Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 1

Experiment title

General introduction about Microbial Ecology and Pollution

Aim and objective

To study the microbes in the surrounding environment, and types of pollution.

Introduction

Microbial ecology is the study of microorganisms in their natural habitats and how they interact with each other and with the environment. Environmental microbiology, on the other hand, is the study of microorganisms in various environmental settings, such as soil, water, air, and sediments. Both fields are closely related and have important implications for understanding microbial diversity, evolution, and ecosystem functioning. Microbial ecology focuses on the interactions between microorganisms, their environment, and other organisms, including plants and animals. These interactions can be either beneficial or harmful, depending on the context. For example, some microorganisms are essential for nutrient cycling and soil fertility, while others can cause disease in plants or animals. Microorganisms play critical roles in many ecosystem processes, such as carbon and nitrogen cycling, and are often used as indicators of environmental health. Understanding the distribution and abundance of microorganisms in different environments can provide insights into the biogeochemical cycles of elements and the effects of human activities on the environment. Both microbial ecology and environmental microbiology have important applications in biotechnology, agriculture, and medicine.

Microbes and types

Most types of microbes remain unknown. It is estimated that we know fewer than 1% of the microbial species on Earth. Yet microbes surround us everywhere -- air, water, soil. An average gram of soil contains one billion (1,000,000,000) microbes representing probably several thousand species.

- Archaea: single cell microorganisms
- Bacteria: can be found in soil, water and the Earth crust. They also live in symbiotic and parasitic relationships with plants and animals.
- Fungi: microorganisms such as yeasts and molds, as well as the more familiar mushrooms.

- Protists: primitive algae, amoebas, slime molds and protozoa.

Importance: The study can help us improve our lives via the use of microbes in environmental restoration, food production, bio-engineering of useful products such as antibiotics, food supplements, and chemicals. It helps measure the effects of climate change and land usage.

Diverse Habitats: Microbes inhabit the widest range of habitats, including, sub-freezing temperature, deep sea, and hot volcanoes.

Pollution: The term "pollution" may be described as "infiltration of vitality or substances into the environment by either natural or anthropogenic means that may compromise human health, decimate ecosystems and living beings. These harmful substances are referred to as pollutants.

Types of pollutants: Pollutants are two types, they are primary pollutants and secondary pollutants. Primary pollutants are harmful in their original form, while secondary pollutants are generated through chemical reactions of relatively benign precursor substances present in the environment.

Types of pollution

Air Pollution: Air pollution refers to the exceeding of predetermined concentrations of certain substances in the atmosphere that result in a harmful phenomenon for the ecological system and disrupt normal conditions for human existence and development. The substance responsible for this contamination is referred to as an atmospheric pollutant/contaminant. The major contaminants are sulphur dioxide (SO2), ozone (O3), carbon monoxide (CO), as well as nitrogen oxides suchas nitrogen dioxide (NO2) and NOx.

Water Pollution: Water pollution is defined as the presence of substances in water bodies that change their chemical, physical, or biological properties and can have adverse effects on living organisms and their environment. Water pollution is caused by various contaminants, such as chemical pollutants (pesticides and heavymetals), mcrobiological pollutants (bacteria and viruses), nutrient pollutants (nitrogen and phosphorus), thermal pollution and waste products (sewage and industrial waste).

Soil Pollution: Soil pollution pertains to the infiltration of noxious substances into the composition of the soil, leading to detrimental consequences on the environment and human health. This phenomenon is typically initiated by anthropogenic activities such as industrial processes, agricultural practices, andimproper disposal of hazardous waste materials. The presence ofcontaminants in the soil can result in significant soil degradation and decreased fertility, negatively affecting the growth and viability of vegetation, fauna, and microorganisms.

Noise Pollution: Noise pollution, also known as anthropogenic noise, pertains to the amplification of inherent background noise levels caused by human activities that produce sound.

Thermal Pollution: Thermal pollution is the harmful alteration of water or air temperature caused by human activities, such as the discharge of heated water from industrial processes or the use of cooling systems in power plants.

Radiation Pollution: Radiation pollution refers to the existence of ionizing or nonionizing radiation from the nuclear power generation, medical procedures, and industrial processes.

Conclusion

Detailed information related to Ecology and pollution was studied

Reference

T. R. G. Gray, Methods In Mlcroblology, Methods for Studying the Microbial Ecology of Soil; Volume 22 ISBN 0-12-521522-3

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 2

Experiment title

Isolation of Nitrogen fixing bacteria

Aim and objective:

Isolation of nitrogen fixing bacteria from the rhizosphere.

Brief Introduction

Nitrogen (N) is abundant in the atmosphere in the form of a dinitrogen gas (N_2), but only a small number of prokaryotic organisms, known as diazotrophs, are capable of using it directly as a nitrogen source through biological nitrogen fixation (BNF). Several organisms rely directly or indirectly on diazotrophs and establish symbiotic interactions with them for proper growth. Nitrogen fixation is a process that implies the transformation of the relatively non-reactive atmospheric N_2 into its more reactive compounds (nitrates, nitrites, or ammonia). Why is nitrogen fixation important? Such reactive forms are suitable for crops and support their growth. One of the most extensively studied interactions is the endosymbiotic relationship established between the roots of legumes and diazotrophic organisms called rhizobia, which provide fixed nitrogen to plants in exchange for protection, nutrients, and energy

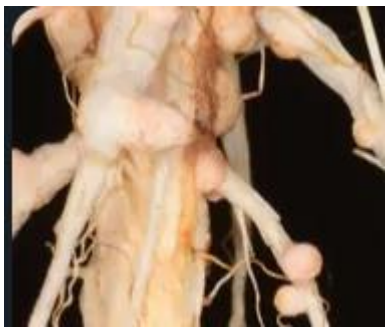
. 

Fig: Root nodules

Legumes are known as the best nitrogen-fixing plants and can be grown either as cash or cover crops. Nitrogen-fixing cover crops bring multiple benefits to farmers:

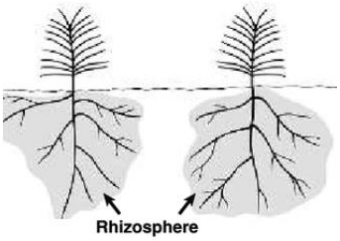
- participate in N fixation
- protect soil from erosion by covering it or holding in place with strong root systems
- improve soil fertility when used as green manure

- retain soil moisture
- help in weed management with crop residues
- serve as forage and grazing material for poultry and cattle
- attract pollinators at the crop flowering time

Nitrogen-fixing bacteria examples comprise Rhizobium (formerly Agrobacterium), Frankia, Azospirillum, Azoarcus, Herbaspirillum, Cyanobacteria, Rhodobacter, Klebsiella, etc. N-fixing bacteria synthesize the unique nitrogenase enzyme responsible for N fixation. Nitrogen-fixing bacteria convert gaseous N from the air to inorganic compounds. Even though the role of legumes in N fixation is undeniable, the task is too hard for them alone. In fact, the fixation process occurs thanks to the symbiosis of legumes and nitrogen-fixing bacteria. It is common for Rhizobium, colonizing legume roots. However, their symbiosis is not the only option: there exist free-living and associated N fixation organisms as well. N-fixing bacteria provide crops with ready-to-use N that they need as part of chlorophyll molecules. Chlorophyll is critical for photosynthesis – transforming the energy of sunlight into chemical energy. Simply put, plants need it to get food. Also, they require N as part of amino acids to build proteins that participate in metabolism and energy storage. A lack of N fixation leads to food deficiency, which results in vegetation yellowing, thinning, withering, overall growth delay, and decay.

Rhizosphere

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root.



General features of *Frankia*

- Filamentous
- Gram-positive and aerobic
- Made up of thick cell walls (peptidologlycan)
- It grows best at around 30 degrees Celsius with an environment pH between 6.5 and 7
- It has a symbiotic relationship with many plants

- Produce three cell types: sporangiospores, hyphae, and diazo-vesicles (spherical, thick walled, lipid-enveloped cellular structures)
- The diazo-vesicles are responsible for the supplying of sufficient Nitrogen to the host plant during symbiosis

Materials

- Autoclave
- pH meter
- Petri plates
- Inoculation loop
- Incubator
- Water testing kit
- Root nodules
- 50 mL Beaker
- Test tubes
- Wash bottles
- Jensen's Medium

Composition of Jensen's Medium

Sucrose	20.0 g
Dipotassium phosphate	1.0 g
Magnesium sulphate	0.50 g
Sodium chloride	0.50 g
Ferrous sulphate	0.10 g
Sodium molybdate	0.005 g
Calcium carbonate	2.0 g
Agar	20.0 g
Water	1000 ml
pH	7.0

Note: Due to presence of calcium carbonate, the medium forms opalescent solution with white precipitate

Procedure

1. Suspend 39.1 grams of the medium in one liter of distilled water.
2. Heat the medium and dissolve completely.
3. Autoclave the medium at 121°C, 15 psi pressure, for 15 minutes.
4. Mix well and dispense into Petri dishes (pour approximately 20 mL/plate).
5. Take 1 gram of rhizosphere associated soil or root nodules and serially dilute it up to 10^{-7} dilution.
6. Take 0.1 mL sample at various dilutions and spread on Jensen's medium.
7. Incubate the plates at 37 °C for 3-5 days and observe colony development.
8. Pick the isolated colony and further streak on Jensen's medium (quadrant streaking).
9. Repeat the step 7 and store the culture at 4 °C.

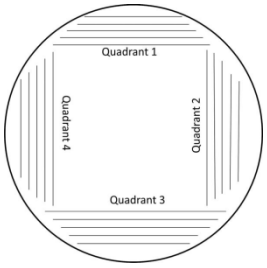


Fig: Diagram of the four-quadrant streak technique

Results and observations

From the root nodules, after 24 hour of incubation, microbial colonies were observed and the colony morphological features were similar to nitrogen fixing bacteria *Frankia*

Conclusion

Nitrogen fixing bacteria was isolated from the root nodules

Reference

Barry E. Smith;nRaymond L. Richards; William E. Newton. Catalysts for Nitrogen Fixation. Nitrogenases, Relevant Chemical Models and Commercial Processes.2004.

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 3

Experiment title

Microbial ecology and survey of microorganisms in different leaves decay areas

Aim and objective

To study effect of different decay leaves on the diversity of the microorganisms on the soil

Introduction

It is often necessary to determine the rate at which different leaves or twigs or components of them decay in different soils. Direct observation of microorganism in the soil provides a basis for ecological studies since it gives direct evidence of the occurrence of microbes in particular environments. This is because microorganisms are not uniformly distributed throughout the soil and their precise arrangement varies spatially and temporally. Methods employed for studying microbial population in soil include;

1. Microscopic examination of stained soil
2. Haemocytometer method
3. Contact slide method
4. Soil enrichment method

General features of Actinomycetes

- Gram positive
- Filamentous, dry powdery in appearance
- Possess both aerial and substrate mycelium
- It grows best at around 30 degrees Celsius with an environment pH between 6.5 and 7
- Capable of producing diffusible pigments
- Actinomycetes produce many enzymes such as protease, amylase, lipase, pectinase, cellulase, xylanase, glutaminase and asparaginase
- It produces many bioactive compounds such as streptothricin, streptomycin, actinomycin, chloramphenicol, tetracycline, erythromycin, leucomycin, vancomycin, gentamicin, teicoplanin, fortimicin, rosamicin, nocardicin and salinosporamide A

General features of Fungi

- Most fungi are multicellular organisms.
- They display two distinct morphological stages: the vegetative and reproductive
- The vegetative stage consists of a tangle of slender thread-like structures called hyphae
- The mass of hyphae is a mycelium
- Most fungal hyphae are divided into separate cells by endwalls called septa

General features of Bacteria

- They are either Gram positive or Gram negative
- They are either motile or non-motile
- Most bacteria are either coccus, rod-shaped or spiral
- Bacteria grow and divide by binary fission, with a wide range in growth rates

Materials required

- Autoclave
- pH meter
- Petri plates
- Inoculation loop
- Incubator
- Water testing kit
- Soils from different leaf decay area
- 50 mL Beaker
- Test tubes
- Wash bottles
- Czapek Dox agar (for fungi)
- Nutrient Agar (for bacteria)
- Starch casein Agar (for actinomycetes)

Reagents

Fixative solution

The fixative solution is prepared by dissolving 0.15 g of gelatin in 1 litre of distilled water.

The staining solution consist one gram of erythrosine or Rose Bengal dissolved in 100 ml

of 5 % aqueous solution of phenol containing sufficient CaCl2 (0.001-0.1 %) to give a very faint precipitate.

Procedure

1. Prepare and sterilize the medium as per the standard protocol (121°C, 15 psi pressure, for 15 minutes).
2. Mix well and dispense into Petri dishes (pour approximately 20 mL/plate).
3. Take 1 gram of leaf litter soil and powder it using the blender and serially dilute the samples to 10^{-7} dilution.
4. Take 0.1 mL sample at various dilutions and spread on Czapek Dox agar, Nutrient Agar and Starch Casein Agar.
5. Incubate the plates at 37 °C for 3-5 days and observe colony development.
6. Pick the isolated colony and further streak on the respective medium for the picking the pure colony.
7. Repeat the step 7 and store the culture at 4 °C.
8. Identify the morphology of the microorganisms based on the appearance in the plates and record it for further study.

Determination of bacterial population

To Calculate CFU (cells/mL) do the following

Step 1: Determine the concentration of cells in the diluted sample:

(# of colonies counted on the petri plate) ÷ (amount of diluted sample added to the petri plate in mL) = CFU in diluted sample (cells/mL)

Step 2: Determine the concentration of cells in the original sample:

(CFU in diluted sample) ÷ (dilution of the petri plate counted) = CFU in original sample (cells/mL)

Example

- **# of colonies counted =** 129
- **amount of diluted sample added to the petri plate in mL** = 200 µL = 0.2 mL
- **dilution of the petri plate counted =** 10^{-6}

Step 1: Determine the concentration of cells in the diluted sample (Figure 1):

(# of colonies counted on the petri plate) ÷ (amount of diluted sample added to the petri plate in mL) = CFU in diluted sample (cells/mL)

(129 colonies) ÷ (0.2 mL diluted sample added to petri plate) = 645 cells/mL in the diluted sample

Step 2: Determine the concentration of cells in the original sample:

(CFU in diluted sample) ÷ (dilution of the petri plate counted) = CFU in original sample (cells/mL)

(645 cells/mL in diluted sample) ÷ (10^{-6} dilution of the petri plate counted) = 645,000,000 cells/mL in original sample

CFU = 645,000,000 cells/mL in original sample

Procedure for Microscopic examination of stained soils

This method consists of preparation of a suspension of soil in a dilute fixative solution, 1 or 2 drops of the suspension is spread upon a clean slide which is then dried and stained with acid dye and finally examined with a high magnification microscope. The fixative solution is prepared by dissolving 0.15 g of gelatin in 1 litre of distilled water. The staining solution consist one gram of erythrosine or Rose Bengal dissolved in 100 ml of 5 % aqueous solution of phenol containing sufficient CaCl2 (0.001-0.1 %) to give a very faint precipitate

Results and observations

The soil and leaf decay samples guided to identify different characteristics features containing bacteria, fungi and actinomycetes. The actinomycetes were pigment producing and the pigments were diffused in the starch casein medium.

Conclusion

Bacteria, fungi and actinomycetes were isolated from the leaf decay samples

Reference

T. R. G. Gray, Methods In Mlcroblology, Chpater,10: Methods for Studying the Microbial Ecology of Soil; Volume 22 ISBN 0-12-521522-3

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 4

Experiment title

Effect of Chemical Factors on the growth and Distribution of microorganisms

Aim and objective

The Effects of Chemical Factors on Growth of microorganisms

- Effect of antibiotics
- Effect of chemical elements
- Effect of organic and inorganic inhibitors
- Effect of heavy metals

Brief Introduction

Antibiotics

They are substances produced by microorganisms that effective in killing or inhibiting the growth of other microorganisms.

They are important in the treatment of microbial diseases.

The mechanism of action of antibiotics in microbes is divided into two parts:

1- Bacteriostatic.

2- Bactericidal.

Some antibiotics are produced by fungi (e.g., penicillin).

Some antibiotics are produced by Streptomyces's (a group of bacteria) (e.g., tetracycline).

Antibiotics are divided according to the way they affect microbes:

1- Affect the composition of the cellular wall.

2- 2- Inhibition of protein building inside the cell.

3- 3- Inhibition of building nucleic acids DNA/RNA.

4- 4- Affect the selective permeability of the cell membrane.

5- 5- Affect some metabolic processes.

Methods of resistance of bacteria to antibiotics:

1- Some bacteria produce enzymes that break down the antibiotics.

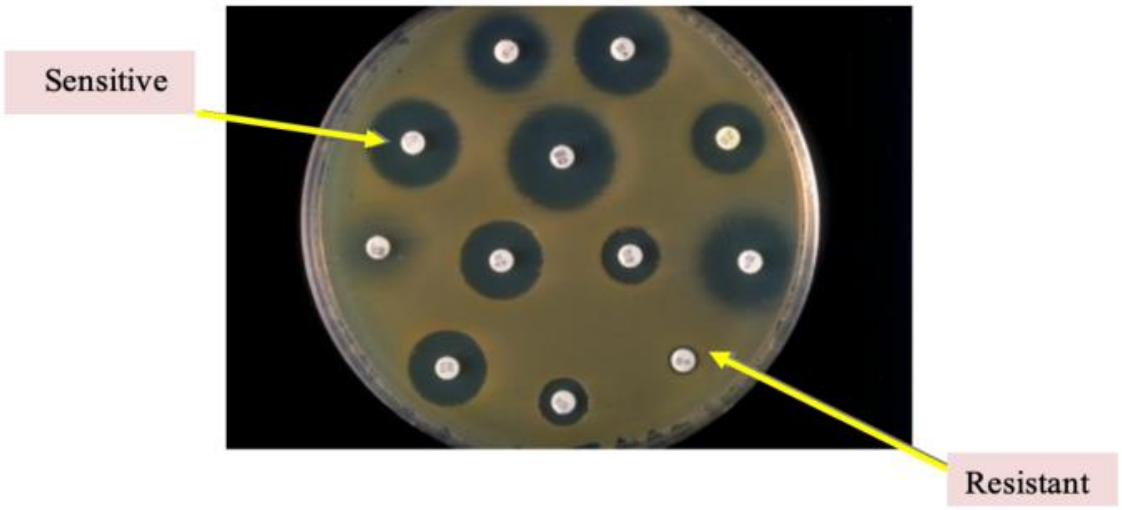
2- Some bacteria secrete enzymes that alter the chemical composition of the antibiotics.

3- change membrane permeability of bacterial cells, which inhibits the entry of the antibiotics.

4- The bacteria change the nature of some of its components that the antibiotics targeted.

Antibiotic sensitivity test:

This method depends on the diffusion method of the paper disk that contains it to the agar media that containing the microbe, which fails to grow in the area of the antibiotic.



Materials

- Autoclave
- pH meter
- Petri plates
- Inoculation loop
- Incubator
- Water testing kit
- Root nodules
- 50 mL Beaker
- Test tubes
- Wash bottles
- Agar medium for the cultivation of microbes
- Different antibiotic discs (Ampicillin (Amp), Amoxycillin (10 µg), Chloramphenicol (30µg), Nalidixic Acid (NA), Nitrofurantoin (300 µg), Gentamicin (10µg), Ciprofloxacillin (5 µg), Cephalothin (30µg), Ceftriaxone (30 µg), Norfloxacin (NOR), Doxycycline (30 µg), Trimethoprim-Sulfamethoxazole (25 µg), and Tetracycline (30 µg).)

Procedure

1. Swab the microbes on the respective agar medium
2. On the top of the microbes, place the antibiotic discs
3. Incubate the plates for 24 to 48 hours and measure the zone of hihibition.

Results and Observation

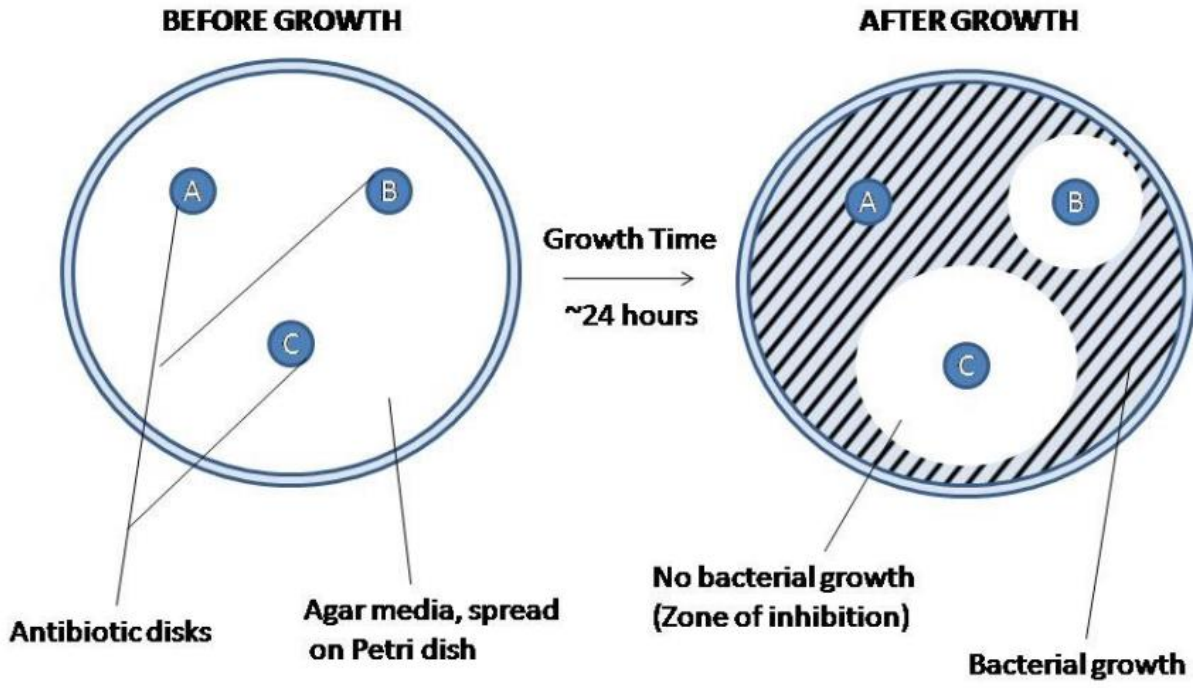


Fig: The effect of antibiotics before and after growth was recorded

Conclusion

The antibiotics inhibited the growth of the microorganisms.

Reference

T. R. G. Gray, Methods In Mlcroblology, Chapter,10: Methods for Studying the Microbial Ecology of Soil; Volume 22 ISBN 0-12-521522-3

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 5

Experiment title

Demonstration Of Ammonification of soil

Aim and Objective:

To detect the ammonification using an unknown bacterial slant culture.

General Information:

During ammonification, some organic nitrogen compounds (amino acids, carbamide, etc.) are deaminated to form ammonium ions and as a function of pH, ammonia is released into the environment. For such deamination reactions, bacteria use many enzymes with different substrate specificity (one way of getting rid of excess organic nitrogen). The remaining part of the molecule can be used for e.g. energy generation. Within the nitrogen cycle, ammonification is considered as a mineralization step. Ammonia and ammonium ion can be taken up and used for amino acid synthesis by other organisms (plants, microbes) or it can be absorbed in the soil by humus-colloids.

Materials Required

1. Peptone broth
2. Urea broth
3. Inoculating loop
4. Sterile distilled water in test tubes
5. Pipette
6. Sterile pipette tips
7. Bunsen burner
8. Incubator
9. Nessler's reagent (an alkaline solution of potassium-tetraiodo-mercurate)

Work Procedure:

1. Make a suspension from the unknown bacterial strain in sterile distilled water.
2. Inoculate the peptone/urea broth tubes with 0.1-0.1 mL bacterial suspension.

3. Incubate the tubes at 28°C for one week.
4. The presence of accumulated ammonia in the broth can be demonstrated by adding a few drops of Nessler's reagent.

Observation and Comments:

A weak positive reaction yields a yellow colour while strong positive reaction results in a yellowish brown colour and precipitation.

Conclusion

The ammonification reaction was confirmed

Reference

T. R. G. Gray, Methods In Mlcroblology, Methods for Studying the Microbial Ecology of Soil; Volume 22 ISBN 0-12-521522-3

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 6

Experiment title

Role of lithotrophic bacteria in sulfur metabolism

Aim of the experiment:

To detect and understand lithotrophs and to analyze lithotrophic bacteria via sulphur utilization

General information

A lithotroph is a microorganism that uses inorganic substrates as a source of electron donors to drive energy acquisition. In nature, photolithotrophic bacteria, and chemolithotrophic bacteria and archaea, work in tandem to carry out the oxidative half of the sulfur cycle. Chemolithotrophy is the oxidation of inorganic chemicals for the generation of energy and is another form of cellular respiration. These organisms are exclusively bacteria and archaea. Although lithotrophs may perform aerobic or anaerobic respiration, because of energy constraints, they are typically aerobic. Chemolithotrophs use a variety of inorganic compounds as electron donors, including, sulphur compounds (such as sulphide and sulphur), nitrogen compounds (such as ammonium and nitrite), and ferrous iron. Lithotrophic bacteria play a key role in the sulfur cycle by oxidizing inorganic sulfur compounds into organic molecules:

Sulfur oxidation: Lithotrophic bacteria use inorganic substrates to produce energy, and sulfur is one of the many substrates they can use. They use a variety of pathways to oxidize sulfur compounds, such as sulfide or elemental sulfur, into sulfate.

Sulfur reduction: Some bacteria, such as those in the phylum Thermodesulfobacteriota, reduce sulfur.

Sulfur cycling: Lithotrophic bacteria work with photolithotrophic bacteria to complete the oxidative half of the sulfur cycle.

Biomass production: Lithotrophic bacteria are primary or secondary producers of biomass in some environments.

General features of Thiobacillus

- Gram-negative Betaproteobacteria

- They are obligate autotrophs
- Widespread in marine and terrestrial habitats
- Chromatiaceae (purple sulfur bacteria) and Chlorobiaceae (green sulfur bacteria) utilize energy from light in an oxygen-free environment to transform sulfur and its compounds to sulfates.

Materials

Sample (wastewater)

Starkey broth

Thiosulfate broth medium

Composition of Starkey broth

Starkey broth is a medium used to isolate and maintain *Thiobacillus* species. The composition of Starkey broth is:

- 3.0 g KH_2PO_4
- 0.2 g $MgSO_4 \cdot 7H_2O$
- 0.2 g $CaCl_2 \cdot 2H_2O$
- 0.5 g $(NH_4)_2SO_4$
- Traces of $FeSO_4$
- pH adjusted to 8.0

After sterilization, 10 g of elemental sulfur is added to the broth.

Composition of thiosulfate broth medium

- 1 g NH_4Cl
- 0.1 g $CaCl_2.2H_2O$
- 10 g NaCl
- 0.5 g KH_2PO_4
- 2 g K_2HPO_4
- 0.8 g $MgSO_4.7H_2O$
- 3 g $Na_2S_2O_3.5H_2O$
- 2 mL of 0.5%

Procedure

Isolation of sulfur-oxidizing bacteria (SOB)

- Serially dilute the wastewater sample
- Take any three dilutions and inoculate into thiosulphate agar media containing 1.5% bacteriological agar and 2 mL of 0.5% phenol red.
- Incubate the culture for 7 days at 30 ºC under aerobic conditions.
- Purify the isolated colonies by streak plate method.

Screening of SOB isolates base on pH increasing and decreasing of sulfur concentration

- Prepare thiosulfate broth medium and adjust the pH to 6.0.
- Inoculate the SOB isolates into thiosulfate broth medium.
- Incubate for 7 days at 30 ºC in a shaker incubator.
- Check the pH of the medium after 7 days and the reduction of pH indicated sulphur oxidation.

Observation and findings

Sulphur oxidizing bacteria is isolated from the sample

The changes in the medium pH indicated sulphur oxidation.

Conclusion

The lithotrophic bacteria from the water sample has been isolated.

Reference

T. R. G. Gray, Methods In Mlcroblology, Chapter,10: Methods for Studying the Microbial Ecology of Soil; Volume 22 ISBN 0-12-521522-3

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 7

Experiment Title

Effect of physical and chemical factors on the growth and distribution to microorganisms-survey of microorganisms.

Aim and objective

To select the maximum tolerance level of the bacteria, fungi, and actinomycetes under different concentrations of crude oil, petrol and diesel

Introduction

The growth profile of bacteria in crude oil, petrol, and diesel can vary depending on several factors, including the organism, the concentration of the hydrocarbon, and the incubation period. The rate of growth or death of a particular microbial species is influenced by a variety of physical factors in its environment including **temperature**, **osmotic pressure**, **pH**, and **oxygen concentration**. Exposure to environmental toxins can change the metabolic activity of microorganisms. The petroleum hydrocarbons inhibited microbial biomass, and that the greatest negative effects were observed in the gasoline-polluted sandy soil.

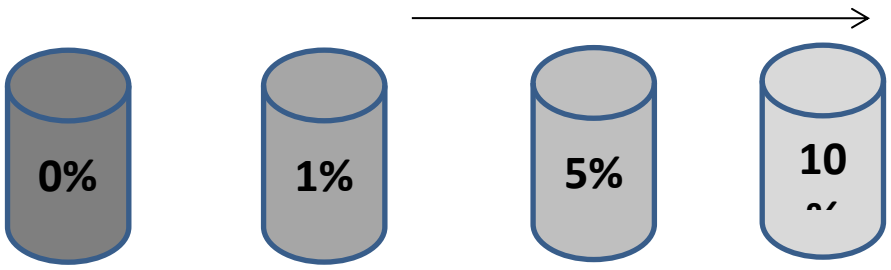
Materials

- 250 ml conical flask
- Nutrient broth (for bacterial culture)
- Potato dextrose broth (for fungal culture)
- Actinomyces broth (for Actinomycetes culture)
- Petrol
- Diesel
- Crude oil
- Pure culture of bacterium (e.g., *Pseudomonas* sp.,), actinomycetes (e.g., *Streptomyces* sp.), and fungus (e.g., *Trichoderma* sp.).

Procedure

- Suspend 2.5 grams of nutrient both medium in 100 mL double distilled water.
- Suspend 2.4 grams potato dextrose broth medium in 100 mL double distilled water.

- Suspend 5.72 grams of actinomycetes broth medium in 100 mL double distilled water.
- Then add 1.0 mL (1%), 5 mL (5%), and 10 mL (10%) diesel, or petrol, or crude oil separately.
- To the blank do not add petrol, diesel or crude oil.
- Inoculate 0.5 mL of the broth culture of bacteria, fungi, and actinomycetes culture (prepare inoculums by inoculating the organism in liquid medium and incubate up to reach optimum growth) individually.
- Incubate the bacterial, fungal, and actinomycetes culture at 30±1 ºC for 1 day, three days, and five days respectively.
- Visually observed the turbidity in the culture.
- Take optical density of the sample to determine cell density at 600 nm and compare the result with control.
- Determine the percentage inhibition and compare the result with control.



Control Growth inhibition at increasing concentration of petrol, or diesel, or crude oil

Observation and Results

The growth pattern of microorganisms was analyzed and the inhibitory effect of hydrocarbon was studied. The results were compared with the control samples.

Conclusion

The effect of different factors on the growth and distribution to microorganisms were studied.

Reference

T. R. G. Gray, Methods In Mlcroblology, Methods for Studying the Microbial Ecology of Soil; Volume 22 ISBN 0-12-521522-3

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 8

Experiment Title

The microorganisms in the extreme habitat

Aim and objective

Isolation of actinomycetes from thermophilic environment for antibiotic production

Introduction

Extremophiles are microorganisms that inhabit a wide range of ecological niches across the globe, from polar regions, extreme temperature, high salinity, and underground mines to oil fields and even the stratosphere. Extremophilic actinomycetes species are capable of surviving in extreme environment and producing antibiotics. Extremozymes, including proteases, and amylases isolated from extremophiles have now become an alternative to chemical catalysts in a broad range of applications.

Materials

- Actinomycetes isolation agar (AIA) medium
- Nutrient agar
- Petri dishes
- Conical flask
- Indicator bacterium (e.g., *E. coli* or *Bacillus*)
- Starch Agar medium
- Mueller Hinton Agar medium

Procedure

Isolation of actinomycetes and bacteria

- Take 1 gram of soil sample from the extreme thermophilic environment, and mix with 99 mL of double distilled water and serially dilute it.
- Then load 0.1 mL of sample (in at least two dilutions) on AIA plates, and nutrient agar plates.
- Incubate the plates for 3-5 days for the isolation of actinomycetes and 24 h for the isolation of bacteria.
- Repeat this procedure until to get pure actinomycetes and bacterial colonies.

Inoculum preparation

- Take a loopful culture of indicator bacteria and inoculate into 50 mL of sterilized nutrient broth medium and incubate for 24 h at 30±2ºC.
- Take a loopful culture of actinomycetes and inoculate into 50 mL of sterilized **AIA medium** and incubate for 6-8 days at 30±2ºC.
- Take the optical density of the broth at 600 nm and record in your note book.

Production of secondary metabolites by actinomycetes

- Take 0.5 mL of actinomyces inoculums and inoculate into 50 mL of sterilized broth medium
- Incubate the culture for 5-8 days and centrifuge the culture at 10,000 rpm for 10 min.
- Store the cell free extract (supernatant) as the source of secondary metabolites.

Antibacterial screening

- Suspend 3.8 grams of Mueller Hinton Agar medium in 100 ml distilled water.
- Then pour 20 mL approximately into each Petri disc and solidify the plates.
- Dip the cotton swab into indicator broth medium and spread on Mueller Hinton Agar plates.
- Take a sterile plain antibiotic disc (6 mm diameter) and load 20 µL of crude secondary metabolite or antibiotic sample.
- Incubate the plates at 30±2ºC for 24 h and determine the zone of inhibition around the sample.

Observation and results

Secondary metabolite production potential of actinomycetes was detected by antimicrobial screening.

Conclusion

Thermophilic microorganisms were isolated and its biological importance were studied.

Reference

Noha F. Omar, Studies on Amylase Produced By Some Actinomycetes: Thermoactinomyces vulgaris alpha-amylase. LAP LAMBERT Academic Publishing

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 9

Experiment Title

The microorganisms in the extreme environment for enzyme production

Aim and objective

Isolation of actinomycetes from thermophilic environment for amylase production

Introduction

Extremophiles are microorganisms that inhabit a wide range of ecological niches across the globe, from polar regions, extreme temperature, high salinity, and underground mines to oil fields and even the stratosphere. Actinomycetes are Gram-positive filamentous bacteria which produce various commercial enzymes. Among actinomycetes, Streptomyces sp. is the very important industrially useful organisms because of their ability of producing various enzymes. *Streptomyces* sp. that produce various proteases include S. griseus, S. clavuligerus, S. thermoviolaceus, S. rimouses and S. thermovulgaris utilize both simple and complex molecules as nutrient source.

Proteases and lipases have lot of potential and are frequently exploited for various industrial processes. These two enzymes have been widely used in leather, detergents, baking, dairy and pharmaceutical industries. Industrial processes like leather and bating and cleaning of various slaughter house equipment generally require a mixture of lipase and proteases. Hence concomitant production of lipase and protease has industrial significance. Proteolytic enzymes from Streptomyces spp. are widely used in processing of various agro industrial wastes such as, nails, feather, plant wastes and hair.

Materials

- Actinomycetes isolation agar (AIA) medium
- Starch Agar medium
- Soil samples from high temperature area
- Petri dishes

- Conical flask

Procedure

Isolation of actinomycetes

- Take 1 gram of soil sample from the extreme thermophilic environment, and mix with 99 mL of double distilled water and serially dilute it.
- Then load 0.1 mL of sample (in at least two dilutions) on AIA plates, and nutrient agar plates.
- Incubate the plates for 3-5 days for the isolation of actinomycetes and 24 h for the isolation of bacteria.
- Repeat this procedure until to get pure actinomycetes and bacterial colonies.

Inoculum preparation

- Take a loopful culture of indicator bacteria and inoculate into 50 mL of sterilized nutrient broth medium and incubate for 24 h at 30±2ºC.
- Take a loopful culture of actinomycetes and inoculate into 50 mL of sterilized **AIA medium** and incubate for 6-8 days at 30±2ºC.
- Take the optical density of the broth at 600 nm and record in your note book.

Protease screening

- Suspend 5.15 grams of skim milk agar medium in 100 ml distilled water.
- Heat to boiling to dissolve the medium completely.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
- Cool to 45-50°C. Mix well and pour into sterile Petri plates.
- Streak the isolated bacteria from the thermophilic sample.
- Incubate the plates for 24 h at 30±2ºC and observe clear zone (zone of hydrolysis) around the bacterial colonies.

Observation and results

Clear zone was observed around the actinomycetes colony indicating the presence of protease enzyme

Conclusion

Thermophilic microorganisms were isolated and its biological importance were studied.

Reference

Noha F. Omar, Studies on Amylase Produced By Some Actinomycetes: Thermoactinomyces vulgaris alpha-amylase. LAP LAMBERT Academic Publishing

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 10

Experiment Title

Determining the growth pattern of bacteria from the polluted area

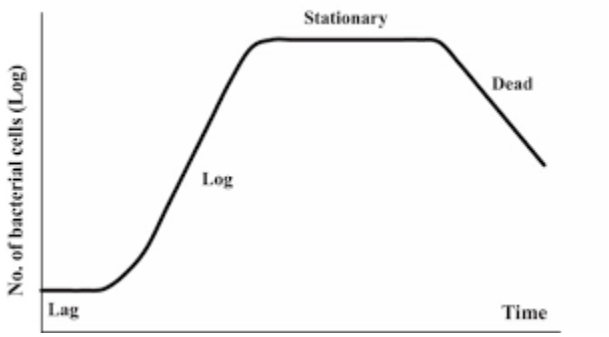
Aim and objective

To select the maximum tolerance level of the bacteria under different concentrations of petrol and diesel.

General Introduction

The growth profile of bacteria in crude oil, petrol, and diesel can vary depending on several factors, including the organism, the concentration of the hydrocarbon, and the incubation period. Since bacteria are easy to grow in the lab, their growth has been studied extensively. It has been determined that in a closed system or batch culture bacteria will grow in a predictable pattern, resulting in a growth curve composed of four distinct phases of growth: the lag phase, the exponential or log phase, the stationary phase, and the death or decline phase. All four phases are given below.

Lag phase: The lag phase is an adaptation period, where the bacteria are adjusting to their new conditions. The length of the lag phase can vary considerably, based on the isolates.



Log Phase or Exponential Phase: Once cells have accumulated all that they need for growth, they proceed into cell division. The exponential or log phase of growth is marked by predictable doublings of the population, where 1 cell become 2 cells, becomes 4, becomes 8 etc. Steeper slope is observed in this phase.

Stationary Phase: At this phase lack of essential nutrients or its growth is inhibited by its own waste products. At this point the number of new cells being produced is equal to the number of cells dying off or growth has entirely ceased, resulting in a flattening out of growth on the growth curve.

Decline phase: In the last phase of the growth curve, the death or decline phase, the number of viable cells decreases in a predictable (or exponential) fashion.

Materials:

- 50 ml conical flask
- Cultivation medium with 0.1% of yeast extract and peptone
- Petrol
- Diesel
- Half dose MH medium and nutrient agar with 1 % petrol

Methods:

- ✓ Shake flask cultivation
- ✓ Volume of the cultivation medium: 25 ml
- ✓ Different concentration of petrol: 0.25 ml to 1.25 ml
- ✓ Different concentration of diesel: 0.25 ml to 1.25 ml

Procedure:

- Inoculate a loop full of pure microbial culture into the sterile medium containing different concentrations of petrol and diesel.
- Incubate the flask under 37 degrees for 24 to 72 hour.
- Measure the cell growth using a spectrophotometer at 600 nm for every 12 hours or 24 hours up to 72 hours.
- Plot a growth curve. In the “X” axis label as “Incubation time” (independent variable), and in the “Y” axis label as Absorbance at 600 nm (dependent variable) or Optical density at 600 nm.

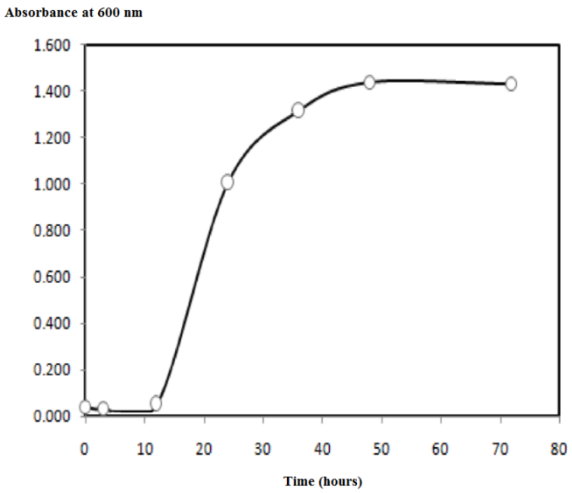


Fig: Schematic presentation of bacterial growth curve. Optical density was measured in terms of absorbance (dimensionless quantity) at a wavelength of 600 nm.

Observation and findings

Growth curve of the bacteria was determined and all four phases were identified.

Conclusion

The growth pattern of the bacteria from the polluted samples were studied

Reference

E. Rosenberg, Microorganisms to Combat Pollution, Book

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 11

Experiment Title

Production of biosurfactant from the crude oil degrading microbes

Aim of the experiment:

To produce biosurfactant in liquid culture using crude oil degrading bacteria and to study basic biosurfactant screening methods.

General Introduction

Biosurfactant is one of the emerging compounds in the industrial sector that behaves similarly with their synthetic counterparts, as they can reduce surface and interfacial tension between two fluids. The structure of a biosurfactant contained hydrophilic moiety made up of amino acids, peptides, (poly)saccharides, or sugar alcohols and a hydrophobic moiety consisting of fatty acids. Surfactant is a chemical compound that can reduce the surface tension and interfacial tension between two surfaces such as air and water. It is differentiated as low molecular weight (lipopeptides, rhamnolipids, trehalolipids and sophorolipids) and high molecular weight (polymeric molecules and lipoprotein) depending on their chemical structure. They used in the bioremediation on oil spills in the marine environment and bioremediation for contaminated soil and water. Biosurfactants show lower toxicity and are biodegradable. Bacteria such as *Bacillus megaterium*, *Franconibacter*, *Aeromonashydrophila*, *Lactobacillus acidophilus, Bacillus thuringiensis, Bacillus toyonensis*, and *Pseudomonas aeruginosa* produce biosurfactant.

Materials

- **Mineral salt medium:** The composition for mineral salt broth media were consist of 1000 ml distilled water (K_2HPO_4 , 1.73g; KH_2PO_4 , 0.68g; $MgSO_4$-$7H_2O$, 0.1g; $FeSO_4$-$7H_2O$, 0.03g; NH_4NO_3 , 1g; $CaCl_2$ -$2H_2O$, 0.02g)**.**
- **Crude oil**
- **Tensiometer** (**can be used to measure the surface tension of liquids)**
- **Iodine vapour**
- **Molish's reagent** (Molisch's reagent is a solution of α-naphthol in 95% ethanol used to detect carbohydrates in a substance. It's used in the Molisch test, a colorimetric method named after Austrian botanist Hans Molisch)
- **1% ninhydrin solution**

Procedure

Isolation of bacteria for biosurfactant production

- Inoculate 1 gm of hydrocarbon contaminated soil samples in mineral salt medium (MSM) containing 2% (v/v) crude oil as a carbon source for enrichment.
- Adjust the pH of the medium to 7.0 ± 0.2.
- Incubate the conical flask at 30°C at 150 rpm for 7 days.
- After 7 days, take this culture and inoculate into a newly prepared MSM medium.
- Incubate it for 7 days at 30°C at 150 rpm.
- Serially dilute the broth culture (10^{-4}-10^{-6}) and spread on nutrient agar plates.
- Incubate the plates at 35°C for 24 h.
- Pick the morphologically different bacterial colonies.

Inoculum preparation

- ✓ Inoculate the bacterial strains in nutrient broth medium.
- ✓ Incubate the culture at 35°C for 24 h.
- ✓ Test the absorbance of the sample at 600 nm.
- ✓ The absorbance of the sample was read at 600 nm (The optical density value of 1.0 (at 600 nm) is considered as optimum for inoculation).

Biosurfactant production

- ❖ Inoculate the bacterium (1 mL) in an Erlenmeyer flask containing MSM medium and 2% (w/v) glucose.
- ❖ Incubate the culture at 30°C with shaking at 150 rpm.

Qualitative assay of biosurfactants

Drop collapse assay

- Drop collapse assay is useful for the detection of biosurfactants using crude oil.
- A single drop of crude oil was set on a glass slide
- Drop culture broth onto the crude oil drop.

Surface tension reduction assay

- Measure surface tension reduction for every 24 h up to 5th day of culture
- The isolates that could reduce surface tension of the culture medium below 35 mN m^{-1} are considered as efficient biosurfactant producers.

Results and Observation

Biosurfactants are produced and basic analytical methods are used to detect biosurfactants.

Conclusion

Bio surfactants were checked from the polluted samples

Reference

E. Rosenberg, Microorganisms to Combat Pollution, Book

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 12

Experiment Title

Microbial equilibrium in soil

Aim of the Experiment:

To determine the microorganisms from garden soil samples.

Brief introduction:

Soil microorganisms, including bacteria, actinomycetes, fungus, and algae, are important for enhancing crop productivity, soil texture, and nutrient levels. Bacterial are the important soil microorganism responsible for many enzymatic transformations like nitrification, ammonification etc.

Materials, Methods & Equipment:

Different types of media - Bacteria – Nutrient agar**; Actinomycetes-** Starch Casein Agar media**; Fungi-** Potato Dextrose Agar media; Petri dish; Pipette, Inoculation loop; Autoclave; Incubator.

Procedures:

- ✓ The pour plate method for counting microorganisms from soil sample is more precise.
- ✓ Take 1 gm of soil sample and make serial dilution using serial dilution protocol.
- ✓ After serial dilution, Transfer 1ml from dilution by pipette to sterile petri dish.
- ✓ Pour sterilized agar (45°c) and mixed with the dilution sample
- ✓ Leave petri dish to solidify

- ✓ Incubate the plate at 37°c for 24 hours
- ✓ Calculate the CFU value of the sample.

Note: Different types of media and dilutions are used depending on the type of microorganism isolation.

Results & Observations:

Count the colonies, multiply by the appropriate dilution factor to determine the number of CFU/mL in the original sample.

Conclusion:

Result indicates that surface soil is rich in microorganism population.

References:

E. Rosenberg, Microorganisms to Combat Pollution, Book

Course no. & Code : MBI 340

Course name : Microbial Ecology & Pollution

Experiment No : 13

Experiment Title

Biogeochemical cycles of nitrogen.

Aim of the Experiment:

Describe the importance of microorganisms in the biogeochemical cycles of nitrogen.

Brief introduction:

Prokaryotes play essential roles in the nitrogen cycle. Nitrogen fixing micro-organisms, interacting with leguminous plants, fix aerobic nitrogen into soil. Rhizobium is the most well-known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen.

Materials, Methods & Equipment:

Yeast mannitol agar media; 0.1 % mercuric chloride (HgCl2); Ethanol; Nodules from leguminous plants; Petri dish; Inoculation loop; Autoclave; Incubator.

Procedures:

- ✓ Collected Leguminous plants are rinsed with tap water to remove soil from nodules.
- ✓ After washing, 5 to 10 nodules are removed from a plant using forceps.
- ✓ Nodules are dipped in 0.1 % mercuric chloride (HgCl2) solution for 30 second and then wash successively eight to ten times with sterilized distilled water.
- ✓ Surface sterilized nodules are crushed in sterilized distilled water by glass rod to obtain a suspension.
- ✓ The suspension is streak on YEMA medium

- ✓ Finally, incubate streaked petri plate at 28±2°C for 2-5 days

Results & Observations:

The colour of the colonies was observed, the pink colonies indicated the Rhizobium.

Conclusion:

In conclusion, the nitrogen-fixing bacteria of *Rhizobium spp*. was isolated from the root nodules.

References:

Reference

Barry E. Smith;nRaymond L. Richards; William E. Newton. Catalysts for Nitrogen Fixation. Nitrogenases, Relevant Chemical Models and Commercial Processes.2004.