Semester – VI

16MBU612B MICROBES IN SUSTAINABLE AGRICULTURE AND DEVELOPMENT- PRACTICAL (4H – 2C)

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 6 Hours

SCOPE

This paper adds information about the role of microorganisms in many food, beverage and pharma industries both in production and spoilage processes. Provides detailed idea about biofertilizer, production and plant disease.

OBJECTIVES

- To encode the importance of the role of microorganisms in food industries both in beneficial and harmful ways.
- To obtain a good understanding of food microbiology and become qualified as microbiologist in food industries.
- To know the role of microbes which make crop output more and increase the fertility of crops.

EXPERIMENTS

1. Study soil profile
2. Study microflora of different types of soils
3. *Rhizobium* as soil inoculants characteristics and field application
4. *Azotobacter* as soil inoculants characteristics and field application
5. Design and functioning of a biogas plant
6. Isolation of cellulose degrading organisms

SUGGESTED READINGS

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2. Singh RS. (1998). Plant Diseases Management. 7th edition. Oxford & IBH, New Delhi.
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6. Barton LL & Northup DE (2011). Microbial Ecology. 1st edition, Wiley Blackwell, USA.
7. Campbell RE. (1983). Microbial Ecology. Blackwell Scientific Publication, Oxford, England.
8. Coyne MS. (2001). Soil Microbiology: An Exploratory Approach. Delmar Thomson Learning.
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10. Mahendra K. Rai (2005). Hand Book of Microbial Biofertilizers, The Haworth Press, Inc. New York.
11. Reddy, S.M. et. al. (2002). Bioinoculants for Sustainable Agriculture and Forestry, Scientific Publishers.

Experiment: 1

STUDY OF SOIL PROFILE

Aim:

To check the vertical section of the soil showing the various layers from the surface to the unaffected parent material

Introduction:

A soil horizon is a layer, approximately parallel to the surface of the soil, distinguishable from adjacent layers by a distinctive set of properties produced by the soil-forming processes. In the soil description, the horizon or layer designation is listed and is followed by the values that represent the depths from the soil surface to the upper and lower boundaries, in that order. The depth to the lower boundary of a horizon or layer is the depth to the upper boundary of the horizon or layer beneath it. Th e variation in the depths of the boundaries is recorded in the description of the horizon or layer. The depth limits of the deepest horizon or layer described include only that part actually seen. The various layers are known as horizons. A soil profile contains three main horizons. They are named as horizon A, horizon B and horizon C.

They are named as horizon A, horizon B and horizon C. The surface soil or that layer of soil at the top which is liable to leaching and from which some soil constituents have been removed is known as horizon A or the horizon of eluviation. The intermediate layer in which the materials leached from horizon A have been redeposited is known as horizon B or the horizon of illuviation. The parent material from which the soil is formed is known as horizon C . A Study of soil profile is important as it is historic record of all the soil forming processes and it forms the basis for the study in pedagogical investigations. Soil profile is the key for the soil classification and also forms the basis for the practical utility of soils. A hypothetical mineral soil profile will include O, A, B, C and R master horizons and all the possible sub-horizons. Master horizons and sub horizons O horizon - It is called as organic horizon. It is formed in the upper part of the mineral soil, dominated by fresh or partly decomposed organic materials. This horizon contains more than 30% organic matter if mineral fraction has more than 50 % clay (or) more than 20 % organic matter if mineral fraction has less clay. The organic horizons are commonly seen in forest areas and generally

absent in grassland, cultivated soils. O1 - Organic horizon in which the original forms of the plant and animal residues can be recognized through naked eye. O_2 - Organic horizon in which the original plant or animal matter can not be recognized through naked eye.

A horizon - Horizon of organic matter accumulation adjacent to surface and that has lost clay, iron and aluminium.

A1 - Top most mineral horizon formed adjacent to the surface. There will be accumulation of humified organic matter associated with mineral fraction and darker in Colour than that of lower horizons due to organic matter.

A2 - Horizon of maximum eluviation of clay, iron and aluminium oxides and organic matter. Loss of these constituents generally results in accumulation of quartz and other sand and silt size resistant minerals. Generally lighter in Colour than horizons above and below.

A3 - A transitional layer between A and B horizons with more dominated properties of A1 or A2 above than the underlying B horizon. This horizon is sometimes absent.

B horizon - Horizon in which the dominant features are accumulation of clay, iron, aluminium or humus alone or in combination. Coating of sesquioxides will impart darker, stronger of red Colour than overlying or underlying horizons.

B1 - A transitional layer between A and B. More like A than B.

B2 - Zone of maximum accumulation of clay, iron and aluminium oxide that may have moved down from upper horizons or may have formed in situ. The organic matter content is generally higher and Colour darker than that of A2 horizon above.

B3 - Transitional horizon between B and C and with properties more similar to that of overlying B2 than underlying C. C horizon - It is the horizon below the solum (A + B), relatively less affected by soil forming processes. It is outside the zone of major biological activity. It may contain accumulation of carbonates or sulphates, calcium and magnesium

R - Underlying consolidated bed rock and it may or may not be like the parent rock from which the solum is formed. Besides, lower case letters are used to indicate the special features of master horizons.

Procedure:

1. Clean off the pit face to expose fresh soil. Dig out the bottom of the pit to expose parent material.
2. Describe soil horizons from top to bottom, as follows:

A. Identify O Horizons, if present, measure depth.

B. Locate preliminary horizon boundaries based upon differences in texture, structure and color and mark, Assign each horizon a master horizon designation.

Observation and Results:

Experiment: 2

STUDY MICROFLORA OF DIFFERENT TYPES OF SOILS

Aim:

Isolation of Algae, Fungi, Bacteria and Actinomycetes from Soil Contents

Principle:

Few environments on earth provide as greats variety of microorganisms as fertile soil. Bacteria, fungi, algae, protozoa and viruses make up this microscopic menagerie, which may reach a total of billions of organisms per gram of soil.

Various methods are available for counting the organisms in soil as well as for isolating various species in pure culture. This is generally done by plating method following dilution. Different kinds of organisms are isolated in specific media. The developing colonies are then counted to get an idea of the microbial population of the soil.

Procedure:

Isolation of Algae, Fungi, Bacteria and Actinomycetes from Soil:

A. Details of Soil Sample:

Samples of soil arc collected from different places. The color of the soil is noted and its water content is determined.

20 g of agar is weighed, taken in a flask containing 500 ml distilled water and heated in a water-bath until the agar melts. In another flask all the other ingredients are taken along with 250 ml. of water. This solution is added to the melted agar solution and the pH of the medium is adjusted to 7.4.

The medium is then dispensed in tubes, each containing about 18 c.c. of the medium. The tubes are then plugged and autoclaved at 15 lbs. pressure for 20 min.

B. Isolation of Actinomyces:

(Dextrose-casein-agar Medium)

Composition:

Dextrose – 2.0 gm. Caesin – 0.2 gm.

(dissolved in 10 ml. of 0.1(N) NaOH soln.) K_2HPO_4 – 0.5 gm.

$MgSO_4$, $7H_2O$ – 0.2 gm.

$FeCl_3$, $6H_2O$ – trace.

Agar agar – 20 gm. Distilled water – 1000 ml. pH – 6.5 to 6.6.

20 g of agar is weighed and added to 500 ml. of distilled water in a flask and heated in a water-bath until the agar melts. All the other ingredients except casein are weighed and added to 490 ml. water in a flask. Casein is dissolved in 10 ml. of 0.1 (N) NaOH solutions and then added to the solution containing all the ingredients.

The agar solution is mixed thoroughly with the other solution. Now the pH is adjusted to 6.5 – 6.6. The medium is then dispensed in tubes and each tube is plugged and autoclaved at 15 lbs. pressure for 20 minutes.

C. Isolation of Fungi:

(Streptomycin-peptone-dextrose Medium of Johnson 1957)

Composition:

KH_2PO_4 – 1.0 gm. $MgSO_4$, $7H_2O$ – 0.5 gm.

Peptone – 5.0 gm. Dextrose – 10.0 gm. Distilled Water – 1000 ml.

Rose Bengal – 10.0 ml. (1:30,000) Streptomycin – 30 µg/ml.

or Aureomycin – 20 µg/ml.

20 g of agar is dissolved in 250 ml. of distilled water in a flask by heating on a water-bath. All the other ingredients except Rose-Bengal and antibiotics are dissolved in 750 ml. water in a flask and then this solution is poured in the agar solution.

The mixture is then heated and stirred continuously, till it boils. After removing from the heater 10 ml. of Rose-Bengal (1:30,000) dilution is added at the rate of 1 ml/100 ml. of medium.

The medium is then dispensed in 100 ml. conical flasks at the rate of 100 ml. per flask. The flasks are then plugged and autoclaved at 15 lbs. pressure for 20 minutes. The antibiotic solution (streptomycin) is added before plating the medium at nearly 45°C (streptomycin inhibits the growth of bacteria while Rose-Bengal is a growth retardant).

D. Isolation of Algae:

(Chou-10-Medium)

Composition:

K_2HPO_4 – 0.01 gm. $Ca(NO_3)_2$, $4H_2O$ – 0.04 gm. $MgSO_4$, $7H_2O$ – 0.025 gm. Na_2SiO_3, $9H_2O$ – 0.025 gm. $FeCl_3$ – 0.008 gm.

Agar agar – 15.0 gm. Distilled Water – 1000 ml.

15 g. of agar is dissolved in 500 ml. distilled water. The other ingredients are dissolved in 500 ml. distilled water. The two solutions are mixed; the pH is adjusted and then dispensed in tubes. They are autoclaved at 15 lbs. pressure for 15 minutes after plugging.

Procedure:

(a) Preparation of Soil Suspension:

10 gms. of sieved soil sample is added to 90 ml. of sterilised distilled water. The flask is shaken vigorously to ensure thorough mixing. Serial dilutions are made by transferring 1 ml. of suspension to 9 ml. of sterilised water in a culture tube.

This is soil suspension of 10^{-1} dilution. After mixing this solution in a vortex mixer, 1 ml. of this suspension of 10^{-1} dilution is transferred to 9 ml. of sterilised water. This is suspension of 10^{-2} dilution. This process is repeated until 10^{-7} dilution is obtained. The whole process is carried out aseptically.

(b) Inoculation and Plating:

(i) Incorporation:

The stabs containing different isolation media are heated in a water-bath for uniform melting. These are cooled to nearly 45°C and 1 ml. of soil suspension of definite dilution is poured in each of the tubes by a pipette. Each tube is rolled in a vortex mixer for thorough mixing.

The medium with soil suspension of each tube is plated in sterilised petridishes aseptically and allowed to solidify. After solidification, the petridishes are incubated at 30°C in an incubator inversely except petridishes for algae isolation which are placed in an open air lighted environment keeping them straight (upper lid upward). Soil suspension of 10^{-6} and 10^{-7} dilution are used for isolating actinomycetes and bacteria; 10^{-4} and 10^{-5} for fungi and 10^{-2} and $10^{-3}$for algae.

(ii) Spreading:

Some selected media are inoculated in this technique. In this method, the media contained in, the flasks are melted and antibiotic added after cooling to 45°C. After thorough mixing, these are plated in sterilised petridishes and allowed to solidify.

After solidification, 0.2 ml. of soil suspension of 10^{-4} and 10^{-5} dilution are added separately to the medium by a pipette and spread out by a glass spreader. The whole thing is done aseptically. Then they are incubated invertedly at 30°C or at 27°C.

(c) Plate Reading:

After 48 hrs. and 96 hrs. The plates are taken out from incubator, the number of colonies in each plate is counted by a colony counter and at the same time the nature of the colonies is observed with the naked eye .

Observation and Results:

Experiment :3

Rhizobium as soil inoculants characteristics and field application

Aim

To isolate and identify *Rhizobium* sp. from root nodule of leguminous plants and its application.

Background

Rhizobium is a soil habitat bacterium, which can able to colonize the legume roots and fixes the atmospheric nitrogen symbiotically. This belongs to bacterial group and the classical example of symbiotic nitrogen fixation. The bacteria infect the legume root and form root nodules within which they reduce molecular nitrogen to ammonia which is reality utilized by the plant to produce valuable proteins, vitamins and other nitrogen containing compounds. The site of symbiosis is within the root nodules. The morphology and physiology of *Rhizobium* will vary from free-living condition to the bacteroid of nodules. They are the most efficient biofertilizer as per the quantity of nitrogen fixed concerned. They have seven genera and highly specific to form nodule in legumes, referred as cross inoculation group. Rhizobium inoculum contains the viable cells of Rhizobium which fixes the atmospheric nitrogen when the roots of higher leguminous plants are injected by Rhizobium.

Materials required

Sample – Root nodules

Media – Yeast Extract Mannitol Agar

(YEMA) Chemicals – 0.1 acidified KCl or sodium hypochlorite

Apparatus – Morter and pestle, petriplates, conical flasks, forceps.

Procedure

- The leguminous plants are uprooted and tested of any nodule is present in the root.
- The root nodule which are white brown to pink green in color and washed in water in order to eradicate the soil particles.
- Then a pinkish green nodule is selected and washed in distilled water.
- The washed root nodule is kept immersed in 0.1 acidified Kcl solution for 5 min. Then again wash the nodule to remove the disinfectant.
- Finally the nodule is immersed in ethyl alcohol and later washed with sterile H_2O.
- The Rhizobium is isolated either by washing the nodule in pestle and morter or by

cutting the nodule and streaking. The washed juice is collected by a sieve and serially diluted and plated.

- The nodule is streaked in a solid media to obtain proper growth of the bacteria. The media
- used for the growth of *Rhizobium* is yeast extract mannitol agar medium and incubated at 30 °C, 3 to 10 days depending on the nature of the *Rhizobium*.
- The correct strain of Rhizobia is identified by nodule formation, cultural tests, microscopic observation and staining techniques.
- The rhizobial cells from the culture identified are mass cultured for the preparation of inoculums.
- The selected rhizobial strain is cultured in YEMA medium for about 7 days in order to establish better growth.
- The Rhizobium culture is tested and the tested Rhizobial culture is transferred to a large container containing the sterile YEMA medium are incubated at 30 C for 9 days.
- Sufficient nutrients should be supplied at regular intervals of 24 h.

Observation and Results:

Experiment :4

***Azotobacter* as soil inoculants characteristics and field application**

Aim

To isolate and identify *Azotobacter* sp. from soil sample and its application.

Background

Azotobacter are free living bacteria which grow well on a nitrogen free medium. Azotobacter is one of the most dominant non-symbiotic nitrogen fixing heterotrophic bacteria. These bacteria utilize atmospheric nitrogen gas for their cell protein synthesis. Azotobacter is gram negative polymorphic bacteria. The species of Azotobacter are known to fix on an average 10 mg of N/g of sugar in pure culture on a nitrogen free medium. The population of Azotobacter is mostly influenced by other microorganisms present in soil. There are some microroganisms which stimulate the Aztobacter population in soil thereby increasing the nitrogen fixation by Azotobacter. On the other hand there are some microorganisms which adversely affect the Azotobacter population and hence nitrogen fixation process is hampered. Azotobacter naturally fixes atmospheric nitrogen in the rhizosphere. There are different strains of Azotobacter each has varied chemical, biological and other characters. The medium used for the growth of Azotobacter is required to have the presence of organic nitrogen, micronutrients and salts in order to enhance the nitrogen fixing ability of Azotobacter.

Materials required

Soil samples, petriplates, conical flask, L-rod, pipette, Ashbys medium. Ashbys Mannitol medium (Nitrogen free medium)

Ingredients gm/L

Sucrose 20

Dipotassium phosphate 1.000

Magnesium sulphate 0.500

Sodium chloride 0.500

Ferrous sulphate 0.100

Sodium molybdate 0.005

Calcium carbonate 2.000

Agar 15.000

Procedure

- Soil samples were collected from soils and rhizosphere of plants like cotton, sunflower, onion, tomato and sugarcane.
- The collected soil samples were kept in polythene packets and brought to the laboratory for analysis.
- About 1gm of soil samples was added into 99 ml of the sterile distilled water and considered as stock solution.
- Then 1 ml of the stock solution is added to 9 ml of sterile distilled water to obtain 10^{-1} dilution, the sample was serially diluted up to 10-7 dilution.
- Then spread plate technique was performed using Ashbys mannitol medium for the isolation. 01 ml of the sample were inoculated on Ashbys mannitol agar medium and incubated at 28 °C for 3 days.
- After incubation the growth on the medium were presumed to be Azotobacter are the sub cultured on Ashbys mannitol agar medium for conformation.
- Isolated Azotobacter was identified on the basis of morphological, cultural and biochemical characteristics such as gram staining and motility.
- Then isolated Azotobacter was mass culture in Ashbys Mannitol broth medium and was incubated at 28 °C for 3 days.
- After 3 days the cultures were mixed with carrier material for biofertilizer production.

Observation and Results:

Experiment: 5

Design and functioning of a biogas plant

Introduction:

In Asia, biogas is used mainly for cooking and lighting purposes. In addition, there are many other advantages in installing the biogas plants. It is used in internal combustion engines to power pumps and electric generators. Sludge is used as fertilizer. The most economical benefits are minimising environmental pollution and meeting the demand of energy for various purposes.

Feedstock Materials:

There are two sources of biomass i.e. plant and animal for biogas production. The biomass obtained from plants is aquatic or terrestrial in origin, while biomass generated from animals includes cattle dung manure from poultry, goat, sheep and slaughter houses, fisheries waste, etc. Cattle dung is most potent for biogas production. Besides dung (gobar), agricultural residue, apple pomade and deteriorated or dumped wheat grains are also proved to be good source for biogas production.

Biogas Production (Anaerobic Digestion):

The anaerobic digestion is carried out in an air tight cylindrical tanks which is called digester. A digester is made up of concrete bricks and cement or steel. It has a side opening (charge pit) into which organic materials for digestion are incorporated. There lies a cylindrical container above the digester to collect the gas. A diagram of single stage digester for gobar gas plant is shown in Fig. 33.2. It is noticed that after 50 days, sufficient gas is produced in gas tank, which is used for house hold purposes. Usually, digesters are burned in soil in order to benefit from insulation provided by soil. In cold climate, digester can be heated.

Metabolic activities of methanogens are quite peculiar. Carbon dioxide fixation, Calvin cycle, serine or hexulose pathways are absent in them. Several coenzymes such as methyl coenzymes M, hydroxymethyl coenzyme M, coenzyme F420, coenzyme F430, component B, methanofuran or carbon dioxide reducing factor, methanopterin and formaldehyde activating factors are present.

The primary reaction in which carbon dioxide formation occurs is given below.

$CO + H_2O \rightarrow CO_2 + H_2$

The secondary reaction takes place in the presence of sufficient hydrogen.

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$.

Other reactions showing methane formation from various substrates are given below:

$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$

$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$

$CH_3COOH \rightarrow 12CH_4 + 12CO_2$

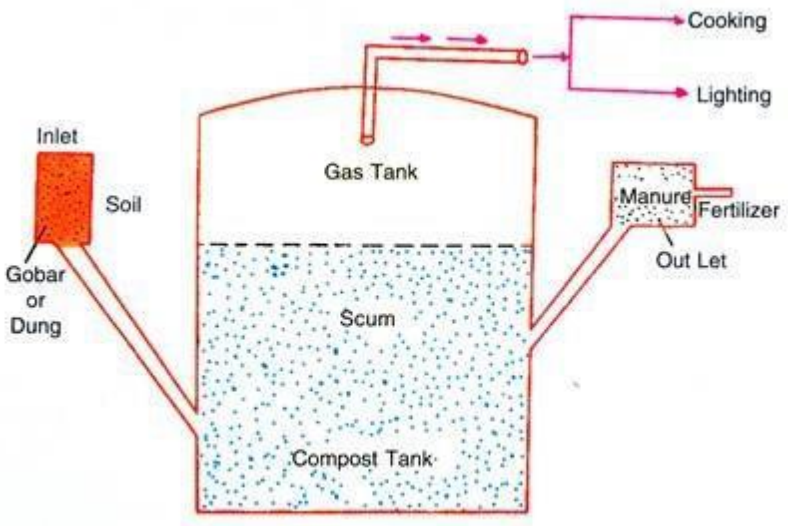

Fig. 33.2 : Single stage digester for gobar gas production.

Experiment 6:

ISOLATION OF CELLULOSE DEGRADING ORGANISMS

Aim:

To isolate cellulose degrading organism

Introduction:

With decades of studies on cellulose bioconversion, cellulases have been playing an important role in producing fermentable sugars from lignocellulosic biomass. Usually, cellulases are mainly composed of three types of synergistic enzymes: endoglucanases (EC 3.2.1.4) that hydrolyze the exposed cellulose chains of the cellulose polymer, exoglucanases (cellobiohydrolases, EC 3.2.1.91) that act to release cellobiose from the reducing and nonreducing ends, and β-glucosidases that help to cleave the cellobiose and short-chain cello-oligosaccharide into glucose.

Numerous microorganisms that are able to degrade cellulose have been isolated and identified. However, many studies have put more emphasis on fungi because the cellulases that they produce are abundant and easy to extract, and some of the fungal cellulases have been used as commercial cellulose. Although fungi such as Trichoderma, *Aspergillus, Penicillium, Phanerochaete,* and *Fomitopsis* have been widely studied in recent years, researchers have also been paying attention to various

bacteria that produce cellulases because of their fast growth, expression of multienzyme complexes, and resistance to extreme environments. Bacteria belonging to the genera *Clostridium, Cellulomonas, Cellulosimicrobium, Thermomonospora, Bacillus, Ruminococcus, Erwinia, Bacteriodes, Acetovibrio, Streptomyces, Microbispora, Fibrobacter*, and *Paenibacillus* have been observed to produce different kinds of cellulase when incubated under anaerobic or aerobic conditions.

Collection of Soil Samples

Collect the soil samples from organic-rich soil.

Strain Isolation and Screening

Inoculate the soil sample suspensions on Czapek's medium containing sugarcane bagasse pulp (in g/L: $NaNO_3$, 2; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; KH_2PO_4, 1.0; yeast extract, 0.4; pulp, 5 (containing 80% water); and agar, 15.0; pH 5.0) and incubated at 28°C.

Subsequently, pick the single colonies using an inoculating needle and inoculate onto Mandels and Reese medium containing carboxymethyl cellulose sodium salt (CMC-Na; in g/L: KH2PO4, 2.0; (NH4)2SO4, 1.4; MgSO4·7H2O, 0.3; CaCl2, 0.3; yeast extract, 0.4; FeSO4·7H2O, 0.005; MnSO4, 0.0016; ZnCl2, 0.0017; CoCl2, 0.002; CMC-Na, 5.0; and agar, 15.0; pH 5.0). After incubation at 28°C for 48 h, Stain with all the plates with 1% (w/v) Congo-red solution for 15 min and discolored with 1 M NaCl for 15 min. The degradation zones will be visible around the bacteria, showing that the strains could hydrolyze CMC.

Cellulolytic bacteria can be isolated and using Carboxy Methyl Cellulose (CMC) agar medium as a selective medium. Production of clear zones by the bacterial isolates on CMC agar medium supplemented with 1% CMC will be considered as indicative of extracellular cellulase activity. The size of transparent zone diameter will considered as proportional to the level of cellulase production. The possible bacterial isolates will be *Bacillus* sp., *Pseudomonas* sp. and *Serratia* sp. based on morphological, cultural and biochemical characteristics.

Observation and Results: