

METHODS OF STUDYING SOIL MICROBIAL ECOLOGY

Much of the study of microorganisms is concerned directly or indirectly with assessment of biochemical reactions brought about by microorganisms. In order to study these reactions, methods have evolved in which organisms are grown in pure culture under conditions which encourage maximum growth. The techniques which give information on the conditions of organisms in soil are divided into four broad groups. They include;

1. Determination of form and arrangement of microorganisms in soil
2. Isolation and characterization of soil microorganisms
3. Detection of microbial activity in soil
4. Determination of microbial biomass

Detection of form and arrangement of microorganisms in soil

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

Microscopic examination of stained soils

This method consist 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 CaCl_2 (0.001-0.1 %) to give a very faint precipitate

Haemocytometer method

A known quantity of soil is suspended in molten agar gel, small drops of agar are removed and placed in the haemocytometer slide of a known depth and allowed to solidify. The films are dried and stained in a solution of acetic aniline blue followed by dehydration in alcohol. Differential counts of measured area of the film will give a quantitative estimation of the microorganism in a given amount of soil.

Contact slide method

This consists of making a slit in the soil with a sharp knife and inserting into the slit a clean slide. The soil is then pressed gently to bring it in contact with the slide, which is left in

position for about 1-3 weeks. The slide is removed, clean on one side and fixed on the other side by passing it over a flame. It is washed gently in clean tap water to remove coarse soil particles, followed by distilled water and then stained with phenol erythrocin for 30 minutes at room temperature. The slide is washed, dried and examined under the microscope. The merits of this method include;

1. It is effective for determining the influence of lime, pH, nutrients, especially NPK and salts on the numbers of microorganisms.
2. It is good for the study of specific soil treatment upon the rhizosphere, or the relationship between plants and microorganisms.

Limitation

The method is inadequate for determining the function of microorganism in the soil

Direct examination of unstained soil

This method employs the use of a strong microscope to examine the soil directly. The microscope was developed by Kubiena. It has a special surface illumination; it has not been extensively used.

A frequently reported limitation of microscopic method is that the number of the microorganisms can only be estimated as small particles of soil may also be counted along with the microorganisms.

Isolation and characterization of soil microorganisms

Microorganisms more often, have to be isolated from the soil and grown in culture before they can be identified and their activities assessed. Microbial Isolation can be considered in three ways.

- i. Growth media
- ii. Transfer of organism to growth media
- iii. Isolation of organisms

Growth media

Two types of growth media are used; (a) non-selective media (b) selective media.

Non-selective media

These are also called broad spectrum media they are designed to isolate broad groups of microorganisms. In practice the media are prepared to favour the development of fungi, bacteria, actinomycetes, algae or other autotrophic forms.

Normally fungi develop best on media with a high carbon: nitrogen ratio, e.g. Czapek Dox agar (sucrose 50 g, NaNO_3 2 g plus mineral salts).

While bacteria grow better in media with a low carbon: nitrogen ratio, e.g. nutrient agar (peptone 5 g, beef extract 3 g with no sugar).

Actinomycetes are able to attack resistant and complex nutrients and are often isolated by incorporating carbon and nitrogen sources like starch, casein, chitin, humic acid into the media.

Most algae are able to synthesize their own nutrients and therefore are grown in extremely simple inorganic salt solutions.

Selective media

These are also called selective media and are designed to encourage the growth of one or a few organisms at the expense of all the others. Media may be selective in the following ways.

1. By adding a substance used by a particular organism but not by others e.g. cellulose as a sole carbon to select cellulolytic organisms
2. By omitting substances required by most organisms but not the one being isolated, e.g. leaving out all organic matter, while supplying ammonium ions as a source of energy and nitrogen encourages the growth of nitrifying bacteria.
3. By altering the reaction (pH) of the media e.g. by acidification with acetic acid allows the slow growth of lactobacilli and inhibiting many other bacteria.
4. By adding selectively microbiocidal substance e.g. P-chloro-nitro-benzene kills most fungi but allows *Fusarium* to grow.
5. By altering the condition of incubation e.g. exposure to high temperature allows the growth of thermophilic organisms, while the exclusion of oxygen favours the development of anaerobes.

Different methods may be successfully combined to isolate very specific fraction of the microflora. For example it is possible to devise a medium for the isolation of a thermophilic anaerobic cellulose decomposing bacterium capable of utilizing inorganic nitrogen.

Selective media are used mostly for the isolation of bacteria. This is because bacteria are most conveniently studied by grouping them according to their biochemical properties. Fungi and algae are easier to identify because of their morphological diversity and biochemical uniformity. Fungi are all aerobic heterotrophic while bacteria may be aerobic or anaerobic, heterotrophic or autotrophic.

Transfer of organism to media

This can be achieved either by or indirect method.

Direct Isolation of soil microorganisms: This method involves the use of micromanipulator techniques the method is difficult because the organisms are so small. Often these methods isolate a more representative selection of the soil microflora since one is removing from morphologically visible and different organisms.

Isolates may be biased in favor of clearly visible organism e.g. dark pigmented forms while transparent hyaline forms are under-represented.

Another problem is that often the organisms failed to grow on media either because they are dead or the medium is unstable.

Indirect transfer methods: This involves the preparation of a soil suspension in water or mineral solution and the addition of this suspension or a dilution of it to the isolation medium. Often the suspension is mixed with an agar medium, poured into a petri dish and allowed to set. This method also allows the determination of microbial populations e.g. the dilution plate method.

Alternatively, a small volume of the suspension is spread over the surface of a plate of solid agar medium so that colonies develop only on the surface.

This method is useful where low numbers of organisms are present in the soil as it is often the case with fungi.

Lumps of soil or pieces of plant root may be placed on the surface of the agar or dispersed in small amounts in molten agar.

Isolation of organisms

Once the soil organisms have transfer to agar media, isolation can be achieved by sub-culturing morphologically recognized and differentiated bacteria and fungi colonies into newly prepared solid media.

Fungi develop from pieces of mycelium as well as spores, some methods have been devised to distinguish between organisms present as spores and those present as vegetative cells.

These methods include;

- i. Killing method: Drying the soil and comparing the fungal colonies that developed from isolation plates before and after drying.
- ii. Growth method: substrates are introduced into soil but are separated from the soil particles by air gap. It is assumed that the fungi that have grown across the gap are vegetative.
- iii. Washing method: if soil particles are agitated vigorously in water, saline or dilute solutions of surface active agents, spores which are readily detachable are removed while the

more firmly attached fungal mycelium remains. This increases the chances of isolating fungi present as mycelium, slower growing forms and forms inside organic particles.

Detection of microbial activity in soil

The activity of microorganisms in soil can be estimated by determining rate of mycelial extension or cell division, the rate of respiration, enzyme content of the soil or by determining the rate of substrate disappearance and metabolite accumulation.

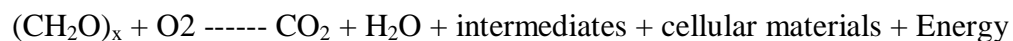
Mycelial extension:

Rate of mycelial growth in fungi can be measured by placing sterile soil in a growth tube and inoculating one end of the tube with that fungus. At a specific interval, the soil is sampled along the tube and the rate of spread is determined.

Alternatively, sterile soil can be placed in a petridish, inoculated centrally and the rate of outgrowth is determined by transferring medium into another petridish.

Rate of respiration:

The overall metabolic activity of soil microflora is determined by oxygen uptake and carbon dioxide output. When organic matter is attacked by microorganisms, the following reaction takes place



Only 60-80 % of the carbon is converted to carbon dioxide even under fully aerobic conditions because of incomplete oxidation of the substrate, giving rise to intermediates and the synthesis of cellular materials.

Fungi and actinomycetes use a greater percentage of metabolized carbon for growth than bacteria.

Theoretically, one molecule of CO₂ is liberated for every molecule of oxygen taken up i.e. the respiratory quotient (RQ) is 1. However, this is rarely observed in the soil for many reasons;

- i. Carbon : nitrogen ratio of substrate can alter the RQ,
- ii. In anaerobic respiration no oxygen is taken up.
- iii. Carbon dioxide may be liberated chemically from soil through the action of microbes producing acids on soil carbonates.
- iv. Oxygen may become bound in soil water and not liberated.

In spite of these limitations respiration still remain the most frequently used method for assessing the activities of microorganism in soil. It can be measured both in the field and laboratory

Measurement of microbial respiration in the field is done by analyzing given volumes of soil atmosphere, usually obtained by pumping air from the soil over a period of time.

It can also be done by determining the amount of carbon dioxide into an enclosed space above the soil surface.

The limitation of these methods is the inability to distinguish microbial respiration from root respiration.

Laboratory measurement of respiration is by removing known volumes of soil and placing them in containers which are incubated under controlled environmental conditions.

Limitation of this method include high rate of respiration at the beginning of the experiment due to the gross disturbance of soil samples, however after a period of equilibration, rate of respiration drops to a low but constant level.

Enzyme content of soil:

During respiration, oxidation of organic substrate is accompanied by reduction of molecular oxygen. In the process hydrogen is removed from the substrate by the action of dehydrogenase enzymes.

Therefore the activity of microorganisms can be assessed by measuring dehydrogenase activity in the soil.

Dehydrogenase activity is measured by reacting the enzymes with tetrazolium dyes which are converted to insoluble red coloured formazan compounds, the intensity of the red colour can be related to enzyme activity.

The main limitations of the technique are (i) obtaining efficient of the enzyme from the microorganisms and then from the soil, (ii) it is rare for enzyme activity to correlate with soil fertility and microbial activity because enzyme activity in the soil is a manifestation of several biological parameters.

Substrate utilization and metabolite accumulation

Rate of substrate accumulation can be measured directly through chemical analysis and indirectly by following the production of metabolites.

In a typical method described by Pochon (1957) a series of soil dilutions are inoculated into media containing the presence or absence of the substrate and its breakdown products.

The activity of the soil population in bringing about this changes is expressed in graphical form by plotting time against the first dilution in which the substrate has disappeared or in which the metabolite have appeared.

Determination of microbial mass

The activity of the soil microflora has often been linked with the number of microorganisms in a soil or occasionally with the microbial biomass.

Biomass is most conveniently determined by counting the number of microbial cells or measuring the length of mycelium present in a soil sample, calculating the volume of such cells and multiplying this figure by a notional value of specific gravity.

There are four methods of determining microbial counts.

- 1. Counting by direct observation:** This includes methods discussed earlier as microscopic methods, the most accurate is the haemocytometer method.
- 2. Counting cells developing in culture:** The number of living cells are usually estimated in culture techniques, the most popular being the dilution plate technique. A known weight of

soil is suspended in a saline solution and shaken or stirred vigorously so that the microorganisms are detached from soil particles and the cells become dispersed. Dilutions of the suspension are made usually in tenfold and known volumes of each dilution are mixed with an agar medium in a petridish. After incubation, cells give rise to colonies and by counting these the numbers of viable cells can be calculated.

3. Bacteria in particular physiological group can be estimated using the **most probable number (extinction dilution) method**. An extended series of tenfold dilution is prepared and 1 ml of each dilution is inoculated into several tubes of medium. After incubation, the number of tubes showing growth is recorded and most probable number of organisms accounting for the result is calculated statistically. The method is less accurate than the dilution plate count.

4. **Determination of biomass without counting:** This is a more method of estimating microbial biomass. When a soil is sterilized with chloroform and then re-inoculated with non-sterile soil a small fraction of the organic matter is rapidly converted to carbon dioxide. This fraction comprising about 2.3-3.4 % of the soil carbon is considered to have been derived from the recently killed microorganism and therefore represent the percentage of carbon in the biomass.