate the plates at 37oC in inverted position in an incubator for 24 h.

6. After incubation, flood the plates with iodine.

7. Clear zone of hydrolysis around bacterial colonies confirm amylase producers.

Practical No: 2 Lab-Scale Production of Alcohol by Yeast

Beer and wine are produced by fermenting glucose in the presence of yeast. Yeast contains enzymes that catalyze the breakdown of glucose to ethanol and carbon dioxide. The active enzyme found in yeast that is responsible for catalyzing the fermentation process is known as zymase.

Glucose + zymase → Ethanol + carbon dioxide

$$C_6H_{12}O_6\ (aq) \rightarrow 2C_2H_5OH\ (aq) + 2CO_2(g)$$

Equipment

- Eye protection
- Conical flask (100 cm3
- Boiling tube
- Measuring cylinder (50 cm3
- Access to a balance (1 decimal place)
- Cotton wool
- Sticky labels
- Warm water 30–40 °C (note 1)

Chemicals

- Glucose, 5 g
- Yeast (as fast acting as possible), 1 g
- Limewater

Apparatus notes

A source of warm water is required. Larger conical flasks can be used, but this dilutes the carbon dioxide concentration, and makes testing for carbon dioxide with limewater more difficult.

Health, safety and technical notes

- Wear eye protection.
- Glucose C6H12O6(s)
- Limewater, Ca(OH)2(aq) – a saturated solution of calcium hydroxide in water.

Procedure:

1. Take 5 g of glucose in a conical flask and add 50 cm3 of warm water in it. Swirl the flask gently to dissolve the contents.

2. Add 1 g of yeast to the solution and loosely close the mouth of the flask with the help of a cotton

plug.

3. Wait for about an hour so that the fermentation reaction can occur.

4. Remove the cotton plug and transfer the liberated gas into the tube containing limewater. Gas will be invisible so; care needs to be taken while shifting. Avoid pouring any liquid in the collecting tube while transferring the gas. Confirmatory test can be performed to ensure that the gas evolved during fermentation was carbon dioxide.

5. Cover the flask containing the fermented solution again with the cotton plug.

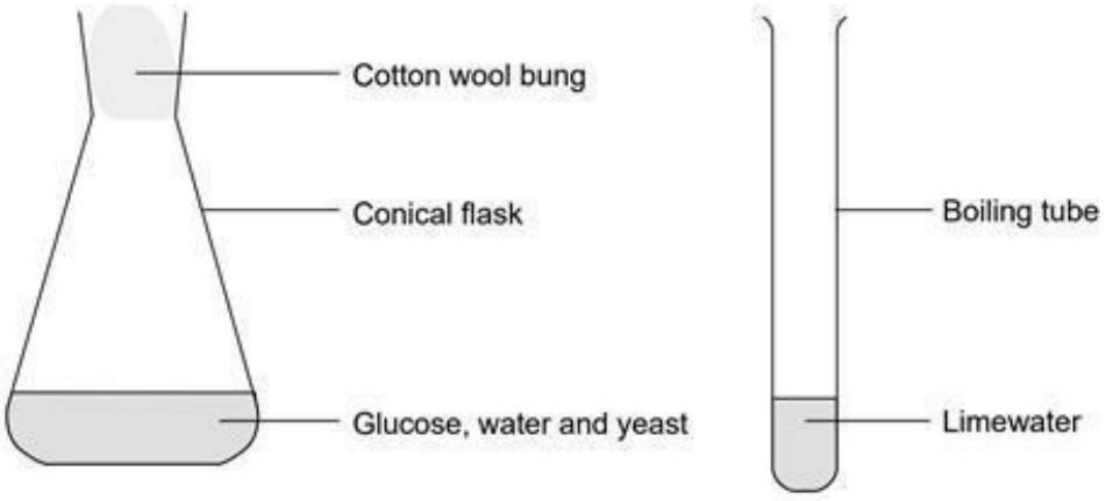


Figure: Apparatus used in lab scale production of alcohol

6. Transfer the fermented solution into the distillation flask connected with the distillation assembly. Boil the mixture thoroughly.

7. Collect the vapor fraction liberated between 77–82 °C. (Ethanol boils at 78 °C.) The obtained vapors will be converted into a liquid while passing through the condenser. This fraction should burn easily compared with the non-flammable original solution. Collect the liquid ethanol in the receiving flask. The ethanol must be poured away immediately. It must not be kept or used.

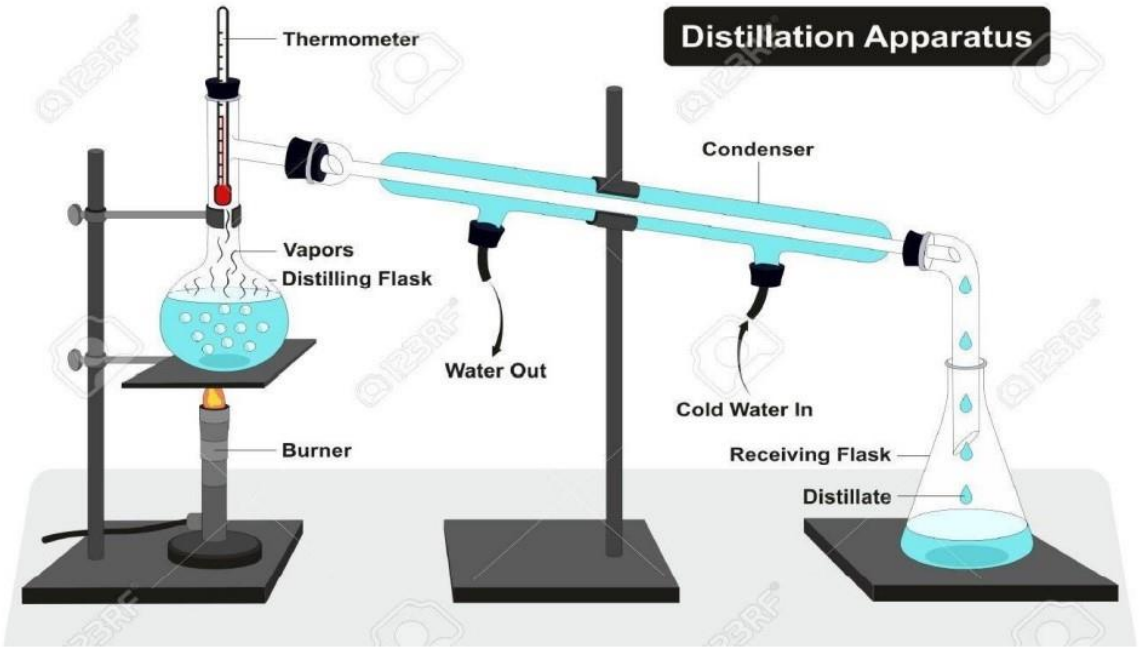


Figure: Distillation apparatus and working

Practical No: 3 Lab-Scale Production of Bacterial Enzymes

Introduction

The term Fermentation comes from Latin word “fervere” which means “to boil”. So, the word “Fermentation” highlights the physical state of boiling or bubbling. Fermentation may be defined as “a process of converting carbohydrates into alcohols or organic acids by using microorganisms (yeast/bacteria) under anaerobic conditions”

Or

Fermentation is a process of mass culturing of cells for the sake of desired product e.g., acids, enzymes, gases, alcohols, hormones etc.

Types of fermentation

There are three types of fermentation on the basis of Moisture (H_2O) content in medium.

1. Solid state fermentation
2. Submerged fermentation
3. Surface fermentation (Organism is allowed to grow on the surface of a liquid medium without agitation. After an appropriate incubation period, the culture filtrate is separated from the cell mass and is processed to recover the desirable product)

Solid-State Fermentation (SSF)

In solid-state fermentation, the microorganisms grow on a moist solid with little or no ‘free’ water, although capillary water may be present.

Examples of this type of fermentation are seen in mushroom cultivation, bread-making, cocoa processing, manufacturing of some traditional foods, e.g., miso (soy paste), soy sauce, tempeh (soybean cake) and gari (cassava), which are now produced by large scale industrial operations.

SSF is also called as Koji Fermentation.

Advantages of solid-state fermentation

1. Cheap
2. Environment friendly

3. High yield

4. Low chances of contamination due to limited water accessibility

Disadvantages

1. Complex downstream processing

2. Difficult to scale up

3. Difficult to monitor and control different parameters

Materials Required

Beaker, Stirrer, 250 ml Erlenmeyer flask, cylinder, wheat bran, Zn $SO_4.7H_2O$, $FeSO_4.7H_2O$, Cu $SO_4.7H_2O$

Procedure

1. Add ten grams of solid substrate such as wheat bran in 250 ml Erlenmeyer flask.

2. Moisten the substrate with 10 ml of suitable diluent (containing mg/L: Zn $SO_4.7H_2O$, 6.2; $FeSO_4.7H_2O$, 6.8; $CuSO_4.7H_2O$, 0.8; Distilled water, 1000 ml).

3. Cover the mouth of a flask with the help of a cotton plug and sterilize it in an autoclave at 121°C, 15 psi for about 15 minutes.

4. Cool the flask at room temperature.

5. Add one ml of the conidial suspension to flask.

6. Incubate the flask at 30/37°C for 48-72 h.

7. After the period of incubation, add 100 ml of distilled water to each flask containing fermented bran.

8. Place the flask in an incubator shaker at 160 rpm for one hour.

9. After one hour, filter the contents of the flasks and use the filtrate for the estimation of the target enzyme (alpha amylase).

Practical No: 4 Use of Microbes in Bioleaching

Introduction

Bioleaching is a simple and effective technology for metal extraction from low- grade ores and mineral concentrates. Metal recovery from sulfide minerals is based on the activity of chemolithotrophic bacteria such as *Thiobacillus ferrooxidans* and *T. thiooxidans,* which convert insoluble metal sulfides into soluble metal sulfates. Non-sulfide ores and minerals can be treated with heterotrophic bacteria and fungi. In these cases, metal extraction is due to the production of organic acids and complex chelating compounds that are later on excreted in the environment. At present bioleaching is used essentially for recovery of copper, uranium and gold, and the main techniques employed are heap, dump and in situ leaching. Bioleaching has also some potential for metal recovery and detoxification of industrial waste products, sewage sludge and soil contaminated with heavy metals.

Microorganisms

Thiobacillus

The bacteria which are frequently used in bioleaching belong to the genus *Thiobacillus*. These are Gram-negative, non-spore forming rods which grow in the presence of aerobic conditions. Most thiobacilli are chemolithoautotrophic species which use carbon dioxide from the atmosphere as their carbon source for the synthesis of new cellular material. The energy is derived from the oxidation of reduced or partially reduced sulfur compounds including sulfides, elemental sulfur compounds and thiosulfate, the final oxidation product being sulfate.

Leptospirillum

Leptospirillum ferooxidans is another acidophilic obligate chemo-lithotrophic bacterium having the ability to oxidize ferrous ions. These bacteria can tolerate low pH values and higher concentrations of uranium, molybdenum and silver as compared to *T.ferrooxidans*, but are more sensitive to copper and unable to oxidize sulfur or sulfur compounds. Therefore, *L.ferrooxidans* cannot attack mineral sulfides by itself. This can only be done with the help of *T. ferrooxidans* or *T. thiooxidans. T. thiooxidans, T. ferrooxidans and L. ferrooxidans* are mesophilic bacteria which

grow best at temperatures of 25-35 degree Celsius.

Thermophilic bacteria

Thiobacillus-like bacteria, so called Th-bacteria, are moderately thermophilic bacteria and grow on pyrite, pentlandite and chalcopyrite at temperatures in the range of 50 Degree Celsius. Ferrous iron is used as an energy source however, growth is observed only in the presence of yeast extract.

Heterotrophic microorganisms

Heterotrophic bacteria and fungi, which require organic supplements for their growth and energy, may contribute to metal leaching. As in the case of manganese leaching, metal solubilization may be due to the enzymatic reduction of highly oxidized metal compounds or cam be affected by the production of organic acids and compounds with at least two hydrophilic reactive groups. These compounds are excreted into the culture medium and dissolve heavy metals by direct displacement of metal ions from the ore matrix and formation of soluble metal complexes and chelates. The heterotrophic microorganisms do not gain any benefit from metal leaching. Among the bacteria, members of the genus Bacillus are most effective in metal solubilization. Among fungi, the genera *Aspergillus* and *Penicillium* are the most important ones.

Bioleaching Mechanisms

In principle, metals can be released from sulfide minerals by direct or indirect bacterial leaching.

Direct bacterial leaching

In direct bacterial leaching, there is physical contact between the bacterial cell and the mineral sulfide surface. The desired oxidation to sulfate takes place via several enzyme catalyzed reactions. In this process, pyrite is oxidized to iron (III) sulfates as shown in the below reactions:

$$4FeS_2+14O_2+4H_2O \xrightarrow{Bacteria} 4FeSO_4+4H_2SO_4 \quad (1)$$

$$4FeSO_4+15O_2+2H_2O \xrightarrow{Bacteria} 2FE_2(SO_4)_3+ 2H_2SO_4 \quad (2)$$

The direct bacterial oxidation of pyrite is best summarized by the following reaction:

$$4FeS_2+15O_2+2H_2O \xrightarrow{bacteria} 2Fe_2(SO_4)_3+2H_2SO_4 \quad (3)$$

Indirect Leaching

In indirect bioleaching, the bacteria generate a lixiviant which chemically oxidizes the sulfide mineral. In an acidic environment, this lixiviant is usually ferric iron, and metal solubilization can be described according to the following reaction:

$$MeS+Fe_2(SO_4)_3 \rightarrow MeSO_4+2FeSO_4+S^0$$

To keep enough iron in solution, the chemical oxidation of metal sulfides must occur in an acidic environment ideally with pH below 5. The ferrous iron generated in this reaction can be reoxidized to ferric iron *by T.ferrooxidans or L.ferrooxidans* and thus, is ready to take part in the oxidation process again. In indirect leaching the bacteria do not need to be in direct contact with the mineral surface. They only have a catalytic function as they accelerate the reoxidation of ferrous iron which takes place very slowly in the absence of bacteria.

Laboratory techniques for leaching

Percolator leaching

In the simplest case, the percolator consists of a glass tube and a sieve plate filled with ore particles at the bottom. The ore packing is irrigated or flooded with a nutrient medium inoculated with bacteria. The leach liquor trickling through the column is pumped up by compressed sterile air to the top of the column for recirculation. Simultaneously, the stream of air takes care of the aeration of the system. To monitor the course of the leaching process, liquid samples are taken at

intervals and the ongoing stage of leaching process is determined on the basis of pH measurements, microbiological investigations and chemical analysis of the metals that have been passed into solution.

Submerged leaching

Submerged leaching uses fine grained material (particle size<100μm) which is suspended in the leaching liquid and kept in a state of motion by continuous shaking or stirring. Higher rates of aeration and an accurate monitoring of various parameters favor the growth and activity of the bacteria so that the reaction times can be considerably shortened and the metal extraction can be increased substantially. Suspension leaching can be carried out in Erlenmeyer flasks or, in a more sophisticated manner, in a bioreactor. Besides mechanically stirred systems, an air-lift reactor has been proven to be suitable for the treatment of ore concentrates, industrial waste products and biodesulfurization of coal.

Column leaching

Column leaching operates on the principle of percolator leaching and is often used as a model for heap or dump size leaching processes. Depending on their size, the columns may be of glass, plastic, lined concrete, or steel. Their holding capacities can range from several kilograms to a few tons. Most column systems have devices for taking samples or special installed instruments for measuring temperature, pH, humidity, oxygen or carbon dioxide levels. This gives information about what has to be expected in heap or dump leaching and how the leaching conditions can be optimized.

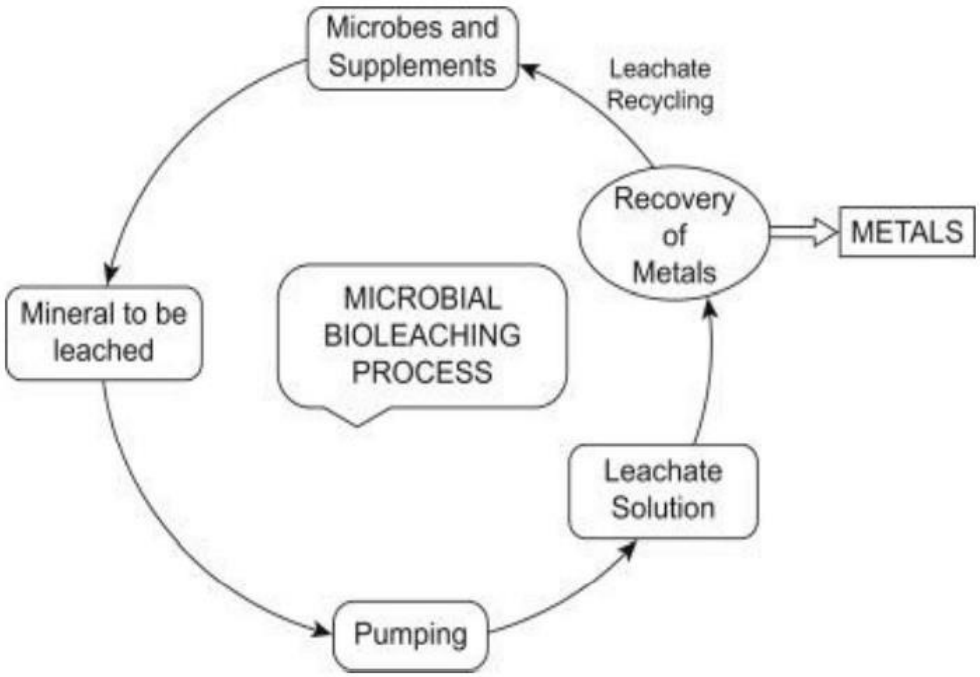


Figure: A flow sheet representing the microbial bioleaching process

Required Chemicals

K2HPO4, KCl, (NH4)2SO4, Ca (NO3)2, MgSO4·7H2O, FeSO4·7H2O, 5M H2SO4, Pyrite concentrates and distilled water

Required Materials

Erlenmeyer flasks, test tubes, aerating assembly, magnetic stirrer, incubator, Perkin-Elmer computerized atomic absorption spectrophotometer and weighing balance.

Media Preparation for the growth of Acidithiobacillus ferrooxidans

- Dissolve the components given in Table in 700ml of distilled water to make Solution A.

Table. Composition of solution A

Component	Quantity
K2HPO4	0.5 g
KCl	0.1 g
(NH4)2SO4	2 g
Ca (NO3)2	0.01 g
MgSO4·7H2O	0.5 g

- Prepare Solution B by dissolving 40 g of FeSO4·7H2O in 300ml distilled water.
- Mix the two solutions together to prevent the formation of unwanted precipitates.
- The major parameters controlling A. ferrooxidans growth are temperature and pH. Stabilize

the acidic conditions of the target solution by adding 5M H2SO4.

- Use a laboratory incubator to maintain an invariable temperature of 30 ◦C after inoculating
- the growth medium with bacteria.

Procedure

1. Prepare 20% pulp using distilled water and pyrite concentrate as a solid sample.

2. Inoculate the pulp with a suitable (5% V/V) bacterial solution (Acidithiobacillus ferrooxidans).

3. Perform the bioleaching experiment in 250ml Erlenmeyer flasks. Take three flasks and label them A, B and C. Add 220ml of the inoculated pulp to each flask.

4. Connect the flasks with air flow assembly and aerate the pulps through air bubbling at the flow rate of 1 liter/min.

5. Agitate the mixture continuously with the help of a magnetic stirrer while maintaining the temperature at 30 Degrees Celsius

6. Maintain the pH at 2.0. The pH tends to decrease while performing the test as the bacteria produce acids during bioleaching but it should not fall below 1.

7. Discard 25% of the leaching solution from each flask every passing day i.e., 55ml/flask/day. Add distilled water to compensate for the discarded solution and raise the total volume to 220ml again.

8. Perform partial re-inoculation using 1% V/V bacterial solution after every three days to make up for the bacteria lost during the time.

9. Take small samples from all three replacement leach solutions separately and confirm the presence of dissolved metals in them with the help of PECAS or Perkin-Elmer computerized atomic absorption spectrophotometer.

10. Collect the metal residues from the flask. Let them dry and weigh them.

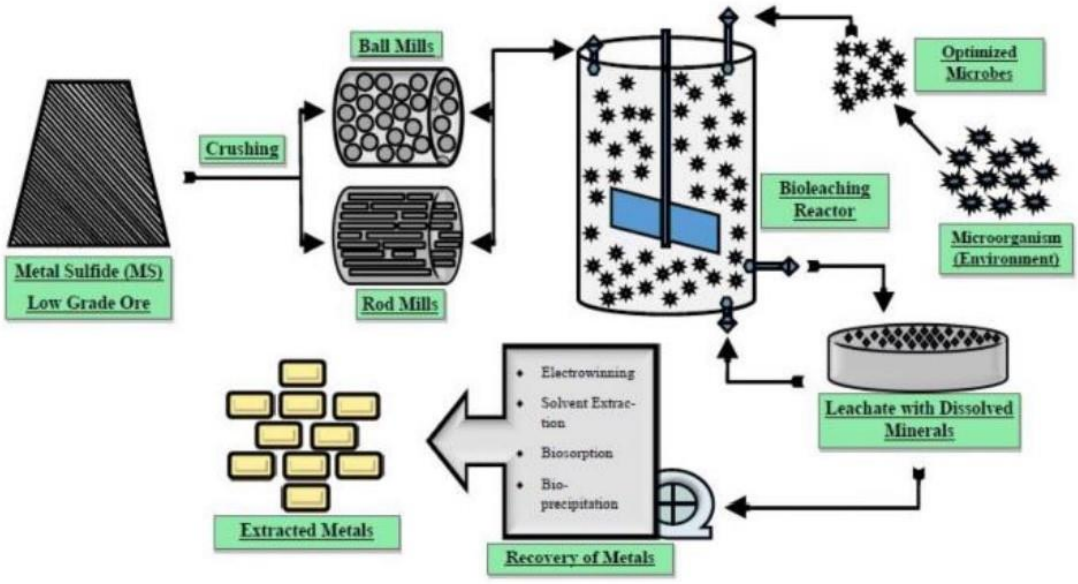


Figure: Bioleaching process

Practical No: 5 Isolation of Microbes Used in Microbial Enhanced Oil Recovery

MICROBIAL ENHANCED OIL RECOVERY

Microbial Enhanced Oil Recovery (MEOR) involves the introduction of viable microorganisms and essential nutrients into an injection well. When favorable environmental conditions are present in the reservoir, the added microbes tend to grow exponentially and their metabolic products mobilize the residual oil. The injected microorganisms can produce many metabolic products during the process which may have many useful applications in EOR. The growth of the microorganisms and their corresponding effects depend on several factors such as:

(i) Pressure, porosity, permeability, temperature, pH, dissolved solids, salinity of the reservoir
(ii) Availability of nutrients for bacteria;
(iii) The type of the microorganisms injected into the reservoir.

MEOR is believed to be able to extract up to 50% of the residual oil left in a reservoir after the primary and secondary recovery processes have been exhausted. In general, this additional recovery is accomplished by the modification of chemical and physical properties of the reservoir rocks and crude oil by the production of metabolites and microbial proliferation during the process. MEOR can, therefore, fulfil fundamental impediments of efficient oil recovery, such as high crude oil viscosity, low permeability of the reservoir, and high oil-water IFTs (inter-facial tension). High interfacial tension creates capillary forces that are sufficiently high to retain the oil within the pores of the reservoir rock.

Mixed microbial populations (principally bacteria and archaea) are commonly used as mixtures or formulations containing metabolic products (e.g., solvents, acids or gases) to increase recovery and prolong the life span of the oil wells. Ideally, metabolic products are produced by the bacteria. For example, solvents such as acetone, butanol and propane-2-diol are produced by bacteria of the genera Clostridium, Zymomonas and Klebsiella. Methane and hydrogen gas are produced by bacteria including Clostridium and Enterobacter, as well as by the archaeon

Methanobacterium. Fermentation gases can re-pressurize wells, leading to the displacement of light crude oil in the well and thus, facilitating its recovery. Among distinct microorganisms used in MEOR, Clostridium is the most suitable because of its highly resistant endospores that promote survival during unfavorable conditions. Some bacilli strains are also effective as they can lead to the in situ production of biosurfactants which are favorable for the MEOR. Nutrients, commonly in the form of fermentable carbohydrates, are injected to promote microbial metabolism.

Advantages

- The injected bacteria, nutrients and/or other natural products can be produced using inexpensive and easy to obtain raw substrates or even waste materials, and they are not affected by crude oil price compared with conventional cEOR processes.
- It is an economically attractive alternative for use in mature oil fields prior to their abandonment.
- No major alteration of the existing field facilities and infrastructure is required to apply the process, making it cheaper and easier to implement than another EOR method.
- Microbial processes consume less energy than thermal EOR processes.

  The process is primarily suited for carbonate oil reservoirs where some EOR technologies would not be able to achieve desired results.
- The benefits of bacterial activity within the reservoir are amplified with time, whereas the opposite is true for other EOR technologies.
- Largely involves fully biodegradable products/additives and hence, is considered an environmentally compatible process.
- Microbial processes can be stimulated in situ within the reservoir, thus minimizing or eliminating the need to accommodate large storage facilities onsite/offshore.

Limitations

- It is a complex process because the desired bacterial activities depend on the physical and chemical characteristics of the reservoir.
- The majority of MEOR field projects are conducted on stripper wells, which renders MEOR a low incremental oil recovery process.
- It is a slower process than chemical or thermal EOR, and usually takes weeks or even months before any benefits can be obtained.
- MEOR is hard to control once implemented in the field, and it is difficult to predict its success rate due to the high heterogeneity of reservoirs.
- The cultivation of microorganisms in the laboratory that can grow and/or produce the desired metabolic products (e.g., biosurfactants) under reservoir conditions has been proven to be difficult.

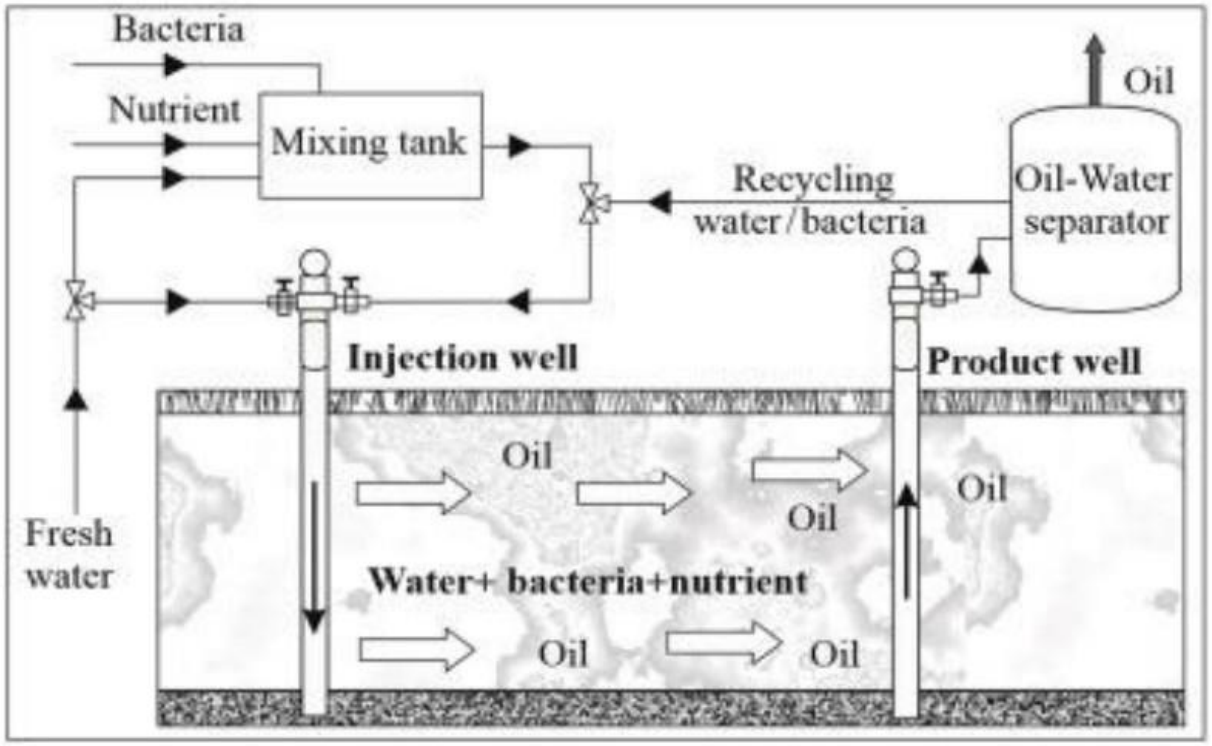


Figure: Procedure of Microbial Enhanced Oil Recovery

Required Chemicals

Molasses, KCl, K2HPO4, (NH4)2 SO4, Na2HPO4, NaCl, Yeast extract, NH4Cl, MgSO4, Tracers, and distilled water.

Required Materials

Test tubes, Incubator, Loop and pipette

Sample and Medium Preparation:

Take MIS oil as a crude oil sample and measure its physical specifications in the laboratory. The specifications are sometimes, mentioned on the label.

Table. Physical specifications of oil sample

Physical Parameter	Value
Viscosity	3.54 cp
Density	42.5 API
IFT	23.3dyn/cm
pH	8

For microorganisms playing a part in oil refining, media enriched with nitrogen has been proven to be a driving force. For isolating the microorganisms from such oil samples, yeast is often added to the growth medium. Prepare the culture medium using the following recipe:

Table: Composition of medium for isolation of microbes associated with oil recovery

Compound	Quantity (g/l)
Molasses	10
KCl	0.5
K2HPO4	1
(NH4)2 SO4	2
Na2HPO4	0.2
NaCl	2
Yeast extract	1.5
NH4Cl	0.25
MgSO4	1
Tracers	10 ml/L
Distilled Water	Enough to make total volume 1 litre

Procedure

1.Take 4 test tubes and add 20ml of microbial cultivation media in each of them.

2. Add about 2ml of crude sample in all four test tubes.

3. The growth media and the crude oil sample will create two distinguishable phases in the test tubes. Place the test tubes in the incubator at 45°C for a period of one month to promote microbial growth.

4. After one month, transfer the viscous residues settled at the bottom of the tubes to the Agar medium as they will be rich in microorganisms. Carefully perform linear streaking on the agar plate.

5. Place the inoculated plates in the incubator again at 45°C for 2 to 3 days.

6. Observe the distinct colonies having different colors and shapes. Identify the bacterial strains.

Practical No. 6

Pure culture study of pickles and acetic acid

Introduction

Acetic acid bacteria (AAB) are a group of microorganisms that fall under the family *Acetobacteraceae*, encompassing various genera and species. Within this family, certain genera such as *Acetobacter, Gluconacetobacter, Gluconobacter,* and *Komagataeibacter* are particularly noteworthy for their significant role in vinegar production. These genera possess exceptional abilities in the oxidation of ethanol to acetic acid, as well as remarkable resistance to the acetic acid released during the fermentation process.

Vinegar production relies heavily on the metabolic activities of these specific AAB species. When ethanol, a primary component of fermented liquids, comes into contact with the acetic acid bacteria, these microorganisms initiate a process known as aerobic fermentation. In this process, they convert the ethanol into acetic acid through the enzymatic action of alcohol dehydrogenase and aldehyde dehydrogenase, resulting in the characteristic sour taste and pungent aroma associated with vinegar.

The selected AAB species possess key traits that make them particularly well-suited for vinegar production. Their exceptional capacity to efficiently oxidize ethanol to acetic acid allows for a rapid and efficient conversion process. Furthermore, these species demonstrate remarkable tolerance to high concentrations of acetic acid, which is released as a byproduct of their metabolic activity. This high resistance enables them to thrive in the acidic environment created during vinegar fermentation, maintaining their enzymatic activity and ensuring the continued production of acetic acid.

The collective contributions of *Acetobacter, Gluconacetobacter, Gluconobacter*, and *Komagataeibacter* species play a crucial role in vinegar production. Their metabolic prowess and ability to withstand the challenging acidic conditions provide the foundation for the successful and consistent production of high-quality vinegar. Through their oxidation of ethanol, these acetic acid bacteria transform the raw materials into a versatile and widely used culinary ingredient, appreciated for its unique tang and versatility in various cuisines and food preparations.

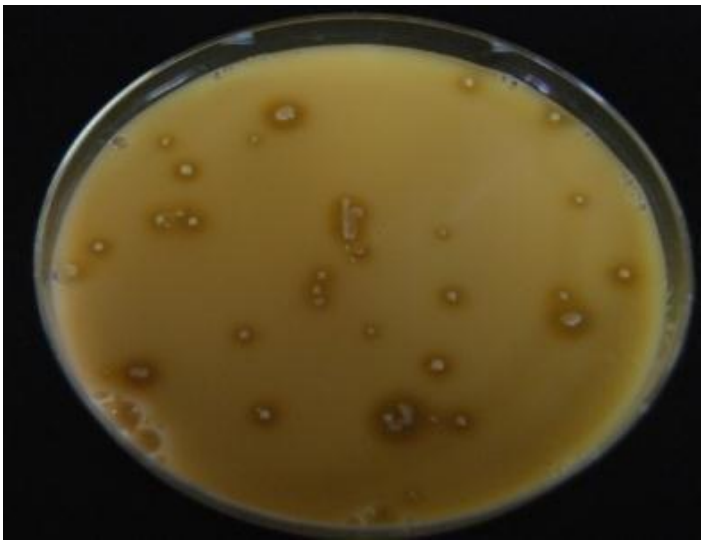
Materials required:

Ripened grapes, Aseptic crushing equipment, Bottles for incubation, Glucose solid GYC medium (10% glucose, 1.0% yeast extract, 2.0% calcium carbonate, 1.5% agar, pH 6.8), Pimaricin antibiotic, Sterile stock solution of Pimaricin, Plates for spreading dilutions, Bromocresol green, Distilled water, Phenolphthalein indicator, 0.5N NaOH solution, Titration equipment, Indole test reagents, Voges Proskauer test reagents, Catalase test reagents

Procedure

1. Collect ripened samples of grapes.
2. Allow the grapes to over-ripen for a few days.
3. Aseptically crush the over-ripened grapes and transfer them into bottles.
4. Incubate the bottles at 30°C for 7 days.
5. Prepare glucose solid GYC medium plates (10% glucose, 1.0% yeast extract, 2.0% calcium carbonate, 1.5% agar, pH 6.8).
6. Supplement the GYC medium with 100 mg of Pimaricin to inhibit yeast and mold growth.
7. Spread 100 µl of different dilutions from the bottles onto the prepared GYC medium plates.
8. After sterilizing the medium, add the antibiotic from the stock solution.
9. Incubate the plates aerobically at 30°C for 3-4 days.
10. Select colonies that exhibit a clear halo on the GYC medium, indicating dominant species.
11. Differentiate Acetobacter and Gluconobacter from the Acetobacteriacea family by their ability to produce acid from calcium carbonate.
12. Examine the morphological and cultural characteristics of the isolated colonies by incubating them on GYC medium at 30°C for 2 days. Differentiate Acetobacter and Gluconobacter using Carr medium and bromocresol green indicator.
13. Observe color changes in the media: Acetobacter turns it from yellow to green, while Gluconobacter turns it yellow.
14. Monitor acetic acid production by performing titration every 24 hours.
15. Mix 5ml of the culture with 20ml of distilled water.
16. Add 3-5 drops of phenolphthalein indicator for acetic acid estimation.
17. Titrate the solution against 0.5N NaOH and record the volume used.
18. Calculate the amount (g) of acetic acid produced in 100ml of medium using the formula: Acetic acid (g/100ml) = Volume of NaOH (ml) used in titration × 0.03 × 20.

19. Perform biochemical tests, including the indole test, Voges-Proskauer test, and catalase test, for further identification of the culture.



***Acetobacter* on GYC media**

Practical No. 7

Preparation of fermented products using bacterial cultures

Introduction

Yoghurt is a food product that is created through the process of bacterial fermentation of milk. The specific bacteria responsible for yoghurt production are referred to as yoghurt cultures, which mainly consist of Lactobacillus bulgaricus and Streptococcus thermophilus. However, other types of lactic acid bacteria can also be utilized. These bacteria have the ability to ferment lactose, a sugar present in milk, and convert it into lactic acid. The lactic acid produced by these bacteria interacts with the milk protein, resulting in the unique texture and tangy flavor of yoghurt.

Yoghurt contains various components such as water, fat, protein, sugar, and minerals (ash). Due to its composition, yoghurt can contribute to the improvement of the gut's microflora. It can be produced using different types of milk, including goat, cow, sheep, horse, water buffalo, as well as skimmed milk, non-fat milk, and even plant-based alternatives like soymilk.

Lactic acid bacteria are well-known and widely used in the food industry, particularly for yoghurt production. These bacteria are characterized by their positive Gram reaction, varying from rod to cocci shaped structures. They have a tolerance to acidic environments and do not produce spores. However, their most notable characteristic is their ability to produce lactic acid, which is essential for the fermentation process involved in yoghurt production.

Materials

Sterile bottles for collecting milk samples, Pour plate technique materials (agar plates, petri dishes, sterile pipettes, incubator), Phenotypic identification tools for lactic acid bacteria (microscope, staining reagents, culture media), Sterile glass bottles, Water bath, Pasteurization equipment, Starter cultures containing lactic acid bacteria, Thermo-controlled water bath, Cold storage facility (refrigerator)

Procedure

1. Collect two samples of raw milk from both cow and goat from the market. Use sterile bottles to ensure cleanliness and prevent contamination during collection.
2. Isolate the lactic acid bacteria (LAB) from the collected raw milk samples using the pour plate technique. This involves plating the milk samples on agar plates and incubating them to allow bacterial growth. After incubation, visually identify and distinguish the LAB colonies based on their characteristics.
3. Screen and select the LAB colonies based on their ability to produce diacetyl (a flavor compound) and lactic acid. Perform tests or observations to assess the production of these substances by the selected colonies.
4. Take sterile glass bottles and pour 100 mL of raw cow milk samples into each bottle. Pasteurize the milk by heating it in a water bath at 85°C for 30 minutes. Then, cool the milk to a temperature of 37°C.
5. Inoculate the pasteurized cow milk samples with 1.0 mL of the selected starter cultures. These starter cultures contain a concentrated amount of LAB, with an inoculum size of 106 colony-forming units per milliliter (CFU/mL).
6. After inoculation, thoroughly mix the contents of the bottles to ensure even distribution of the starter cultures. Place the bottles in a thermostatically controlled water bath set at a temperature of 42°C.
7. Incubate the milk samples in the water bath for 4-6 hours to allow fermentation to occur. Once the incubation period is complete, cool the yoghurt samples to 4°C and store them in cold storage (refrigerator) for future use.

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