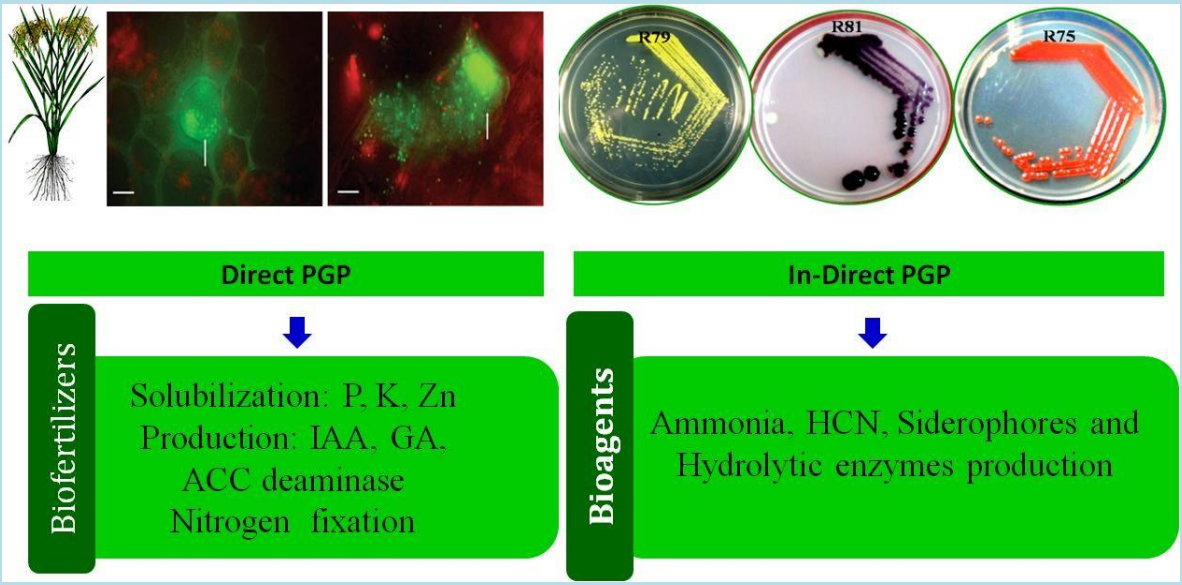
DKSGACA Manual No. 07/2021

Production Technology for Bioagents and Biofertilizers

A Laboratory Manual



Compiled by
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Dr. Khem Singh Gill Akal College of Agriculture
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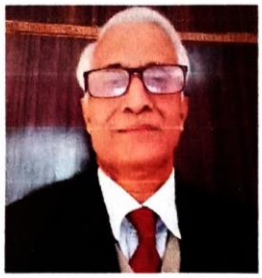
Foreword



Dr. S.K. Sharma

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Microbes are ubiquitous in the environment and can survive in most habitats. They play a major role in the ecosystem and are even excellent for plant growth promotion and protection for agro-environmental sustainability. It is expected that, the fast-growing world population will make food security a big issue in the future. Agricultural sustainability is facing to be a formidable task by using chemical-based fertilizers and pesticides in order to increase the yield of the crop plants. To fulfill the increasing demand of food supply with the problems like shrinking farmlands, and global warming is one of the major challenges. The soil microbiome has found diverse and complex habitats, which consist of billions of bacteria, fungi, and other living organisms. Beneficial microbes play an essential role in nutrient cycling and plant shielding from destructive effects of biotic and abiotic stresses. The macronutrients for plant growth are generally provided via chemical fertilizers. In comparison to the chemical and synthetic fertilizers, biofertilizers and biopesticides improve plant growth and crop productivity in eco-friendly way.

I recommend this "Laboratory Manual" to students working on the emerging and fascinating field of agricultural microbiology, microbial biotechnology, and related subjects. The laboratory manual "Production Technology for Bioagents and Biofertilizers" has four sections *viz.* Laboratory Equipments, Biofertilizers for Sustainable Agriculture, Bioagents for Sustainable Agriculture and Molecular Characterization of Biofertilizers/Bioagents. Section "Laboratory Equipments" deals with all instruments and tolls required for development of biofertilizers and bioagents. The remaining three sections content 22 experiments describing the details about isolation, characterization, identification, development and applications of biofertilizers and bioagents.

(S. K. Sharma)
Dean

Preface

Nitrogen, phosphorus and potassium are the three major plant nutrients. Fertilizer industry greatly depends on petroleum reserve that will be almost exhausted. It estimated that annually 25.1 mt of nutrients (N, P, K) are removed from the soil, whereas, only 15.0 mt are supplied from soil sources including organics. The fertilizer production in our country is less than the required amount. To fill this gap alternate sources of nutrients have to be looked for. Organic wastes and biofertilizers are the alternate sources to meet the nutrients requirement of crops and to bridge the future gaps. Further, knowing the deleterious effects of using only the chemical fertilizers on soil health, use of chemical fertilizers supplemented with organic wastes and biofertilizers could help in efficient and sustainable management of soil ecosystem. Such integrated approach will help to maintain soil health and productivity. Certain microorganisms like bacteria and blue-green algae have the ability to use atmospheric nitrogen and transfer this nutrient to the crop plants. Some of these 'nitrogen fixer' like rhizobia are obligate symbionts in leguminous plants, while others like *Azospirillum* colonize root zones and fix nitrogen in loose association with plants. The third group includes free-living nitrogen fixers such as blue-green algae and *Azotobacter*.

Bacteria such as *Pseudomonas* and *Bacillus* excrete acids into the growth medium and hence solubilize bound phosphates. These organisms are quite useful in the utilization of rock phosphates with low content of P_2O_5. The efficiency of utilization of phosphatic fertilizers is very low due to chemical fixation in soil. The introduction of efficient P-solubilizers in the rhizosphere of crops increases the availability of P from insoluble sources of phosphates. VA-mycorrhizal fungal association with plant roots also helps in improving the nutrients, particularly P-uptake of plants. Rhizospheric bacteria that favorably affect plant growth and yield of economically important crops are designated plant growth promoting rhizobacteria (PGPR). The well-known PGPR include bacteria belonging to the genera *Azotobacter, Azospirillum, Klebsiella, Pseudomonas, Bacillus, Enterobacter, and Serratia*. An attempt has, therefore, been made in this manual to highlight certain pratical aspects dealing with isolation, characterization of N_2-fixer, P-solubilizers, VAM fungi, PGPR, mass production and quality control of biofertilizers.

I am grateful to the Department of Genetics, Plant Breeding and Biotechnology, Dr. Khem Singh Gill Akal College of Agriculture, Eternal University, Baru Sahib and Department of Environment, Science and Technology, Shimla funded project "***Development of Microbial Consortium as Bio- inoculants for Drought and Low Temperature Growing Crops for Organic Farming in Himachal Pradesh***" for providing the facilities and financial support.

My sincere thanks to the research scholars Ms. Tanvir Kaur, Ms. Rubee Devi, Dr. Divjot Kour, and colleagues for their support, love, and motivation in all my efforts during the compilation of this laboratory manual on "Production Technology for Bioagents and Biofertilizers".

Eternal University

Ajar Nath Yadav

Table of Content

Laboratory Equipments

1. Autoclave

An autoclave is a machine that provides a physical method of sterilization by killing bacteria, viruses, and even spores present in the material put inside of the vessel using steam under pressure.

Principle: The autoclave works on the principle of moist heat sterilization where steam under pressure is used to sterilize the material present inside the chamber. The high pressure increases the boiling point of water and thus helps achieve a higher temperature for sterilization.

Instrumentation

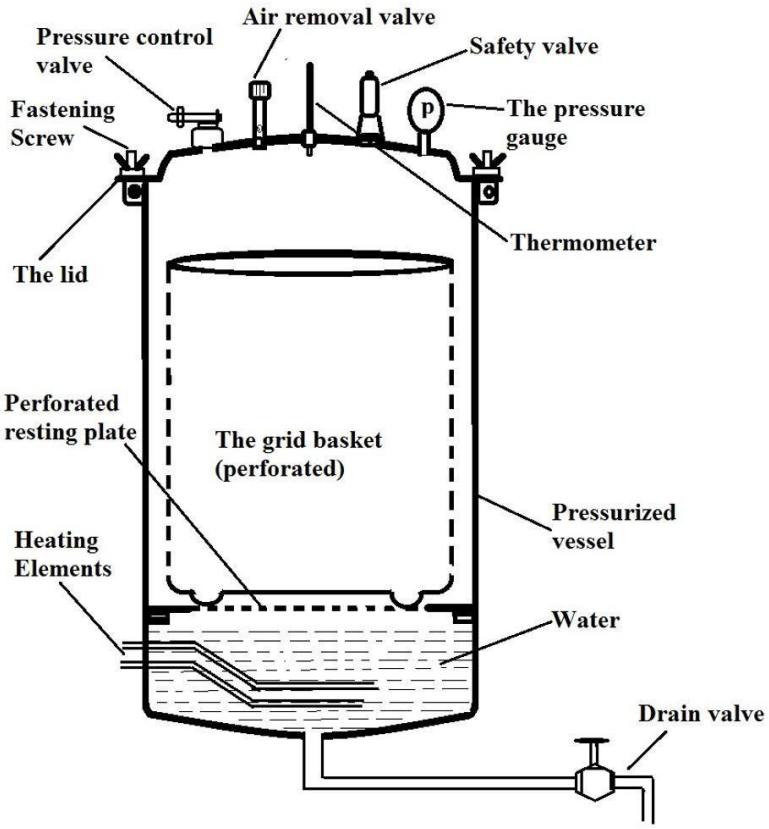
1. Pressure Chamber: The pressure chamber is the main component of a steam autoclave consisting of an inner chamber and an outer jacket. The inner chamber is made up of stainless steel or gunmetal, which is present inside the out chamber made up of an iron case.

2. Lid/ Door: It is next important component. The purpose of the lid is to seal off the outside the atmosphere and create a sterilized condition inside of the autoclave. The lid is made airtight via the screw clamps and asbestos washer. The lid consists of various other components like: pressure gauge (to indicate the pressure created in the autoclave during sterilization), pressure releasing unit (A whistle is present on the lid that ontrols the pressure inside the chamber by releasing a certain amount of vapor by lifting itself) and safety valve (release the pressure and to avoid the danger of explosion).

3. Steam generator/ Electrical heater: An electrical steam generator or boiler is present underneath the chamber that uses an electric heating system to heat the water and generate steam in the inner and the outer chamber.

4. Wastewater cooler: Many autoclaves are provided with a system to cool the effluent before it enters the draining pipes. This system prevents any damage to the drainage pipe due to the boiling water being sent out of the autoclave


Pressure Regulating Device
Pressure Gauge
Safety Valve
Autoclave Lid
Handles
Autoclave Body
Steam Release Valve
Vacuum Release Valve
Outer Stand


Air removal valve
Pressure control valve
Safety valve
Fastening Screw
P
The pressure gauge
Thermometer
The lid
Perforated resting plate
The grid basket (perforated)
Pressurized vessel
Heating Elements
Water
Drain valve

Applications

Autoclaves are important devices to ensure the sterilization of materials containing water as they cannot be sterilized by dry heat sterilization. Besides, autoclaves are used for various other purposes.

1. They are used to decontaminate specific biological waste and sterilize media, instruments, and lab ware.

2. BOD incubator

A BOD (biological oxygen demand) incubator is a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature, humidity and other conditions such as oxygen content of the atmosphere inside. BOD incubators are essential for much experimental work in cell biology, microbiology and molecular biology and are used to culture both bacterial and eukaryotic cells. It is also known as low temperature incubator or refrigerated incubator because it is made with temperature range between 5°C to 80°C or with cooling and heating functions under one unit.

Principle: An axial fan distributes air inside the container. The temperature sensor senses the prevailing temperature and provides data to the PID controller, which furthermore grips the set heat constant till the aspires time.

Components of BOD incubator

Temperature

1. The temperature range of a BOD incubator is 5°C to 80°C.
2. It contains a digital PID controller supported by a PT100 sensor to provide the temperature accurately and uniformly.
3. Temperature correctness of a BOD incubator is ±0.5 °C and uniformity remains ± 1 °C.

Illumination

1. It has fluorescent or LED light, which is door operated.
2. It also contains 3 tubes light on the door with a digital cyclic timer. It controls the illumination 24×7 weekly.

Safety: It contains an over temperature and over current protection and temperature low and high limit buzzer alert for safety.

Cooling: It has a CFC free refrigeration system for cooling temperature under BOD incubator.

Heating: It contains ISI mark U-shaped tabular air heaters which help in heating.

Tray: It contains 2-5 removable trays which are made of steel wire mesh cable.

Air circulations

1. It contains axial fans or a motorized blower system
2. These axial fans help in the uniform circulation of air within the incubator

Construction

1. It is double-walled incubator consist of exterior and interior walls
2. The exterior wall consists of thick mild steel and powder coated
3. The interior wall consists of stainless steel
4. The space within certain two walls in loaded with PUF insulation

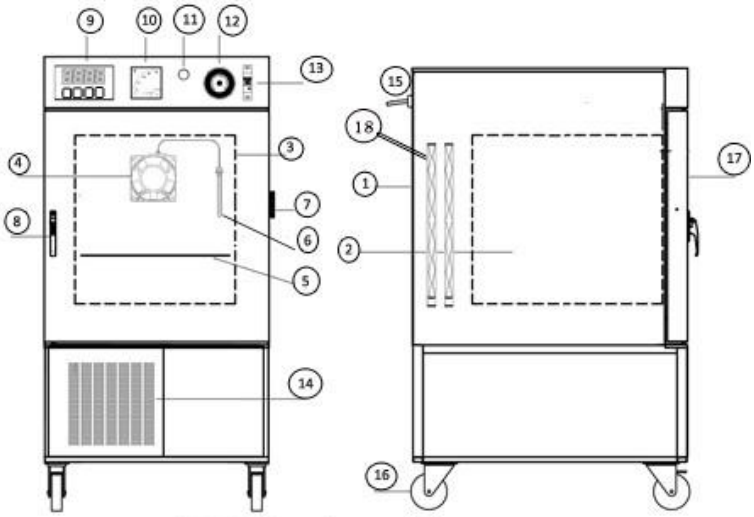
Door

1. It contains double doors such as the outer door and inner door.
2. The outer door is solid and has a mechanical door lock and key whereas the inner door is consisted of glass to view the sample.

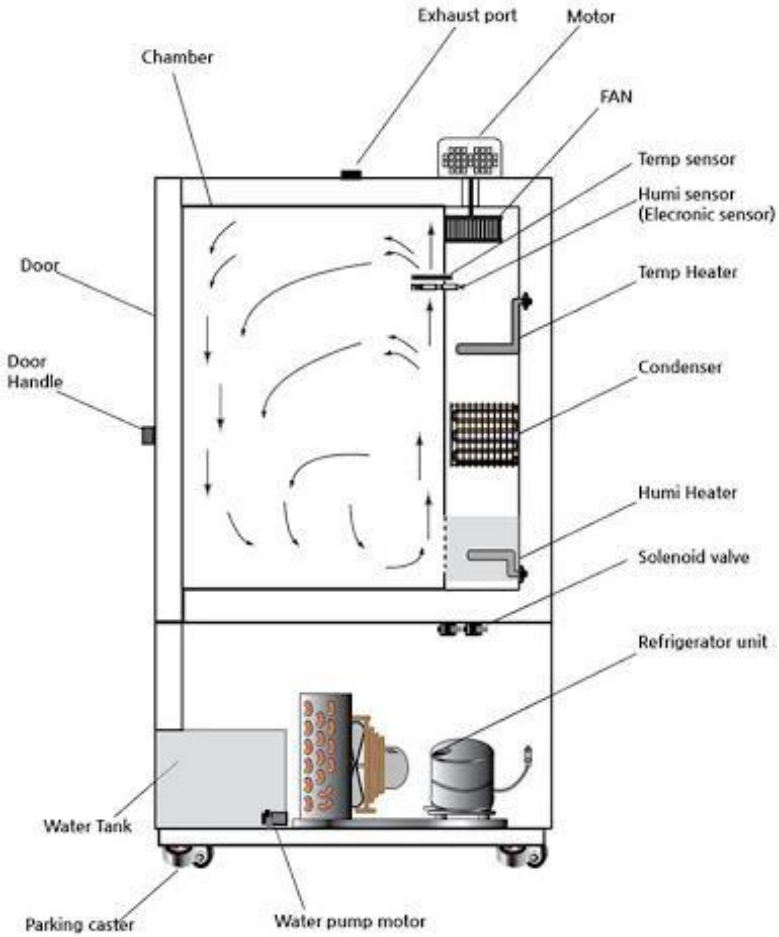
Applications

1. Many industries use BOD incubators such as pharmaceutical, agriculture, beverages and research laboratories.
2. In manufacturers, it is employed in waste processing plants to define the proformance of the treatment system.
3. BOD incubator is employed in liquors to distinguish the nourishment in intended working situation
4. It is employed in the farming industry to define the germination of anaerobic bacteria.

1. Exterior
2. Inner chamber
3. Toughened glass window
4. Air circulation fan
5. Removable tray
6. Temperature sensor
7. Door hinges
8. Door handle with lock & key
9. PID temperature controller
10. Analog ampere meter
11. Pilot lamp
12. Safety thermostat
13. On/Off MCB
14. Refrigeration system
15. Power cord
16. Caster wheels
17. Solid door
18. Heater



BOD Incubator Diagram



3. Laminar air flow

A laminar flow cabinet is defined as enclosed workbench which is used to create a contamination free work environment through installed HEPA filters that capture all the particles entering the cabinet. Air from the room passes through the HEPA (High Efficiency Particulate Absorbing) filters and is fed into the working chamber by a unidirectional vertical descending flow. From the working area the air is moved back to the environment in the following way: partly – through the perforation in the bottom rear area of the cabinet, but most air – through the space between the working surface of the table and the protecting glass. Laminar flow hood involve a unidirectional exhaustion of air to the workplace and personnel whereby filtered air is discharged with a regular and fixed velocity. Some of the basic components of a laminar flow chamber include UV light, glass shield, an air intake fan, a protection plate, windows, etc. Each of the components of a clean bench plays an important role in creating and maintaining sterile conditions. The fan pulls the air through the filter pad where the dust is pinned down; then, the HEPA filter pulls out all airborne contamination to maintain sterile conditions.

Types of laminar flow hoods

The direction in which the air moves is based on the specific laminar cabinet that is being used. The type of cell culture hood needed depends very much on the requirements of the laboratory, the kind of airflow needed, working laminar air flow principle, and the type of operation. Two main types of flow hoods differ according to the functions they can perform: horizontal airflow hood and vertical airflow hood.

1. *Horizontal airflow hood*

The ambient air comes from the behind the laminar air flow bench, then it is projected through a blower towards the HEPA filter, and the filtered air is exhausted in a horizontal direction to the workplace environment. Airflow that is parallel to the workplace cleanses the environment with a constant velocity. Horizontal laminar chamber needs a larger operational space and more depth to provide a germ-free environment and hence is more difficult to handle.

2. *Vertical airflow hood*

The working principle of a vertical laminar unit is quite different from a horizontal airflow hood because it consists of a fan which is placed on the ceiling of the cabinet and the contaminated air is taken in through that fan and directed from the bench top downwards in a vertical direction with a positive pressure.

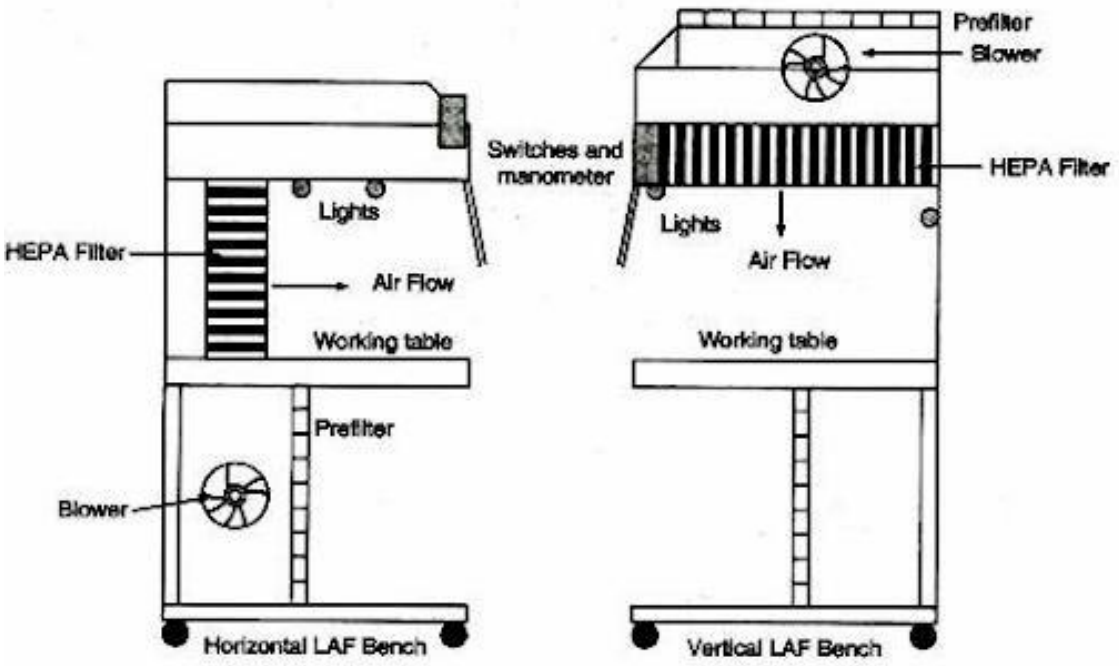
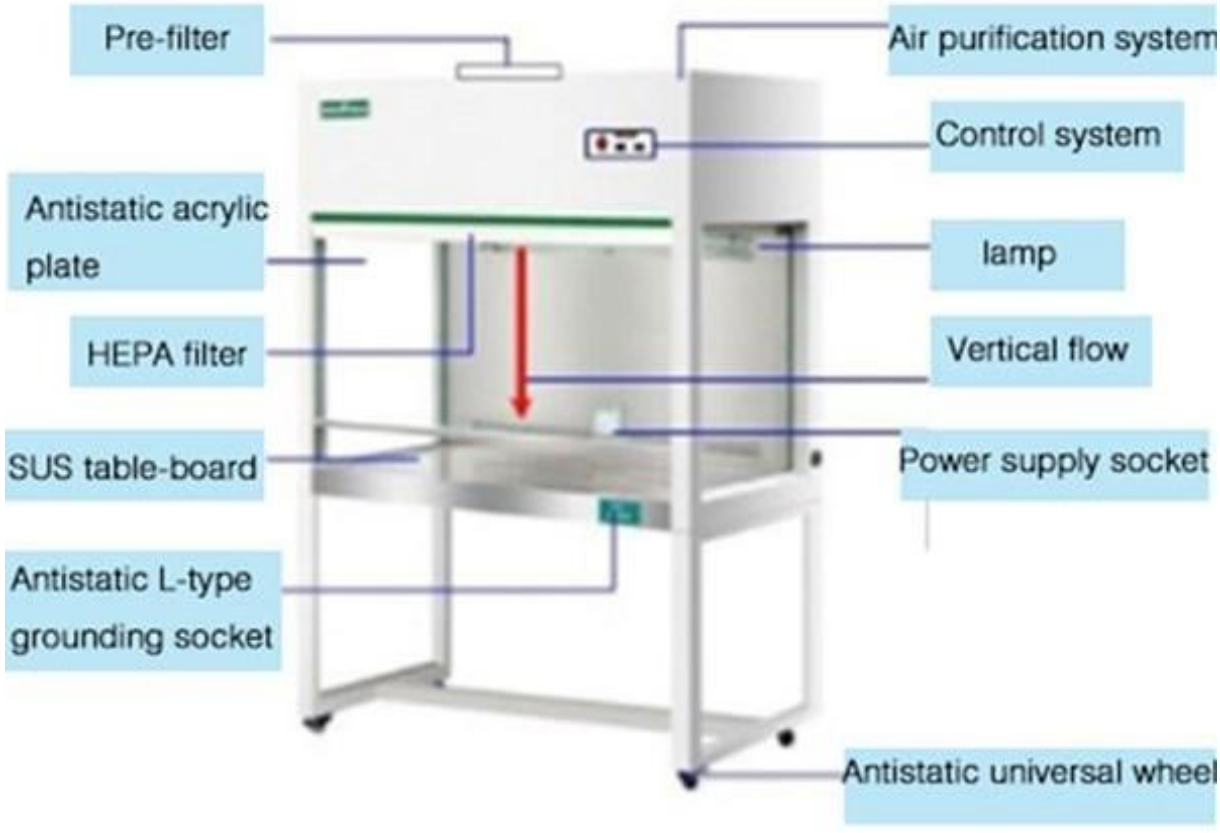


Fig. 25.3. Line diagram of horizontal laminar air flow bench.

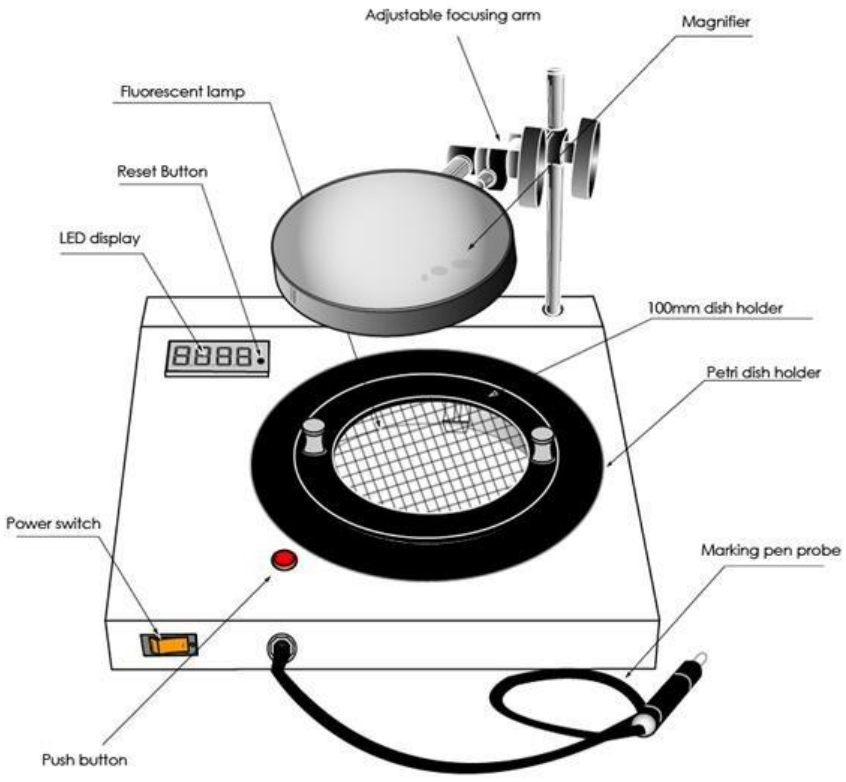


Applications

Laminar Flow Cabinets are suitable for a variety of applications and especially where an individual clean air environment is required for smaller items, e.g. particle sensitive electronic devices. In the laboratory, Laminar Flow Cabinets are commonly used for specialised work. Laminar Flow Cabinets can be tailor made to the specific requirements of the laboratory and are also ideal for general lab work, especially in the medical, pharmaceutical, electronic and industrial sectors.

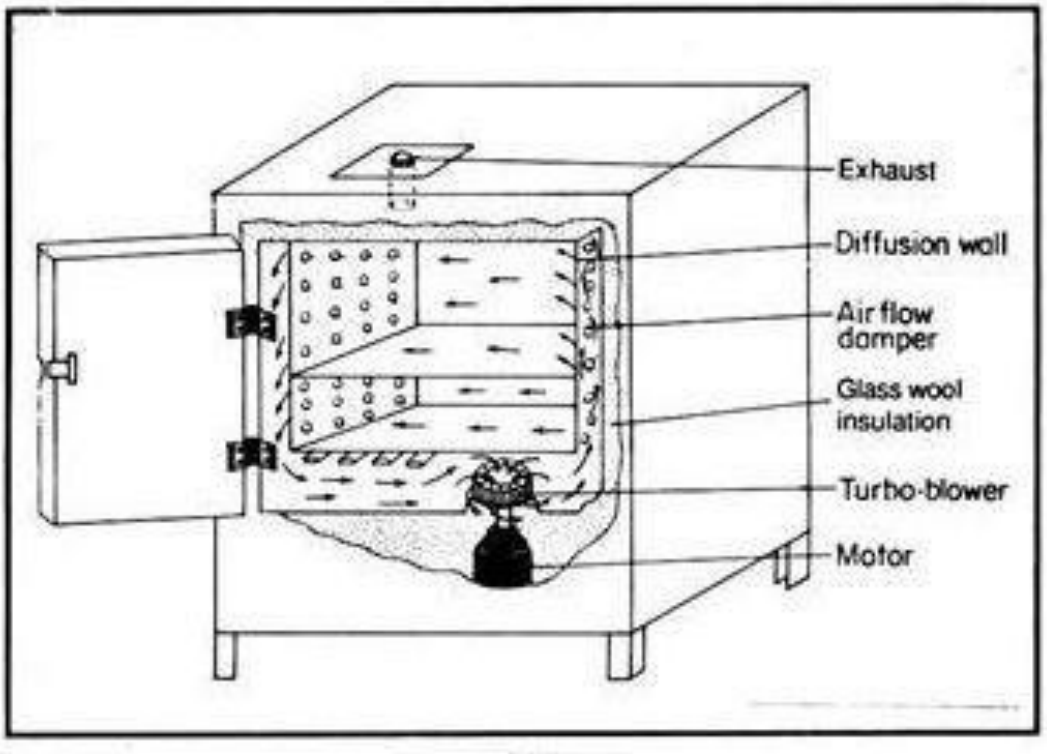
4. Colony counter

Automated colony counters are used to estimate a liquid culture's density of microorganisms by counting individual colonies on an agar plate, slide, mini gel, or Petri dish. Instruments accommodate various sizes and formats, including units that can be used to view plates up to 300 × 300 mm. Some counters can be optimized for use with ultraviolet light illumination, white light, fluorescent, and green fluorescent protein colonies (as might be the case with the pigment produced by *Pseudomonas aeruginosa*). The counting can be accomplished manually, often with touch pressure and a digital counter, or can be semi- or fully automatic.



5. Hot air oven

Hot air ovens are electrical devices which use dry heat to sterilize. They were originally developed by Pasteur. Generally, they use a thermostat to control the temperature. Their double walled insulation keeps the heat in and conserves energy, the inner layer being a poor conductor and outer layer being metallic. There is also an air filled space in between to aid insulation. An air circulating fan helps in uniform distribution of the heat. These are fitted with the adjustable wire mesh plated trays or aluminium trays and may have an on/off rocker switch, as well as indicators and controls for temperature and holding time.



Advantages

They do not require water and there is not much pressure build up within the oven, unlike an autoclave, making them safer to work with. This also makes them more suitable to be used in a laboratory environment. They are much smaller than autoclaves but can still be as effective. They can be more rapid than an autoclave and higher temperatures can be reached compared to other means.

Applications

1. It is used to dry glassware, sterilize N95 mask, general instruments, and packing items in life sciences, microbiology laboratory.

2. It is also used in chemical and pharmaceutical industries, food and beverage industries, textile industries
3. It helps in the elimination of moisture from the material thus it is used in curing, drying, baking and annealing.
4. It is also used for the measurement of mixed liquor suspended solids
5. In certain laboratories and hospitals, it is used to store materials at constant temperatures.

6. Spectrophotometer

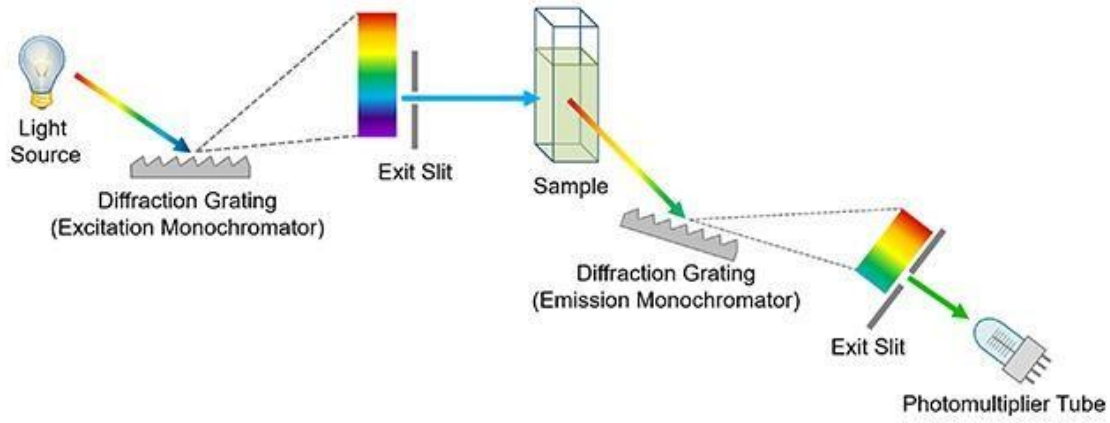
Ultraviolet (UV) and visible (Vis) spectroscopy refers to absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet and visible spectral regions. This means it uses light in the visible and adjacent ranges. To analyses compounds this spectroscopy uses the electromagnetic radiation spectrum from 10 nm to 700 nm. Many atoms are able to emit or absorb visible light, and it is this absorption or reflectance that gives the apparent colour of the chemicals being analyzed.

Principle: UV absorption spectra arise from transition of electron with in a molecule from lower to higher level. Molecules absorb UV radiations of frequency; the electrons in those molecules undergo transition from lower to higher energy level. This spectroscopy is used for the detection of Functional groups, impurities, qualitative analysis, and quantitative analysis. It also helps to show the relationship between different groups, it is useful to detect the conjugation of the compounds.

Instrumentation

Components of Spectrophotometer: Source of light, monochromator, sample compartment, detector, recorder

1. ***Radiation source:*** Deuterium or hydrogen lamp is used that emits radiation in the range 160-375 nm.
2. ***Monochromator or filter:*** monochromator contains many component parts i.e an entrance slit, collimating lens, a dispersing device (prism), focusing lens, an exit slit. Filter is made up from pieces of colored glass which transmit limited wavelength range spectrum. Wide band width 150 nm; gelatin filter consist of mixture of dyes placed in gelatin and sandwiched between glass plates. Band width 25nm and Inter ferometric filter- band width 15nm. Prism helps in bending of monochromatic light and produce non linear dispersion. The amount of deviation depends on the wavelength of the light.
3. ***Sample Container or sample cell:*** the choice of sample cell is based on the path length, shape, size. It is usually transparent and made up quartz or fused silica cuvettes.
4. ***Detectors:*** there are three types of common detectors: Barrier layer, photo cell detector, photomultiplier.



Applications

General applications

1. Qualitative analysis
2. Quantitative analysis
3. Best method for the detection of impurities in organic compounds
4. Can study the chemical kinetics
5. Molecular weight determination
6. Analysis of inorganic compounds
7. Measuring concentration of solution
8. Determine the structure of chloral

Application in organic compounds

1. It helps to show the relationship between different groups and also detect the conjugation of the compound.
2. Detection of geometrical isomers i.e. trans and cis
3. Detects the functional groups.

7. Bioreactor

In one case, a bioreactor is a vessel in which a chemical process is carried out which involves organisms or biochemically active substances derived from such organisms. This process can either be aerobic or anaerobic. These bioreactors are commonly cylindrical, ranging in size from litres to cubic metres, and are often made of stainless steel. It may also refer to a device or system designed to grow cells or tissues in the context of cell culture. These devices are being developed for use in tissue engineering or biochemical/bioprocess engineering.

Components of fermenter

The following components of the fermenter are required for aeration and agitation: (t) agitator (impeller), (ii) stirrer glands and bearings, (iii) baffles, and (iv) sparger (the aeration system).

1. **Agitator (Impeller):** Agitators achieve the following objectives; (a) bulk fluid and gas-phase mixing, (b) air dispersion, (c) oxygen transfer, (d) heat transfer, (e) suspension of solid particles, and (f) maintenance of a uniform environment throughout the vessel.

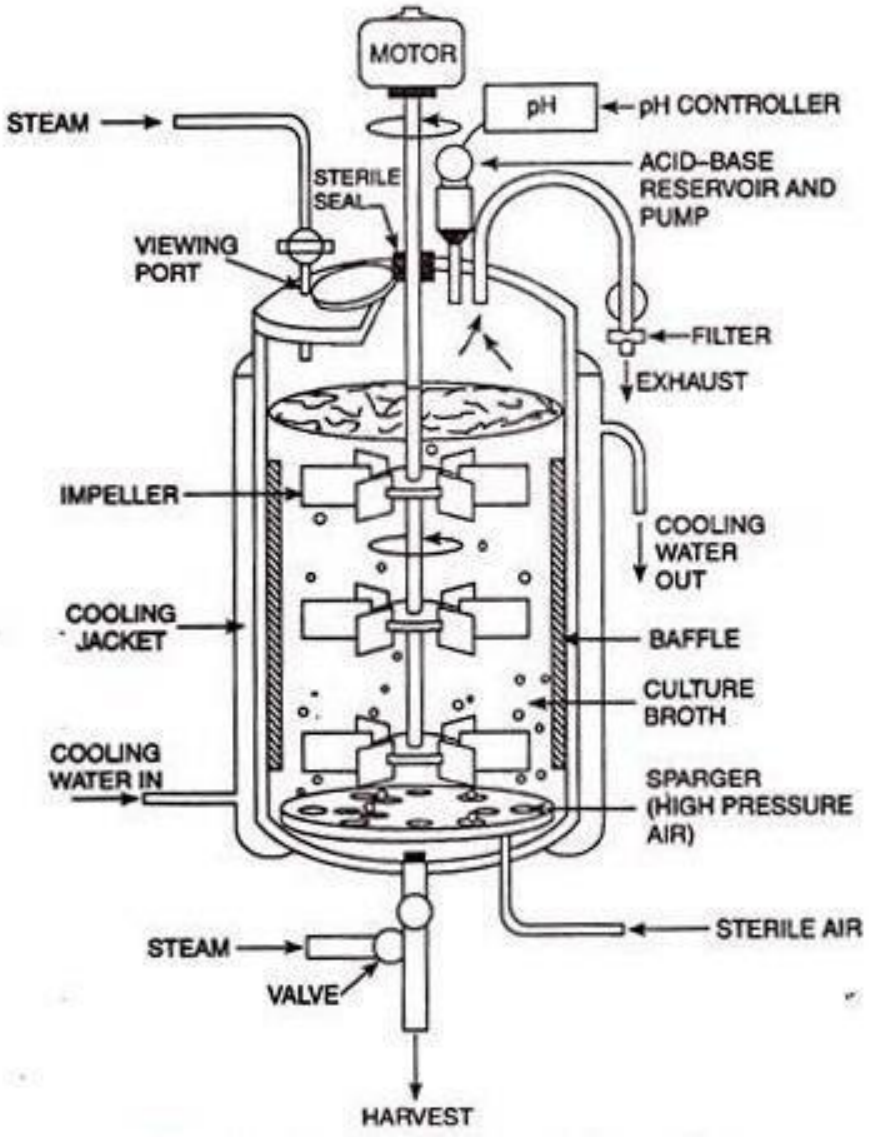
2. **Baffles:** Baffles are metal strips roughly one-tenth of the vessel diameter and attached radially to the fermenter wall. They are normally used in fermenters having agitators to prevent vortex formation and to improve aeration efficiency. Usually, four baffles are used, but larger fermenters may have 6 or 8 baffles. Extra cooling coils may be attached to baffles to improve cooling.

3. **Aeration System (Sparger):** The device used to introduce air into the fermenter broth is called sparger. Spargers are of the following three basic types: (1) porous spargers, (2) orifice spargers and (3) nozzle spargers. Porous spargers may be made of sintered glass, ceramics or a metal. They are used primarily on a laboratory scale in non-agitated vessels. The bubble size from such spargers is always 10 to 100 limes larger than the pore size of the sparger. These spargers have low air throughput because pressure drops across the sparger, and the fine holes often become blocked by microbial growth. Orifice spargers consist of perforated pipes arranged in various ways, e.g., the sparger pipe forming a ring below the impeller. In most cases, air holes are drilled on the underside of the pipe and the holes are arranged in the form of ring or cross. It is desirable that the holes are at least 6 mm in diameter to avoid clogging by microbial growth. These spargers (without agitation) have been used to a limited extent in yeast manufacture, effluent treatment and in air-lift fermenters used for single-cell protein (SCP) production. Nozle sparger consists of an open or partially closed pipe. Most modern fermenters (laboratory to production scale) have a single open or partially closed pipe as a sparger that is ideally placed centrally below the impeller. It provides a stream of air bubbles. The sparger should be as far below the impeller as possible to avoid flooding of the impeller in a stream of air bubbles. These spargers cause a lower pressure loss than the other spargers and they are not easily blocked.

4. **Temperature Regulation:** The fermenter must have an adequate provision for temperature control. Both microbial activity and agitation will generate heat. If this heat generates a temperature that is optimum for the fermentation process, then heat removal or addition may not be required.

5. **Foam Control:** Foam is produced during most microbial fermentations. Foaming may occur either due to a medium component, e.g., protein present in the medium, or due to some compound produced by the microorganism. Proteins are present in corn-steep liquor, pharma media, peanut meal and soybean meal. Foaming may lead to several physical and biological problems for example: the working volume of the fermenter may decrease due to a circulation of oxygen-depleted gas bubbles in the system, the heat and mass transfer rates may also

decline and foaming may interfere with the functioning of sensing electrodes resulting in invalid process data. The biological problems of foaming include (1) deposition of cells in the upper parts of the fermenter, (2 problems of sterile operation as the air filter exits of the fermenter become wet, and (3) increased risk of contamination. In addition, (4) there may be product loss due to siphoning of the culture broth.



Biofertilizers for Sustainable Agriculture

Biofertilizers consists of the microorganisms bringing about the improvement of the nutrients of the soil enhancing their accessibility to the crops. Plant nutrients form the most vital components of the sustainable agriculture. Producing healthy crops for the fulfillment of the demands of the world's growing population is completely dependent upon kind of the fertilizers being used to provide the plants with all the major nutrients but more dependability on the chemical fertilizers is destroying the environmental ecology and negatively influencing the health of humans. Thus, using microbes as bioinoculants is believed to be the best substitute of chemical fertilizers as eco-friendly manner for plant growth and soil fertility. These microbes are known to be the potent tool to provide substantial benefits to crops for sustainable agriculture. The beneficial microbes colonize the plant (epiphytic, endophytic and rhizospheric) systems of crops and plays significant role in nutrient uptake from surrounding ecosystems of plants. The plant associates microbes have ability to promote growth of plant under the natural as well as extreme conditions. These plant growth promoting microbes (PGPM) enhance the plant growth by various direct and indirect PGP mechanisms such as biological nitrogen fixation, the production of various plant growth hormones, siderophores, HCN, various hydrolytic enzymes and solubilization of potassium, zinc, and phosphorus. Extensive work on the biofertilizers has been done and even available which clearly reveals that these microbes possess the potential of providing the vital nutrients to the crops in adequate quantities for the enrichment of yield of the crops without disturbing the environment.

Soil nutrients are very vital for the continuous and efficient production of the crops and healthy food to meet the demands of increasing population. Their sufficiency is very vital component of sustainable agriculture. Agriculture is completely dependent on fertilizers for increasing the yield of the crops. The fertilizers used can either be chemical, organic or biofertilizers and the characteristics of each of the fertilizer are different and each possesses its own ability to the enhance growth of the crop and soil fertility. Chemical fertilizers are used to provide the plant nutrient requirement within a short period and bring rapid results. Their applications provides nutrition in high concentration but entails a lot of disadvantages including water and environmental pollution caused by washed away and evaporated chemical fertilizers respectively. In this view, bio fertilizers are one among the most promising way to add to the productivity of the crops in an eco-friendly manner.

Plant growth promoting microbes play a very significant role in regulating the dynamics of various processes such as the decomposition of the organic matter, the accessibility of various nutrients of plants such as iron, magnesium, nitrogen, potassium, phosphorus, and promote growth of the plants. Now, it has been well-recognized that microbial inoculants make up the main component of integrated nutrient management thereby leading to sustainability. Further, these bioinoculants can be used as cost-effective input for increasing the productivity of the crops by lowering doses of fertilizers and finally, harvesting more nutrients from the soil. Bio-based fertilizers are basically the preparations of living cells or latent of efficient potential

microbial strains that assist the plants in nutrient uptake by their associations in the rhizospheric region when supplied to the plants either through seed or the soil (Fig.1). The technology involved in the production of biofertilizers/bioinoculants is rather simple and installation cost is very less. In last few decades, a huge progress has been made in research and use of different biofertilizers.

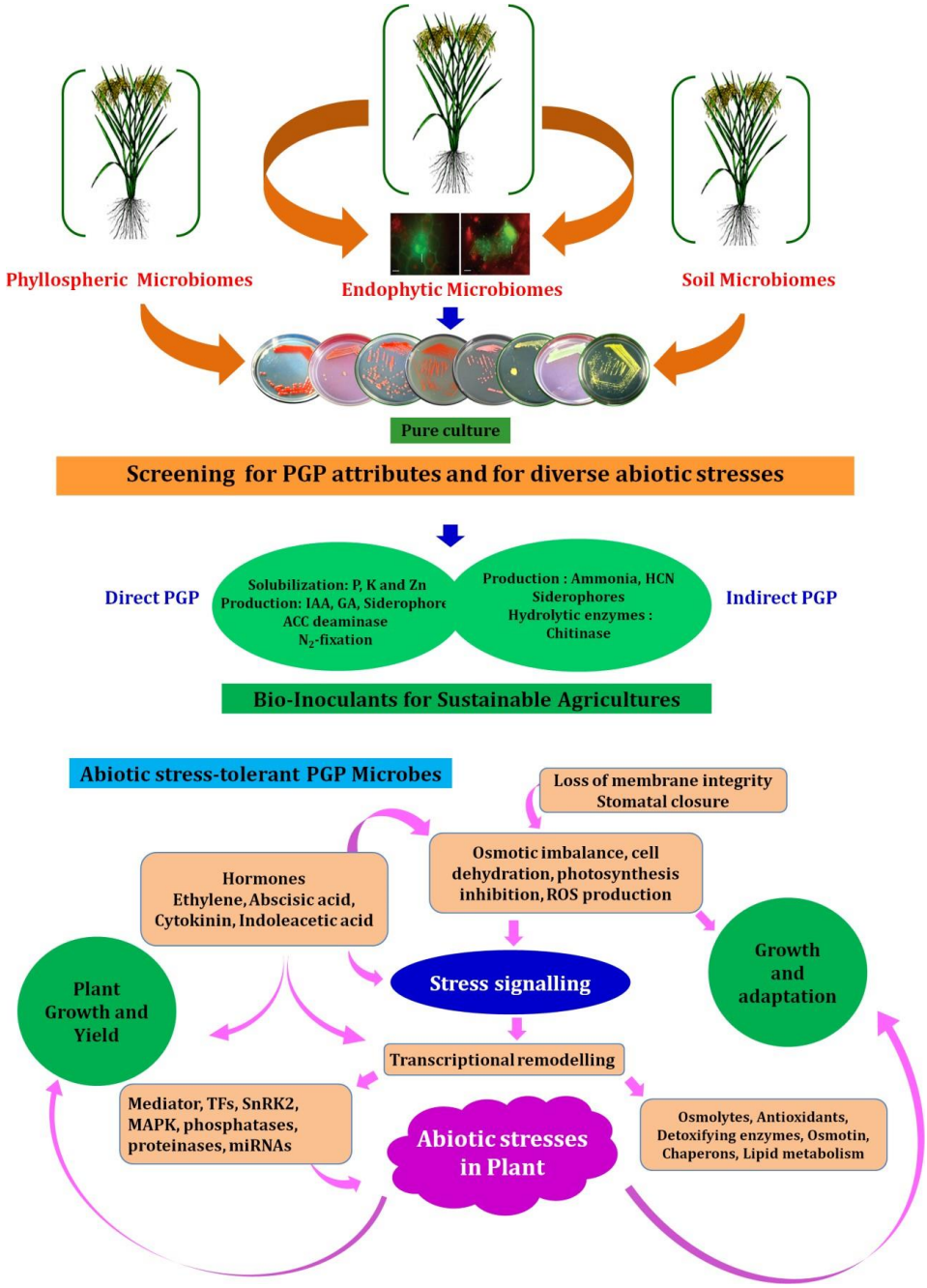


Fig. 1 Plant and soil microbiomes with multifarious plant growth promoting attributes as biofertilizers for PGP and soil fertility under the natural and stressed conditions

Category of microbial biofertilizers

Nitrogen fixing microbes

Symbiotic nitrogen fixing biofertilizers

These are the best known symbiotic nitrogen fixers and among the most exploited ones which belong to the family Rhizobiaceae and mainly consists of different genera such as *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium,* and *Rhizobium. Rhizobium* is symbiotic bacteria and lives in root nodules forming a mutualistic relationship with their host plants. In the root nodules, *Rhizobium* basically fix the atmospheric N_2 gas by reducing the molecular N_2 to NH_3 which is then used by the plants for synthesizing a range of vitamins, proteins, nitrogen containing compounds. The N-fixation is carried out by a complex enzyme nitogenase consisting of dinitrogenase reductase with iron as its cofactor and dintrogenase with molybdenum and iron as its cofactor. The genes for the fixation of the nitrogen i.e., *nif* genes have been demonstrated to be present in both symbiotic as well as free living microorganisms.

Azolla have ability to fix the atmospheric nitrogen in association with the N_2-fixing blue green algae, *Anabaena azollae. Anabaena azollae* is considered to be the most potent biofertilizers as far as its contribution of nitrogen to the rice is considered mostly used for the wetland rice. Rice growing areas in South East Asia are mainly focusing on using *Azolla* as an alternating nitrogen source to the commercial nitrogen fertilizers. It has been estimated that it can fix about 40-60Kg N/ha of rice crop. Further, it has been reported from Vietnam about 10 - ton layer of *Azolla* enables the rice yield to increase by 10-25% over corresponding *Azolla* free rice fields.

Acetobacter is an obligatory aerobic nitrogen fixer. It is an important inoculant for sugarcane. It basically colonizes roots of different varieties of sugarcane. Additionally, it also forms symbiotic association with coffee. It is also known for the production of indole acetic acid as well as gibberellic acid that promotes proliferation and increase the number of rootlets which results in the uptake of mineral, phosphate solubilization and water which promotes growth and sugar recovery of the sugarcane.

Free living nitrogen fixing biofertilizers

Azotobacter is an important free living nitrogen fixing bacteria belonging to family *Azotobacteriaceae* and is mostly present in the neutral as well as the alkaline soils. It fixes atmospheric nitrogen in non-leguminous plants particularly cotton, rice and vegetables but without any symbiotic relationship and do not need a specific host. It can fix 15- 20 kg/ha of nitrogen/year and increases germination as well as vigour in young plants. *Azotobacter* and *Pseudomonas* inoculation plus fertilization decreased the application of the chemical fertilizers by 25-50% in the field. There are number of other species of *Azotobacter* which have also been reported including *Azotobacter beijerinckii, A. insignis, A. macrocytogenes, and A. vinelandii*.

Cyanobacteria are one of the most primitive forms of life on earth accounting for about 33% of total algal population nationwide and reaching upto 50% in some southern and eastern

states. They have been firstly reported in rice fields and since then diverse forms have been reported from variety of habitats. The paddy field ecosystem is the most suitable environment favouring the growth of cyanobacteria in terms of humidity requirements, nutrient availability, light, temperature, and water. Cyanobacteria or blue green algae are basically photosynthetic nitrogen fixers and free living. *Anabaena*, *Nostoc*, *Calothrix*, *Aulosira* are the dominant nitrogen fixers.

Associative symbiotic nitrogen fixing biofertilizers

Azospirillum is among the best-studied associative symbiotic nitrogen fixer reported to be generally associated with different grasses. Presently, there are 17 species of *Azospirillum* which have been described: *Azospirillum zeae*, *A. thiophilum*, *A. rugosum*, *A. picis*, *A. oryzae, A. melinis, A. lipoferum, A. largimobile, A. irakense, A. humicireducens, A. himalayense, A. halopraeferens, A. formosense, A. amazonense, A. fermentarium, A. doebereinerae, A. canadense,* and *A. brasilense.* The most studied and well described among these include *Azospirillum brasilense* and *Azospirillum lipoferum*. The species belong to this genus have been isolated from soil as well as areal part of plant having nitrogen fixing ability. It is known to fix a considerable range of nitrogen i.e, about 20-40 Kg of N/ha/year in non-leguminous plants.

Phosphorus solubilizing microbes

Phosphorus (P) is among the vital macronutrient playing key function in biological growth and development of crops. It is present at the levels 400-1200mg/Kg of the soil. Despite of its high total concentration, its soluble concentration is very low and unavailable for the plants. It exists in two forms in soil i.e, inorganic and organic. Inorganic P in the soil mostly appears after the application of the chemical fertilizers. The plants cannot absorb these precipitated forms. Alternatively, organic matter forms of phosphorus are an important reservoir of immobilized P which accounts for about 20-80% of the soil. The primary minerals for instance hydroxyapatite, apatite, hydrated oxides such as iron, aluminium, manganese are poorly soluble and represent the mineral forms of the phosphorus in the soil.

Phosphate solubilizing microbes (PSM) are well known to promote plant growth as they have the ability of converting the insoluble form of phosphorus to a soluble form. There are different mechanisms which are usually involved in this conversion which includes acidification, exchange reactions and chelation. Various enzymes such as acid phosphatases and phytases which are produced by the P-solubilizers play vital role in the solubilization of soil phosphorus. Various species of *Bacillus* and *Pseudomonas* have been isolated from soil, rhizosphere of different crops which exhibited the P-solubilizing attributes. Additionally, *Achromobacter*, *Brevibacterium*, *Burkholderia*, *Corynebacterium*, *Escherichia freundii*, *Erwinia* sp., *Flavobacterium* sp., *Micrococus* sp., *Mycobactreium* sp., *Rhodococcus* sp., *Sarcina* sp., *Serratia phosphaticum,* and *Xanthomonas* sp., has been also reported as phosphorus solubilizers

Potassium solubilizing microbes

Potassium (K) is among the most vital nutrient which enhances the growth of the plants, playing main role in metabolic activities and the development. Further, due to its involvement with about 60 different enzyme systems in plants, it is also known to be an important regulator. It has key role in drought and disease resistance, besides these important roles, it is also involved in the starch production, controlling the growth of the root, regulation of the stomata movement in plant cells and ultimately contributing to the quality. If the supply of potassium is insufficient, the roots of the plants will be poorly developed, they will have slow growth rates, seeds produced will be small, yield will also be lowered and the plants will become more susceptible to diseases and pest. There are numbers of microorganisms in the rhizosphere playing an important role in the release of the K from different insoluble potassium compounds present in soil and surrounding systems. There are number of potassium solubilizing microbes (KSMs) that have been reported in different studies including *Aminobacter, Acidothiobacillus ferrooxidans, Bacillus circulans, Burkholderia, Enterobacter hormaechei , Paenibacillus* sp*, Pseudomonas, Paenibacillus glucanolyticus, Sphingomonas*

Experiment 1

Aim: Isolation of *Rhizobium* from root nodules

Requirements: Root nodules, 0.1% $HgCl_2$ (Mercuric chloride), 70% ethanol, petridish, sterile distil water, glass rod, test tubes, glass rods, congo red yeast mannitol agar

Theory

Rhizobium is a genus of Gram-negative soil bacteria that fix nitrogen. Rhizobium species form an endosymbiotic nitrogen-fixing association with roots of legumes and Parasponia. The bacteria colonize plant cells within root nodules, where they convert atmospheric nitrogen into ammonia using the enzyme nitrogenase and then provide organic nitrogenous compounds such as glutamine or ureides to the plant. The plant, in turn, provides the bacteria with organic compounds made by photosynthesis. *Rhizobium* can be isolated from the root nodules after cleaning and surface sterilizing the nodules. The nodules can be either crushed in small volume of sterile water or dissected and the nodules contents streaked on the plate containing Congo red yeast extract mannitol agar (CRYMA). Dye Congo red in medium are used to distinguish rhizobia from other contaminating bacteria.

Media composition		**Amount (g/L)**
Yeast extract	:	1
Mannitol	:	10
Dipotassium phosphate	:	0.5
Magnesium sulphate	:	0.2
Sodium chloride	:	0.1
Congo red	:	0.25
Agar	:	20
pH	:	7.0

Procedure

1. Roots with healthy nodules were taken and brought to laboratory.
2. Healthy, pink nodules were separated from roots the washed with distilled water.
3. Transfer nodule to sterile beaker and immersed in 0.1% mercuric chloride and 70% ethanol for 3 min and 30 sec., respectively.
4. The nodules were washed repeatedly in sterile water for 3-4 times.
5. The nodules were placed in small vial containing sterile water and crushed with sterile glass rod.

6. The nodules suspension was plated on CRYMA media.
7. The plates were placed in BOD incubator at 28 ºC till small round, colorless or white with central red dot and entire margin develops.
8. Transfer the isolated colony to fresh CRYMA plate for purification.
9. Maintain the pure culture on YEMA (yeast extract mannitol agar) slants till further use.

Observation: White with central red color colonies of *Rhizobium* obtained.

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Experiment 2

Aim: Isolation of *Azospirillum* from plant roots

Requirements

Soil sample (rhizospheric), screw-capped tubes, sterile water blanks, semi-solid free nitrogen malate culture medium, mercuric chloride ($HgCl_2$), sterile distilled water

Theory

Azospirillum, a Gram-negative and a ubiquitous rhizosphere bacterium is the main group of microaerophilic free living/associative nitrogen fixing bacteria. A vibriod bacteria belongs to the family of Rhodospirillaceae. *Azospirillum* are aerobic, but many can also function as microaerobic diazotrophs, meaning, under low oxygen conditions, they can change inert nitrogen from the air into biologically useable forms. It can be either isolates from the rhizospheric soil or from root interior part. Semisolid culture medium free nitrogen malate (NFb). Malic acid is used as the carbon source, whereas, Dipotassium phosphate provides buffering effect and other inorganic salt ingredients provide necessary growth nutrients. Agar at 0.17% concentrations provides microaerophillic conditions necessary for nitrogen fixation by *Azospirillum* species.

Media composition		**Amount (g/L)**
Malic acid	:	5
Dipotassium hydrogen phosphate	:	0.5
Sodium chloride	:	0.1
Magnesium sulphate	:	0.2
Calcium chloride	:	0.02
Potassium hydroxide	:	4.5
Agar	:	1.75
pH	:	6.8

Procedure

1. Healthy roots of plants were brought to laboratory.
2. Collected plant roots system was washed under running tap water.
3. Cut the washed roots into small pieces of about 0.5 cm.
4. Root pieces were sterilized with 0.1% $HgCl_2$ for one minute and then roots were serially washed with sterile distilled water.
5. Sterile pieces of roots were placed on semi-solid agar medium containing sodium malate in screw-capped tubes.

6. The tubes are inoculated at 28-30 ºC for 2 days.

Observation: Nitrogen-free malate medium becomes blue in color and white pellicles of bacterium are observed.

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Experiment 3

Aim: Isolation of *Azotobacter*

Requirement

Jensen's nitrogen free medium, soil sample (rhizosphere), silica-gel plates, petridish, pipettes, sterile water blank, inoculating needle.

Theory

Azotobacter is free living, nitrogen fixing, heterotrophic bacterium that derives energy by degrading plant residues. They are usually motile, oval or spherical bacteria that form thick-walled cysts and may produce large quantities of capsular slime. *Azotobacter* is found in slightly acidic (*A. beijerincki*) to neutral alkaline (*A. chroococcum*) soil. Jensen's medium is used for isolation of *Azotobacter*. Sucrose acts as the energy source, sodium molybdate in the media increases the fixation of nitrogen, whereas, sodium chloride maintains osmotic equilibrium of the media. Calcium stimulates nodulation when present as chloride or sulphate.

Media composition		**Amount (g/L)**
Sucrose	:	20.0
Dipotassium phosphate	:	1.0
Sodium chloride	:	0.5
Magnesium sulphate	:	0.5
Ferrous sulphate	:	0.10
Sodium molybdate	:	0.005
Calcium carbonate		2.0
Agar	:	20

Procedure

1. Soil sample was collected and brought to laboratory.
2. On the other hand, media (Jensen's media) and dilutions were prepared into a flask and test tube, respectively, were autoclaved.
3. The autoclaved media was poured in to a petridish and allowed them to solidified
4. After pouring, the dilutions blank and pertiplates containing media were labeled as 10^{-1}, 10^{-2}, 10^{-3}.
5. After that, in 10^{-1} blank 1g of samples was mixed and using sterile pipette, transfer 1ml of sample from dilution 10^{-1} to 10^{-2} and mixed. This process is continues till 10^{-3}.
6. 0.1 mL of suspension is spread on the agar plate.
7. The plates were incubated at 28-30 ºC in BOD incubator.

Observations: After incubations, light brown to black pigments on Jensen's medium is observed.

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Experiment 4

Aim: Isolation of plant growth promoting rhizobacterium

Requirement
Petriplates, sterile water blanks, pipettes and different media

Theory
Beneficial free living soil bacteria isolated from the rhizosphere of plants, which have been shown to improve plant health or increase yield are usually referred to as plant growth promoting rhizobacterium (PGPR). A number of different nitrogen fixing, phosphorus, potassium and zinc solubilizing and siderophores producing bacteria may be considered to be PGPR including *Azotobacter, Azospirillum, Rhizobium, Bacillus, Burkholderia, Enterobacter* and *Pseudomonas*.

1. Methyl red agar for isolation of gram positive bacteria

Media composition		**Amount (g/L)**
Beef extract	:	3.0
Peptone	:	5.0
Methyl red*	:	0.15
Agar	:	20
pH	:	7.0

*Dissolve 150mg methyl red in 10 mL water and sterile separately. Add after autoclaving of the medium

2. Crystal violet agar for isolation of gram negative bacteria

Media composition		**Amount (g/L)**
Beef extract	:	3.0
Peptone	:	5.0
Crystal violet*	:	0.15
Agar	:	20
pH	:	6.8-7.0

*Prepare stock solution of crystal violet (0.05 w/v) and filter sterilize; ass after sterilization of medium

3. **King's B for isolation of *Pseudomonas***

Media composition		**Amount (g/L)**
Protease peptone	:	20
Glycerol	:	10
Di-potassium phosphate	:	1.5
Magnesium sulphate	:	1.5
Agar	:	20
pH	:	7.2

4. **T3 Agar for isolation of *Bacillus***

Media composition		**Amount (g/L)**
Tryptone	:	3
Tryptose	:	2
Yeast extract	:	1.5
Manganese chloride	:	0.005
Sodium dihydrogen phosphate	:	0.05
Agar	:	20
pH	:	6.8

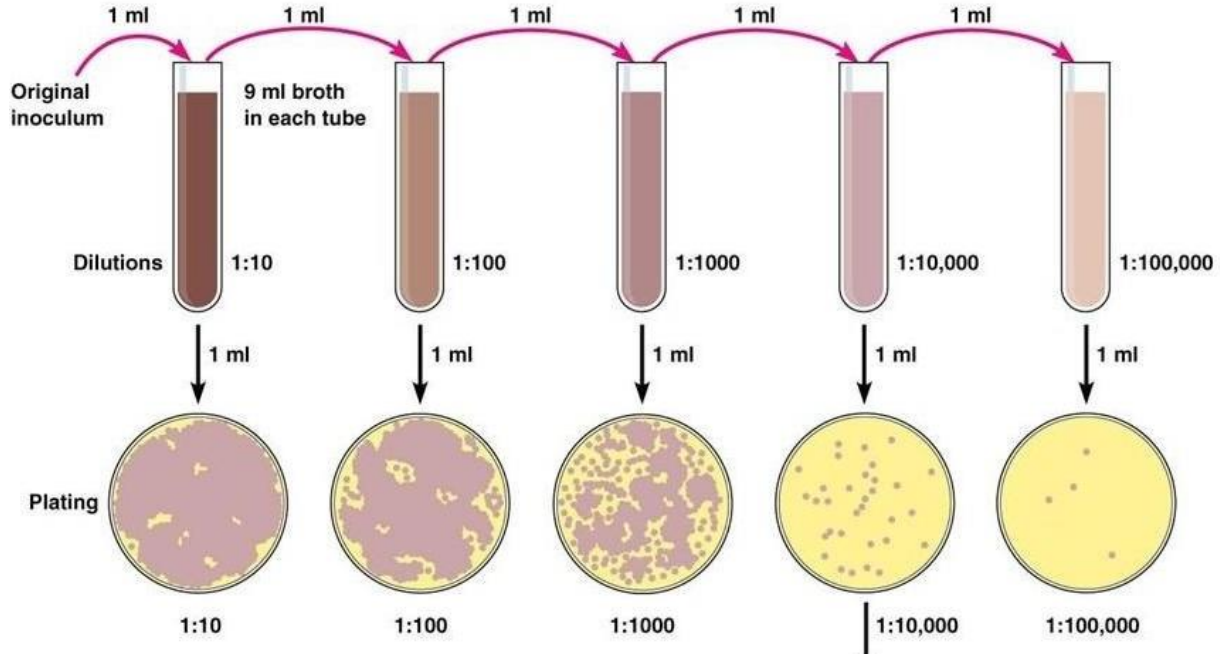
5. Ammonium mineral salt for the isolation of methalotrophs (pH-6.8)

Media composition		**Amount (1L)**
Dipotassium phosphate	:	0.7g
Potassium dihydrogenphosphate	:	0.54g
Magnesium sulphate	:	1.0g
Calcium chloride	:	0.2g
Ammonium chloride	:	0.5g
Ferrous sulfate	:	2mg
Zinc sulphate	:	5mg
Manganese chloride	:	1.5mg
Boric acid	:	15mg
Copper chloride	:	0.5mg
Cobalt chloride	:	10mg
Nickel chloride	:	1mg
Sodium molybdate	:	3mg
Methanol*	:	5mL
Agar	:	20g

*Methanol should be add after autoclaving

Procedure

1. Pour plates of different media and allow them to solidify. Keep them inverted until use.
2. Take 1g soil from the rhizosphere of the host plant and prepare 10 fole siltion series oup to 10^{-5}.
3. Withdraw by sterile pipette, 0.1mL aliquots from dilution (10^{-2} to 10^{-5}) and pour on plates containing respective media.
4. Dip the spreader in alcohol and sterilize it in flame. Cool the spreader and use it for spreading the diluted aliquot by rotating the plates.
5. Incubate at 30 ºC for 2-5 days.
6. Record the visual observation after development of colonies.



Observation: Colonies of different colour, size and texture was observed.

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Experiment 5

Aim: Characterization of plant growth promoting bacteria

Theory

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly. The direct promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR prevent deleterious effects of one or more phytopathogenic microorganisms. The exact mechanisms by which PGPR promote plant growth are production of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene, siderophores, antibiotics and hydrogen cyanide, fixation of nitrogen, solubilization of mineral phosphates and other nutrients.

The isolates can be screened for their plant growth promotion activities by assaying the following attributes

(A) Seed germination
(B) Production of ammonia
(C) Production of IAA
(D) Phosphate solubilization
(E) Nitrogen fixation
(F) Production of siderophores
(G) HCN production
(H) Antibiotics production
(I) Antifungal production

(A) Seed germination assay

The selected PGPR strains can be bioassayed for their ability to promote/inhibit seedling growth by following procedure

1. Surface sterilizes the seeds with 0.1% $HgCl_2$ for 3 min, followed by successive washing with sterile distilled water.
2. Decant the water
3. Add the seeds to cultures grown in their respective medium for 48 hrs containing at last 10^6 cells/mL. Keep for 10 minutes in the culture medium and decant the medium.
4. Pour the plates with 0.8% sterile agar.

5. Keep the seeds on soft agar plates and incubate at 30 ºC for 2-3 days. Keep at least 3 replicates.
6. In control plates, seeds to be treated with sterilized medium alone.
7. After 3 days, record root and shoot length.

(B) Production of ammonia

1. Grow the isolates in peptone water in tubes.
2. Incubate the tubes at 30 ºC for 4 days.
3. Add 1 mL Nessler's reagent in each tube.
4. Presence of a faint yellow colour indicates the small amount of ammonia and deep yellow to brownish indicates maximum production of ammonia.

Peptone water

Media composition		**Amount (g/L)**
Peptone	:	10
Sodium chloride	:	5
pH	:	6.8

(C) Indole Acetic Acid (IAA) production

1. The microbial cultures were grown for 24 h in broth of LB (Luria Bertani) medium.
2. Centrifuge at 10,000 g for 15 minutes
3. Take 2 mL of supernatant and add 2-3 drops of o-phosphoric acid
4. Add 4 mL of reagent to the aliquot
5. Incubate the sample for 25 minutes at room temperature
6. Read the absorbance at 530 nm
7. Record auxin quantification by preparing calibration curve made by using IAA as standard (10-100 µg/mL).

Reagent: 1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$; IAA stock solution-100mg/mL in 50% ethanol

(D) Phosphate solubilization

Requirement: Petriplate, Pikovskaya medium, flasks

Pikovskaya medium

Media Composition		Amount (g/L)
Glucose	:	10.0
Ammonium sulphate	:	0.5
Potassium chloride	:	0.2
Magnesium sulphate	:	0.1
Yeast extract	:	0.5
Tricalcium phosphate/apatite/rock phosphate	:	5.0
Sodium Chloride	:	0.2
ferrous sulphate trace	:	0.0001
manganese sulphate trace	:	0.0001
Agar	:	20
pH	:	7.2±0.2

Procedure

1. Prepare Pikovskaya medium containing three different insoluble phosphorus source. (apatite, tricalcium phosphate, and rock phosphate) and autoclave it.
2. Pour the media into the petripates and allow them to be solidify.
3. On solidify agar plate, inoculate ten microlitres of microbial suspension.
4. Incubated the plates at 30 °C for 48-96h in BOD incubator.
5. Observe for clearing of solubilization zones around the colonies.

(E) Siderophores production

The siderophores production was analysed on chrome-azurol-S (CAS) agar medium.

Procedure

In siderophores studies contaminating iron should be avoided, so treating the chemicals and glassware for removal of iron therefore become essential. The glassware should be soaked in 2N HCl for at least 24 hours and washed with double distilled water to remove acid.

Preparation of media

1. Dissolve 60.5 mg of chrome-azurol-S (CAS) in 50 mL distilled water

2. Prepare iron solution separately by dissolving 1mM FeCl3 and 10 mM HCl in 100 ml distilled water
3. In different beaker, mix 10 mL iron solution and 50mL CAS solution stirrer well
4. Separately, 72.9 mg of CTAB is mixed in 40 mL distilled water and it was mixed with 60mL CAS.
5. Prepare 300mL nutrient agar also.
6. Autoclave, nutrient agar and CAS

Screening

1. Pour the prepared CAS medium in the petriplates
2. On solidify agar plate, inoculate ten microlitres of microbial suspension.
3. Incubate the plates at 28±2 °C for 24 hrs.
4. Occurrence of yellow halo around the cultural colonies is a positive indication of siderophores production.

(F) Hydrogen Cyanide (HCN) production

Cyanogenesis from glycine results in the production of HCN, which is volatile in nature. Reaction of HCN with picric acid in presence of sodium carbonate results in the colour change of the filter paper from deep yellow to orange and finally to orange brown to dark brown. In the case of negative test, the deep yellow colour of the filter paper remained unchanged after the growth of bacteria.

Requirement: King's B medium, glycine, Whatman filter paper, picric acid, sodium carbonate, disc, parafilm, and petriplate

Procedure

1. Prepare King's B medium amended with 4.4 g l^{-1} glycine and sterilize it.
2. Pour the medium in plates
3. After solidification, streak the isolate in plates only single isolated has to be streaked on each plate.
4. Soak Whatman filter paper no. 1 disc (9cm in diameter) in 0.5% picric acid in 2% sodium carbonate.
5. Place the soaked disc in the lid of each petriplate.
6. Seal the petriplates with parafilm and incubate at 28±2 ºC for 4 days.
7. Keep an uninoculated control for comparison of results.

(G) Antibiotics production

Principle: Certain bacteria produce antibiotics which inhibits the growth of other bacteria. This antagonistic property of the bacteria can be used against pathogenic microorganisms.

Procedure

1. Weigh 1g of soil and place it into 9mL dilution blanks. Shake well and make dilutions up to 10-2.
2. Take 1mL aliquot from 10-2 dilution and pour plate it along with TY medium in petridishes.
3. After the solidification of the plates, spot the test cultures on these plates.
4. Incubate the plates at 30±2 ºC for 48 hr.
5. Observe for the appearance of inhibition zones around the test cultures.

(H) Antifungal activity

Principle: Certain bacteria produce antifungal metabolites like NH3, HCN, antifungal antibiotics and fungal cell wall degrading enzymes like chitinase and glucanase which is attribute to their antifungal properties. Bacteria which can inhibit the fungal pathogens can be used as a biocontrol agent.

Procedure

1. Grow the fungal cultures on potato dextrose agar (PDA) medium.
2. Raise broth cultures of rhizobacterial isolates in nutrient broth or their specific medium
3. Cut 2-3 mm agar disc containing fungal growth from previously grown plates and place it at the center of fresh PDA.
4. Streak the test bacteria on the periphery of the plate
5. Incubate the plates at 25±1 ºC.
6. Observe for the inhibition of radial growth of fungus.

Experiment 6

Aim: Mass production of biofertilizers i.e. *Rhizobium*, *Azotobacter* and *Azospirillum*

Requirement: Fermenter, air compressor, boiler autoclave, shaker, chemicals, glassware and glass wool

Theory: Bacteria (*Rhizobium*, *Azotobacter*, and *Azospirillum***)**to be inoculated in soil as biofertilizer need to be multiplied on artificial media to harvest on a large scale so that it can be supplied to farmers.

Procedure

Preparation of starter culture

1. The starter culture of or inoculum *Rhizobium*, *Azotobacter*, and *Azospirillum* for each experiment is prepared in 750 ml conical flask containing 300 ml YEM, Jensen's media and free nitrogen malate (NFb) media, respectively.
2. The flasks are allowed to be shaken on rotary shaker (260 rpm) at 28± 2 ºC for 5-7 days depending upon the growth of cultures.
3. The samples are drawn aseptically for checking of purity

Production of cultures

1. The fermenter is filled with 10 litres of YEM, Jensen's and NFb media
2. Lid is placed in position and sterilized at 15 lb pressure for 30 minutes in horizontal autoclave.
3. The medium is inoculated through inoculation point aseptically with 200 mL suspension of *Rhizobium*, *Azotobacter*, and *Azospirillum.*
4. The rate of aeration is adjusted by manipulating air in-let valve and air bleeder to give a constant pressure.
5. The cultures are grown for 40-72 (fast growing strains) and 96-144 hours (slow growing strains).
6. After the complete process, culture should be recovered by releasing pressure.

Observations: The broth samples are drawn aseptically at 12 hours intervals after inoculation till the end of growth cultures.

Precautions

1. All points of the fermenter are to be checked for hermetically, sealing with soap solution before sterilization of the medium and prior to inoculation
2. Air sterility is to be checked via air sterility assembly before passing air to the fermenter.

3. The air collect shoulf be via approximately I meter of rubber tubing and the air allowed to bubbled through a bactericide (5% phenol solution)
4. At the time of power failure, air-inlet and all entry points may be closed immediately and then 5% phenol solution is to be sprayed.

Experiment 7

Aim: Methods of evaluation of biofertilizers

Requirement: Leonard bottle-jar assembly, washed river sand, calcium carbonate, lighting assemblies, seedling nutrient solution, legume seeds, test species of *Rhizobium*, wick (Absorbent cotton), glass cover (removed after seedlings emergence), water resistant paper bags (to cover assembly during sterilization, remaining to cover junction of bottle and jar).

Theory: To select an effective strains/isolate for particular traits, a number of isolated need to be screened. The screening has to be done under aseptic conditions to rule out the influence of native population Leonard jar assembly has been designed for studying the rhizobial host interactions with big seeded legumes.

Procedure

1. The top half of the unit consists of a bottle (round beer or spirits 800mL capacity) that has had its bottom cut out to provide a level flat ground finish (it is convenient for this to be size that can be covered with a petridish half. Amber bottles usefully protect the roots from light.
2. The lower half (the reservoir) consists of a jar of such dimension that the inverted bottles sits snugly on its rim and the neck of the bottle comes to within 2-4 cms of the bottom of the jar.
3. A wick is provided to help the capillary rise of moisture from the reservoir to the top of the growth vessel. Absorbent cotton wool or lamp wick have all been used. The later might need through pre-washing with detergent to make them wettable. The wick is secured in the neck with a wad of cotton wool.
4. Add moderately well washed coarse river sand, mixed with 1g/kg calcium carbonate, to bottle unit to within 5 cm of the top. In doing this keep the wick roughly central and have it reached practically to the surface.
5. Prepare seedlings nutrients solution, dilute to 1/5 or 1/5 and moisture the jar from the top until liquid commences to drain into the reservoir. Fill the reservoir with the same nutrients solution to within 2 cm of the junction of the two vessels.
6. Cover the top of the growth vessels with a petridish half and the whole unit with moisture-proof paper secured with rubber bands or elastic bands.
7. Place dry sterilize fine gravel or chips (about 4mm) to cover the same in the unit to a depth of 2-3 cm.
8. Autoclave whole units at 121 ºC for two hours and keep covering intact until they are brought into use.
9. Sterilize and prepare the seeds in the usual way (as in agar tube method).

10. Remove the covering paper of bottles leaving junction of bottle and reservoir protected.
11. Plants the seed, with or without prior inoculation (according to purpose), just below the surface of the moist sand aseptically.
12. Replace the petridish half to protect against contamination and maintain the surface in moist conditions.
13. Set the assembly in place favourable for germination and growth, i.e. under lighting assembly with overhead illumination. If the units are of clear glass protect the roots from light by covering it with opaque paper.
14. As soon as plants are established and before their development is restricted by petridish, cover the sand with the dry gravel to a depth of 2-3cm. Inoculation can be conveniently carried out immediately prior to this stage if it has not already been done. The units are now left open.
15. Uninoculated and nitrate (0.05/KNO_3 (w/v) controls are also put in the usual way.
16. Units can be arranged compactly with overhead illumination
17. Nodulations is observed after 2-4 weeks of sowing.

Experiment 8

Aim: Production of BGA biofertilizer in rice crop

Theory: Blue green algae (BGA) are prokaryotic phototrophic organisms that can fix the atmospheric nitrogen biologically, and were directly applied as a biofertilizers in agricultural fields specifically paddy field.

Procedure: Production technology: The BGA production technology has been improved over the years and at present basically two technologies are available. These are

1. Open air soil based biofertilizer production
2. Polyhouse/glasshouse straw based production

Both these methods offer flexibility in size and quantity required and utilise the four strains- *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertilissima*, *Tolypothrix tenuis*

Improved open air production technology
Depending upon the requirement, algal biofertilizer can be produced by individual farmers and or also on commercial basis.

At individuals level

1. Prepare shallow troughs, at least 2 or multiple of two, approximately 2m×1m×23cm of galvanised iron sheet, permanent cement and mortar tank or polythene lined pit on ground. All these structures should be in an open area so as to receive sunlight for the entire day. A light intensity of 1000 to 4500 lux is good enough for BGA growth.
2. Spread about 3kg soil (1.4 kg m^2) per multiplication unit and add 200g single super phosphate. For best growth of BGA soil pH should be between 7.0 to 7.5, however, growth does occur at higher or low soil pH. To bring the pH about 7.5 soils can be amended with gypsum or lime depending upon initial soil pH.
3. Add water upto a height of 3-4 inches to the multiplication units and mix the contents.
4. As soon as the suspended soil settles and water becomes clear, sprinkle/broadcast the starter culture containing *Anabaena* and *Nostoc* (mixture 1) and *Aulosira* and *Tolypothrix* (mixture 2) in two different trough/multiplication units. This is essential to give equal opportunity to all form to grow and multiple equally
5. To prevent insect breeding, add carbofurans (3% granules, 20g/tray or unit) or malathion, BHC or any other suitable insecticide.

6. In hot summer months (temperature more than 30 ºC) BGA grows fast and in about seven to ten days a thick mat is formed. During this period if rate of evaporation is high, add water intermittently. Once the algal growth is thick, stop watering. Allow the algal mat to dry, dried algae form flakes along with soil.
7. Collect the dries algal flakes mix from both the multiplication unit to make composite mixture and stores in polythene lined bags in cool dry place.
8. Repeat the process till the requirement is met. From a single harvest about 3kg dried material per unit is obtained.

Field scale production is scaled up process for the production in large quantity. It can be adopted in cooperative basis by a group of workers. This process is possible in soils where water remain standing after pubblings.

1. Demarcate the area (40 m^2), stubbles are moved and soil is well pubbled to facilitate waterlogging.
2. Bund the area with strong 15 cm earth embakments.
3. Flood the area with water to a height if 1-2 inches.
4. Apply single super phosphate at the rate of 12kg/40 m^2.
5. To control the predators or algal grazers like snails, daphnids, mosquitoes and larvae apply carbofurans (3% granules) or ekalux (5% granules) at 250 g/40 m^2.
6. Inoculate the plot with 5 kg composite inoculum.
7. In clay soil within about 2 weeks good growth of BGA takes place, whereas in loamy soils three to four weeks are required. In between if the plots get dry fill with water. As soon as good algal mat is formed, it is allowed to dry. Dry algal flakes are collected and stores.
8. One continuously harvests BGA from the same area by reflooding the plot and applying super phosphate and pesticides.
9. During summer months (April-June), the average yield of BGA per harvest ranges from 16-30 kg/40 m^2.
10. This production procedure can be adopted at the time of sowing nurseries for rice seedlings and by settings BGA production unit side by side.

Storage of soil based BGA

- The sundried BGA can be stores for a long time (more than 2 years) in a dry state at normal room temperature in shade.
- Storage bags for BGA biofertilizers should be fresh and not the bags earlier containing urea or other chemicals fertilizers and other agrochemicals.

Experiment 9

Aim: Pot and field with inoculated seeds

Requirement: Pots, soil, seeds of legumes, *Rhizobium* culture (peat based or other carrier based) 10% sugar solution, superphosphate, muriate pf potash

Theory: It is necessary to evaluate the performance of selected strains (under asesptic conditions) under native conditions. Many a time the selected strains is unable to do well because of many reasons viz; non-competetive nature, inhibited by microbial metabolites. The selected isolate needs to overcome these hurdles, and form a beneficial association with the plant. The ultimate test is the yield parameter. Experiments with pot and fields design are necessary for recommending an isolate as inoculants.

Procedure

1. Fill the pots with finely processed soil.
2. The soil should be provided with phosphorus and potassium in the form of single super phosphate and muraite of potash, respectively at recommended level.
3. Before the soil should be moist enough for enabling the seeds to germinate.
4. Seeds should be treated with firstly with 10% sugar solutions and then with the culture of *Rhizobium* before sowing.
5. After coating of seeds, they are sown immediately.
6. The effects of seed inoculation can also be tested in the same way under field conditions.

Observations

Nodulation observations: At 30 and 60 days of plant growth, the following observations are to be recorded:

1. Number of nodules/plant
2. Dry weight of nodule/plants

Plant observation: Dry weight of shoot and root at 30 and 60 days of plant growth.
Harvest observations: At the time of plant maturity, the following observation are to be recorded.

1. Dry matter production
2. Number of pods
3. Grain yield

Bioagents for Sustainable Agriculture

Global increase in the population and degradation of environment possess a challenge to crop production worldwide and it is a need of hour to find solutions for abiotic stress, pests and pathogens. The excessive use of synthetic chemical pesticides pertains to cause unsympathetic effect on living beings and on their surrounding ecosystem along with several adjacent effects like emergence of new resistant insect pest species, high residue of pesticides in the produce obtained and diminution in the population of natural enemies. Eco-friendly approach of management by using biopesticides is an alternative against synthetic chemicals. Biopesticides means biological method of control by entanglement and manoeuvring of living organisms. Biopesticides comprises microbial pesticides, biochemical pesticides and plant-incorporated protectants (PIPs). Microbial biopesticides are the products obtained from microorganisms which are beneficial and can be applied against plant diseases and insect pests responsible to cause damage to agricultural crops year after year. Microbial pesticides can play an important role for crop protection in the agricultural based economy of the world. Microbial biopesticides in market comprises board spectrum approx. 90% of entire biopesticides with plenty of scope in public health and agriculture development. It is crucial now to popularise the use of these microbial biopesticides among the farmers worldwide. Entomopathogenic bacteria consist of Bacillaceae, *Burkholderia*, *Chromobacterium*, *Pseudomonas*, *Saccharopolyspora*, *Serratia*, *Streptomyces* and *Yersinia* species, whereas fungi include various strains of *Metarhizium anisopliae*, *Beauveria bassiana*, *Hirsutella, Isaria*, *Lecanicillium, Paecilomyces* and *Verticillium* species. Baculoviruses (insect pathogenic viruses) are species-specific and active against chewing and bitting insects, primarily against Lepidopteran caterpillars. Entomopathogenic nematodes (EPNs) include 2 genera *Steinernema* and *Heterorhabditis* showing mutualistic symbiotic association with bacteria *Xenorhabdus* and *Photorhabdus*. Biopesticides sources easily exist in nature, are naturally biodegradable, show different modes of action, less expensive and possess less toxicity to living organisms. Research on the application and stability of various biopesticides can support the sustainable agriculture worldwide.

Table List of important biopesticides

Entomopathogenic microbes	**Target insect pests**	
	Scientific name	**Common name**
Entomopathogenic viruses (EPVs)		
Cotton bollworm NPV; HezeSNPV	*H. armigera*	Cotton bollworm, pod borer
Corn earworm Nuclear polyhedrosis virus (NPV)	*H. zea* *Helioth virescens*	Tomato fruitworm Tobacco budworm
Diamond back moth GV	*P. xylostella*	Diamond back moth
AngeMNPV	*Anticarsia gemmatalis*	Velvetbean caterpillar
BuzuNPV	*Buzura suppressaria*	Tea moth
AucaMNPV	*Autographa californica*	Alfalfa looper

Entomopathogenic bacteria (EPB)		
B. thuringiensis subspecies *kurstakia*	*P. xylostella, S. exigua, H. armigera* and *Bombyx mori*	DBM, beet armyworm, pod borer and domestic silkworm
B. thuringiensis sub-species *aizawaia*	*Agrotis ipsilon*	Black cutworm
B. thuringiensis sub-species *japonensis*	*Anomala orientalis* and *Cyclocephala borealis*	Oriental beetle and Northern masked chafer
Paenibacillus popilliae	*Popillia japonica*	Japanese beetle
Entomopathogenic fungi (EPF)		
Aschersonia aleyrodis	*Bactericera cockerelli*	Potato psyllid
Beauveria brongniartii	*Pyronota festiva*	Manuka beetle
Lecanicillium longisporum	*Myzus persicae*, *Frankliniella occidentalis* and *B. tabaci*	Green peach aphid, Western flower thrips and Sweet potato white fly
Metarhizium anisopliae sensu lato	*B. cockerelli*	Potato psyllid
Nomuraea rileyi	*H. armigera, S.litura, Tricoplusia ni* and Anticarsia *gemmatalis*	Pod borer, tobacco cutworm, cabbage looper and velvetbean caterbillar
Entomopathogenic Nematodes (EPNs)		
Steinernema carpocapse	*A. ipsilon*	Cutworm
Heterorhabdits sp.	*Hypera postica*	Lucerne weevil
Heterorhabdits sp.	*H. armigera*	Pod borer
Heterorhabdits indica	*Scirpophaga excerptalis*	Sugarcane top borer
Steinernema sp. *Heterorhabdits* sp.	*Spodoptera frugiperda*	Fall armyworm
H. bacteriophora, S. kraussei, H. marelatus, S. feltiae	*Otiorhynchus sulcatus*	Black vine weevil
S. feltiae, S. riobrave	*Conotrachelus nenuphar*	Plum curculio
H. taysearae *Steinernema* sp.	*Bactrocera dorsalis*	Mango fruit fly
S. carpocapse	*S. exigua* and *B. tabaci*	Beet armyworm and sweet potato white fly

Experiment 10

Aim: Isolation of *Trichoderma viride*

Requirements: Soil sample, test tube, distill water, measuring cylinder, spreader, flask, petriplates, Molasses yeast media

Principle

Trichoderma is a genus of fungi in the family Hypocreaceae that is present in all soils, where they are the most prevalent culturable fungi. *Trichoderma* is a patent biocontrol agent and used extensively for soil borne diseases. It has been used successfully against pathogenic fungi. The genuses *Trichoderma* have five species i.e. *Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma pseudokoningii and Trichoderma viride.* The growth of *Trichoderma* has been screened on different culture media for various studies using available, relatively cheaper supporting media such as corn meal agar, oat meal agar, potato dextrose agar, Czapek's Dox agar, special nutrient media, carrot agar, rose Bengal agar, selective media, etc. However, selective media favor growth of Trichoderma strains over other fungi and hence preferred for easy identification of *Trichoderma* isolates over rapidly growing fungi that may overlap it.

Composition of Rose Bengal Agar (RBA) media

Media composition	:	**Amount (g/L)**
Bacteriological peptone	:	5.0
Rose bengal	:	0.05
Glucose	:	10.0
Chloramphenicol	:	0.1
Dipotassium phosphate	:	1.0
Agar	:	15.0
$MgSO_4.7H_2O$	:	0.5

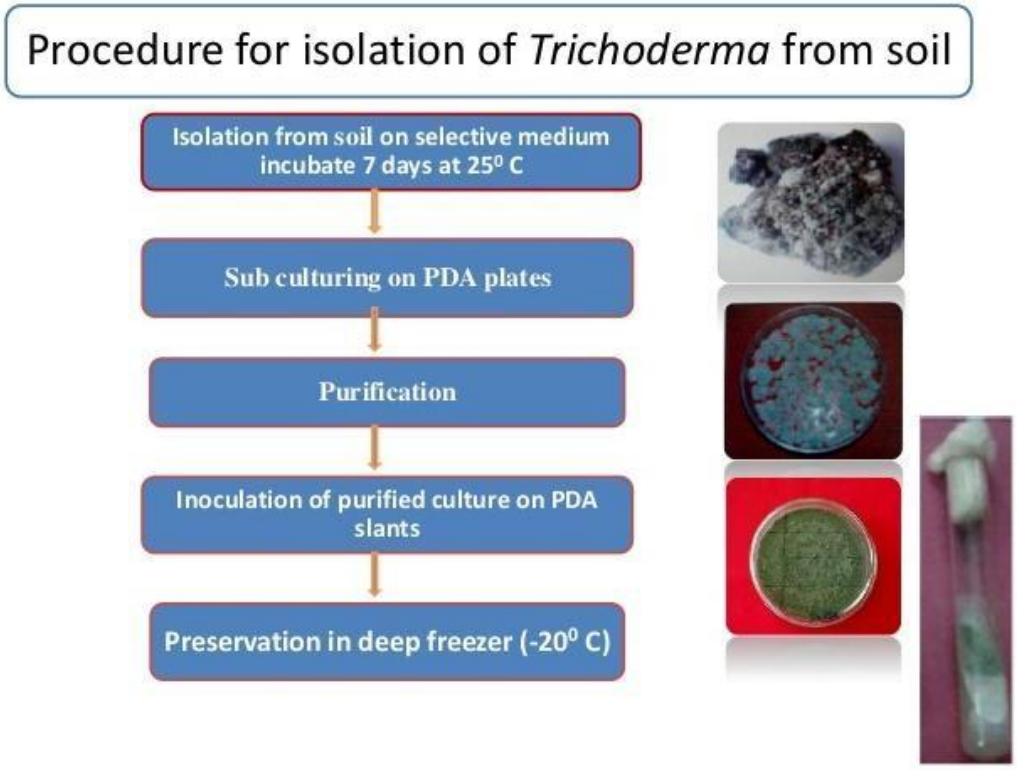
Composition of Potato Dextrose Agar (PDA) media

Media composition		**Amount (g/L)**
Dextrose	:	20
Potato extract	:	4
Agar	:	15

Procedure

1. *Trichoderma* fungal species were isolated from soil samples by using potato dextrose agar (PDA) medium.

2. PDA media were prepared and autoclaved at 121 ^{o}C @15psi for 20 min.
3. Penicillin @100,000 units L-1 and streptomycin @0.2g L1were added to sterilized stock media just before pouring to inhibit the bacterial growth.
4. The media were poured in 9cm diam. Petri plates @ 10 ml per plate.
5. Samples were inoculated over plates by multiple tube dilution technique (MTDT) and the plates were incubated at 26˚C for 4-7 days.
6. Inoculated plate shows a mixture of multiple fungi and bacteria cultures.
7. The desire fungal colonies were then picked and purified by streaking or by single spore and incubated at 26˚C for 7-8 days.
8. 1ml of distilled water spread over the fully grown culture of *Trichoderma* with a spade and then transfer to 99 ml of distilled water to make a suspension.
9. Take a drop from the diluted fungal suspension over the side and observe *Trichoderma* sp. under the microscope at 10-40X magnification.



Observation: Yeasts will grow as creamy to white colonies. Molds will grow as filamentous colonies of various colours

Precautions

3. Media should be prepared carefully.
4. Experiment should be carried in sterile conditions.

Experiment 11

Aim: Isolation of *Pseudomonas fluorescence*

Requirements: Soil sample, test tube, distill water, measuring cylinder, spreader, flask, petriplates, King's B media

Principle

Pseudomonas fluorescence is a genus of Gram-negative, rod shaped bacteria. *Fluorescent Pseudomonas* belong to family Pseudomonadaceae and *Pseudomonas* genus. *Pseudomonas fluorescens* encompasses a group of common, nonpathogenic saprophytes that colonize soil, water and plant surface environments. As its name implies, it secretes a soluble greenish fluorescent pigment called fluorescein, particularly under conditions of low iron availability. Certain members of the *P. fluorescens* have been shown to be potential agents for the biocontrol which suppress plant diseases by protecting the seeds and roots from fungal infection. They are known to enhance plant growth promotion and reduce severity of many fungal diseases.

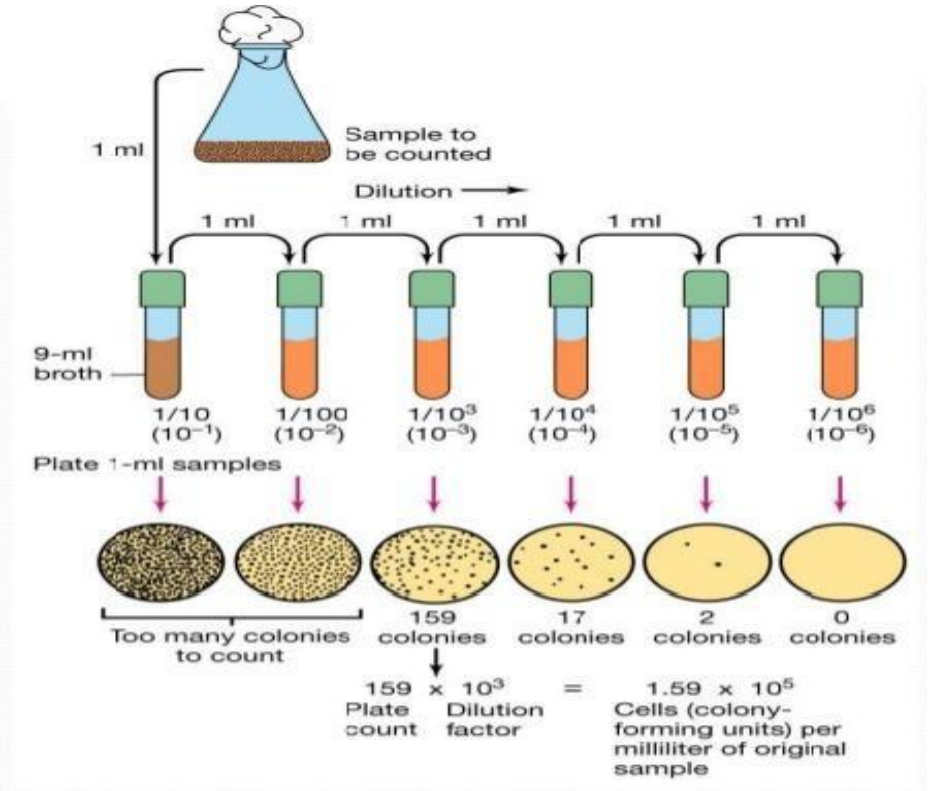
Composition of King's B media

Media composition	:	**Amount (g/L)**
Protease peptone	:	20
Glycerol	:	10ml
Di-potassium phosphate	:	1.5
Magnesium sulphate:	:	1.5
Agar	:	20

Procedure

1. Then the soil samples were collected in a plastic bag and bring these samples to laboratory.
2. On the other hand, media and dilutions were prepared into a flask and test tube, respectively, autoclaved.
3. The autoclaved media was poured in to a petridish and allowed them to solidified
4. After pouring, the dilutions blank were labeled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}.
5. After that, in 10^{-1} blank 1g of samples was mixed and using sterile pipette, transfer 1ml of sample from dilution 10^{-1} to 10^{-2} and mixed. This process is continues till 10^{-5}.
6. Label the agar plates as 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}
7. Then using a sterile pipette, transfer 0.1 ml sample from the 10^{-2} labeled test tube and with the help of a heat sterile spreader transferred liquid was spread evenly and thoroughly until the liquid is being absorbed into the plate.

8. Similarly 0.1 ml from dilution 10^{-3}, 10^{-4}, and 10^{-5} were also spread on to the media
9. Tape the spread plates and incubated upside down at 30ºC.
10. Molecular identification of *P. fluorescens* is generally done by 16S rRNA, intergenic spacer (ITS1) utilizing traditional polymerase chain reactions (PCR).
11. Nowadays, qPCR and multiplex PCR are largely utilized in identification of *P. fluorescens* based on AprX gene (extracellular caseinolytic metalloprotease) in the milk and meat spoilage strains.
12. The available methods still show some disadvantages with accuracy and specificity of detection.
13. Rapid detection of *P. fluorescens* in food samples is the need of hour to improve the detection efficiency.



Observation

Yellowish green colour with light green pigmentation colonies of *Pseudomonas fluorescence* obtained.

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Experiment 12

Aim: Isolation of *Bacillus thuringiensis* from soil

Requirement: T3 agar, dilution blank, soil sample, test tube, distills water, spreader, flask, petriplates, inoculating loop.

Principle

The microorganisms which have capability to kill the pest are known as microbial pesticide which include bacteria, fungi, and protozoans. Among bacteria, *Bacillus megaterium*, *B. subtilis*, *B. thuringiensis*, and *Pseudomonas fluorescence* are well known to be used as bacterial pesticide. *Bacillus* and *Bacillus* derived genera may be isolated using different techniques leaf imprinting, surface sterilization, and serial dilution with help of T3 agar and Mannitol-egg yolk-polymyxin (MYP).

Composition of T3 Agar media

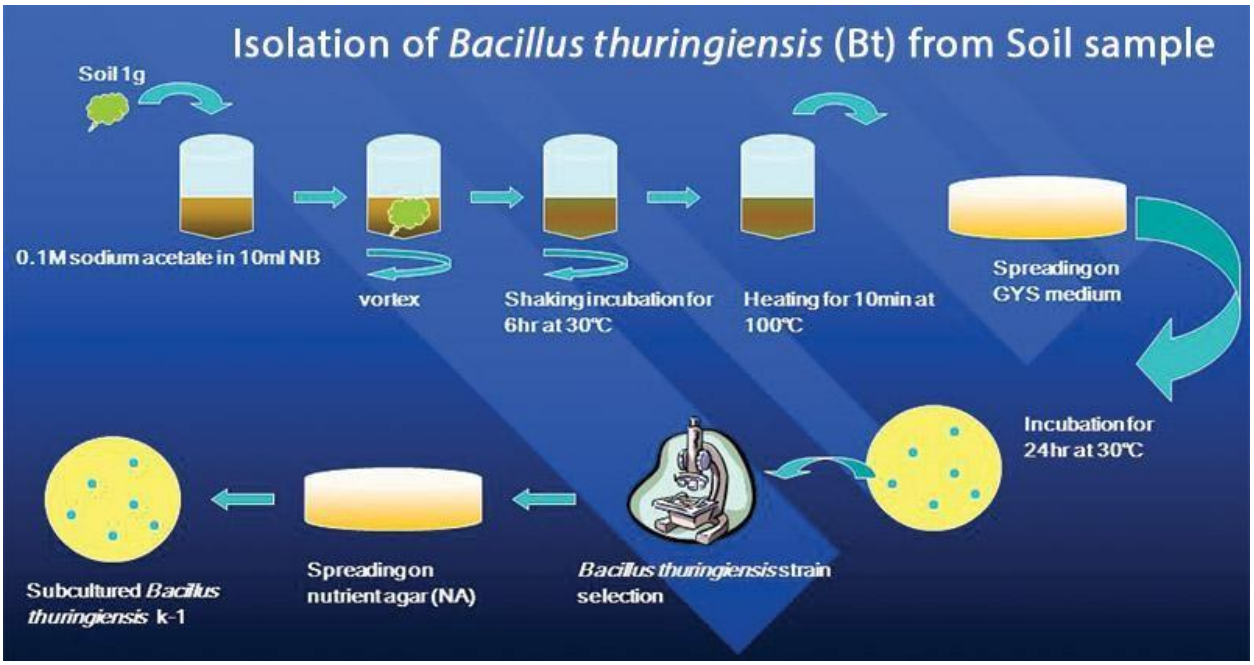
Media composition		**Amount**
Tryptone	:	3
Tryptose	:	2
Yeast extract	:	1.5
$MnCl_2$	:	0.005
Agar	:	20
pH	:	6.8

Composition of Mannitol-Egg Yolk-Polymyxin (MYP) Agar

Media composition		**Amount (g/L)**
Beef extract	:	1
Peptone	:	10
Mannitol	:	10
NaCl	:	10
Phenol red (1% solution in 95% ethanol)	:	2.5 ml
Agar	:	20

Procedure

1. Obtain about 20 g of cultivated or non-cultivated soil sample with a tubular soil sampler after removing the 2-3 cm of the top layer.
2. Place samples at 4 ºC in 50 ml (sterile) centrifuge tubes or zip-lock bags until isolation.
3. Suspend the soil samples of 1 g in 10ml 0.85% NaCl.
4. Heat with shaking at 70°C for 10 min.
5. Plate aliquots of 100µl of suspension onto nutrient agar (0.5% Peptone, 0.3% beef extract, 0.5% NaCl and 1.5% agar).
6. Incubate plates at 30±2°C for 48h.
7. Sub-culture bacterial colonies exhibiting Bt-like phenotype for single-colony isolation again on fresh plates and incubate.
8. Stain the culture with amino black and Ziehl's carbol fuchsin and examine under a standard light microscope for preliminary identification.



Observation

1. Colonies mostly appear matte white colour, flat, dry and with uneven borders.
2. Cultures that show parasporal crystals dyed black on microscopic observation may be of importance and require storing.

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Experiment 13

Aim: Isolation of *Beauveria bassiana* from soil

Requirement: Soil sample, test tube, distill water, measuring cylinder, spreader, flask, petriplates, DOC2 and PDA media.

Principle

Beauveria bassiana is a fungus that grows naturally in soils throughout the world and acts as a parasite on various arthropod species, causing white muscardine disease; it thus belongs to the entomopathogenic fungi. It is being used as a biological insecticide to control a number of pests such as termites, thrips, whiteflies, aphids and different beetles. Its use in the control of bedbugs and malaria-transmitting mosquitos is under investigation.

Composition of DOC2 medium

Media composition		**Amount (g/L)**
Bactopeptone	:	3
$CuCl_2$	:	0.2
Crystal violet	:	0.002
Agar	:	20

Compositions of Potato dextrose agar (PDA) media

Media composition		**Amount (g/L)**
Dextrose	:	20
Potato extract	:	4
Agar	:	20

Procedure

1. Soil samples were collected in a plastic bag and stored at 4–8 °C.
2. DOC2 medium for *B. bassiana*, autoclaved and poured into 15 cm Petri dishes.
3. Taken (1g) of soil sample from was suspended in 10ml sterile distilled water.
4. Suspensions were applied at a concentration of 100 µl/plate and spread using a glass rod.
5. Plates were incubated at 25 °C in the total darkness.
6. For increasing of quantity of *B. bassiana* by using of Potato Dextrose Agar (PDA) medium. Incubation at 25 °C in the total darkness.
7. Cultures were examined periodically and identified when they sporulated.

8. The cultures were separated into groups based on their morphological characteristics including growth pattern, colony texture, pigmentation, and growth rate of the colonies on PDA
9. When fungal colonies sporulated on PDA, small plaques from the edge and the center of each growing colony were transferred onto glass slides, and then were examined using a compound light microscope

Observation: Generally, *B. bassiana* grows as a white mould. It produces many dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells.

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Experiment 14

Aim: Isolation of *Serratia entomophila*

Requirements: Caprylate thallous agar (CTA), Adonitol agar (ADO), DNase-toluidine blue agar, Itaconate agar, Phosphate buffer, Ultrasonic bath, Plastic bag

Principle

Serratia entomophila (Enterobacteriaceae) is currently being developed as a biological control agent for the New Zealand grass grub, Costelytra zealandica (White) (Coleoptera: Scarabaeidae). Soil dilutions were plated on caprylate thallous agar (CTA), selective for Serratia spp. Most strains of Ser. entomophila grew well on CTA; the mean efficiency of colony formation on CTA was 94 f 3% of that on a non-selective medium. Adonitol agar (ADO) was included to distinguish between *Serratia entomophila* and *Serratia proteamaculans* strains commonly isolated from soil. The identity of colonies growing on CTA was determined on the basis of their growth reactions on DNase-toluidine blue agar, adonitol agar and itaconate agar. Serratia entomophila could be distinguished from other Serratia spp. found in New Zealand soils, in particular Serratia proteamucufans, another causal agent of amber disease of grass grub. The identification scheme allowed the selective recovery of *Serratia entomophila* from field soils containing a diverse microflora.

Compositions of caprylate thallous agar (CTA)

Media composition		**Amount (g/L)**
H_3PO_4,	:	1.96
$FeSO_4.7H_2O$	:	0.0556
$ZnSO_4.7H_2O$	:	0.0287
$CuSO_4.\ 5H_2O$	:	0.0025
$Co(NO_3)_2\ 6H_2O$	:	0.003
H_3BO_3	:	0.0062
$MnSO_4.4H_2O$	:	0.0223

This solution was stored unsterilized at 4°C. Then, two solutions designated A and B were prepared, autoclaved separately, and mixed aseptically to yield a liter of the final medium.

Solution A

Media composition		Amount (500 mL)
$CaCl_2.2H_2O$	:	0.0147 g
$MgSO_4.7H_2O$	:	0.123 g
KH_2PO_4	:	0.680 g
K_2HPO_4	:	2.610 g
Caprylic (n-octanoic) acid	:	1.1 ml
yeast extract	:	2 ml of a 5% (wt/vol) solution
Thallous sulfate	:	0.25 g
Trace elements solution	:	10 ml
pH	:	7.2

This solution A was autoclaved at 110°C for 20 min.

Solution B

Media composition		Amount (500mL)
NaCl	:	7g
$(NH_4)_2SO_4$	:	1g
Agar	:	15g
pH	:	7.2

The pH was adjusted to, and this agar base (solution B) was autoclaved at the same time and under the same conditions as solution A. After autoclaving, solutions A and B were mixed aseptically, and the resulting CT agar medium was poured into sterile plastic petri dishes (25 ml when the diameter of the dish was 9 cm and 80 ml when the diameter was 14 cm). The dishes were stored at 4°C until used; for some as yet undetermined reasons, remelted medium was not satisfactory. Plates of CT agar were dried before use by placing them in a 30 to 37°C incubator for a few hours.

Composition of Adonitol solution

Media composition		Amount
Bactopeptone	:	10g
NaCl	:	5 g
Bromothymol blue solution	:	12ml

Agar	:	15g
Adonitol solution	:	3%

Two hundred ml of 3% adonitol solution was added after autoclaving. Bromothymol blue solution was prepared by mixing 0.2 g of bromothymol blue and 5 ml of 0.1 mol 1-’ NaOH with 95 ml of distilled water.

Procedure

1. Soil samples were randomly collected in a plastic bag and stored at 4–8 °C.
2. Four different specific media were used to grown *Serratia entomophila* like caprylate thallous agar
3. (CTA), DNase agar (DNA), chitin salts casamino acid agar (CHSA) and adonitol agar (ADO)
4. 10g soil samples were diluted in 90 ml of sterile water, phosphate buffer and treated for 5 min in an ultrasonic bath (Branson Cleaning Equipment Co., CT, USA) before dilutions were prepared.
5. Suspensions were applied at a concentration of 100 µl/plate and spread using a glass rod.
6. Plate was incubated for 24 h before presence or absence of a red halo around the colony was determined. Strains were assessed as chitinase positive if a clear halo formed around the colony after incubation on CHSA for 4 d. ADO was incubated for 24-28 h, before colony colour was recorded. Growth on ITA was recorded after incubation for 4 d.
7. Efficiency of colony formation on CTA was measured by preparing suspensions of *Serratia* spp. in sterile phosphate buffer (pH 7.2, 100 mmol I-’) and plating dilutions in duplicate on parallel plates of nutrient agar (NA) and CTA.
8. Colonies were counted after 1 d at 30°C for NA plates and 4 d incubation at 30°C for CTA plates.
9. Cultures were examined periodically and identified when they sporulated.
10. The cultures were separated into groups based on their morphological characteristics including growth pattern, colony texture, pigmentation, and growth rate of the colonies on CTA.
11. When fungal colonies sporulated on CTA, small plaques from the edge and the center of each growing colony were transferred onto glass slides, and then were examined using a compound light microscope

Observation: Red halo around (CHSA) with small cream colonies forming after 4 d incubation on CTA, the colons of *Serratia entomophila* was absorbed

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Experiment 15

Aim: Isolation of *arbuscular mycorrhizae*

Requirements: Coarse soil-sieve, Distilled water, Centrifuged, Glycerine, Glass container, Lactic acid, Pipette, PVLG and Sodium hexametaphosphate

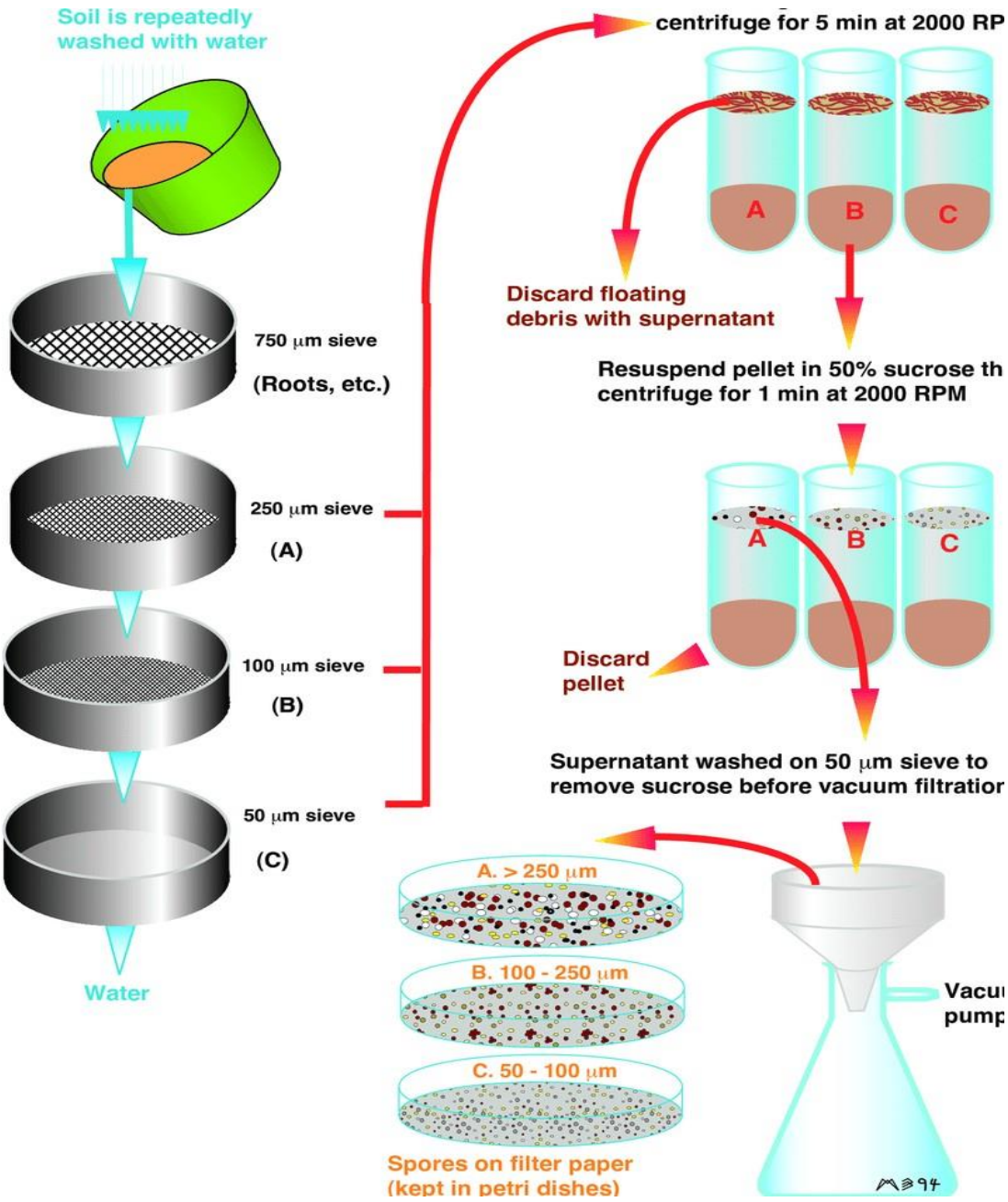
Principle

Arbuscular mycorrhizas (AM) are symbiosis between plant roots and fungi belonging to the family Glomeraceae and phylum Glomeromycota. AM fungi colonize the plant roots and penetrate into surrounding soil, extending the root depletion zone and the root system. These are two types "endomycorrhizal and ectomycorrhizal". AMF helps in improving soil aggregation by influencing beneficial bacteria communities. The mutualistic nutrient exchange in this symbiosis is characterized by the transfer of phosphorus from the mycosymbiont to the host plant and by the reverse transfer of carbon compounds derived from photosynthates. Several techniques have been used for the isolation of VAM fungal spores from the soil such as floating technique, decanting and sieving, gradient centrifugation, monoclonal antibodies and polyclonal antibodies methods but techniques wet sieving method & sucrose gradient method are mostly using to Isolation of AM (Arbuscular Mycorrhizae/ VAM) fungi.

Composition of important reagents used in mycorrhizal techniques: 1. PVLG mountant

Media composition		**Amount**
Polyvinyl alcohol	:	8.33 g
Distilled water	:	50 ml
Lactic acid	:	50 ml
Glycerine	:	5 ml

Polyvinyl alcohol (24–32 centripose viscosity) can be used and dissolved in water by heating (90 °C) overnight



Procedure

1. Mix a volume of soil (250 gm) in water (l000 ml) and allow heavier particles to settle for a few seconds.
2. A dispersant such as sodium hexametaphosphate can be used with clay soils

3. Pour liquid through a coarse soil-sieve (500-800 ml) to remove large pieces of organic matter. Collect the liquid which passes through this sieve. Wash the sieve in a stream of water to ensure that all small particles have passed through.
4. Next, the suspension is passed through a 100 μm sieve and then through a 63 μm sieve.
5. The spores and small amount of debris that remain on the 63 μm sieve are poured into a centrifuge tube containing water and centrifuged at 1800 rpm.
6. The upper solution is poured off, 40% sucrose is added to the debris at the bottom and the mixture is then centrifuged for 2 min at 1800 rpm.
7. Pick out 40–100 typical, clean spores with a pipette or other device and place them in a watch glass containing distilled water.
8. A drop of PVLG is then placed on a clean and dry microscope slide. Add 10–25 spores with a minimum amount of water to the mountant. Gently mix the spores and mountant together with a needle or other object to slightly disperse the spores.
9. Allow the mountant to set for 4–5 min to become more viscous and then add a coverslip gently onto the mountant without the formation of any air bubble.
10. Do not apply pressure to the coverslip in this process. Let the mountant with spores dry overnight in a flat position.
11. Clean off any excess mountant with a muslin cloth moistened with a solvent such as ethanol. Seal the edges of the coverslip with a clear fingernail polish or other sealant and allow it to dry.
12. Repeat the steps with a second drop of PVLG on another slide. Gently break the spore walls under cover slip of this second slide by applying light pressure on the cover slip with the back of the needle.
13. It is very important that the break of the spore wall be adequate. The spores should not be crushed during the process. The spores can also be stained in a drop of Melzer's reagent by mixing with PVLG in a ratio of 1:1.
14. The upper solution is separated for examination under the stereoscopic microscope. The spores which are collected under the microscope are stored in Ringer's solution for identification.

Observation: The AM species can be identified using spore color, size wall structure and other morphological structures

Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

Wet Sieving methods for isolation of *Arbuscular mycorrhizal* fungi

Experiment 16

Aim: Isolation of *Entomopathogenic nematodes*

Requirements: Dish cover, Petri dish, Soil sample, Plastic bags, cooling box, larvae, distilled water, filter paper and container

Principle

Entomopathogenic nematodes (EPN) represent a group of soil-inhabiting nematodes that parasitize a wide range of insects. These nematodes belong to two families: Steinernematidae and Heterorhabditidae. Until now, more than 70 species have been described in the *Steinernematidae* and there are about 20 species in the *Heterorhabditidae*. The nematodes have a mutualistic partnership with *Enterobacteriaceae* bacteria and together they act as a potent insecticidal complex that kills a wide range of insect species. The life cycle of EPNs includes an egg stage, 4 juvenile stages, and an adult stage. The 3rd juvenile stage of EPNs is referred to be the "infective juveniles" (IJs). Once the IJs penetrate the host, they release bacteria that live symbiotically within the EPNs' gut. Once released into the host, the bacteria multiply quickly and under optimal conditions causing host mortality within 24 to 48 h. EPNs represent an alternative control method to insecticides as it is an environmentally safer option, not harmful to humans, animals, plants, or earthworms.

Procedure

1. Soil sample were collected
2. Soil samples were taken a depth of 5-20 cm by 1.5 kg of soil and packaged in a plastic bag.
3. The samples were transported to the laboratory in a cooling box.
4. Each sample was divided into three replicates each of them inside the plastic box by half a kg of soil per pack, 3-5 larvae of wax worms added to each box with wetting of the soil moisture to become 6 soil : 1 water (w : w).
5. The samples were kept at a temperature of 25±2 ºC for a week. The samples examined to extract the dead larvae.
6. Dead larvae were transferred to white traps which is a large petri dish inside it a small petri dish cover inverted.
7. Dead larvae were placed on the inside cover with a little distilled water.
8. The larvae were placed on the filter paper and incubated at 25 ± 10^{0}C until all nematode progeny had emerged and moved down into the water in the container.
9. Note to record infection and nematodes migration after the completion of the life cycle and reproduction in these larvae.

10. After confirming that each isolate belonging to the (EPNs) collected in plastic cans and recorded all the data for each isolate and kept in a temperature of 10°C and the infection repeated every month for each sample.
11. For identification, at generic level (on the basis of change of colour of dead insect larva: *Heterorhabditis* spp. - red/ brownish pink and *Steinernema* spp.-grey/white) and at species level, identification was done using morphological characters.

Observation

Red/ brownish pink with grey/white colonies of EPNs obtained.

Experiment 17

Aim: Mass production of *Trichoderma viride*

Requirements: Soil sample, sterile distilled water, Martin Rose Bangle agar

Theory

Entomopathogenic fungus *Trichoderma* species usually recorded in worldwide and it mostly used as biological control agent of soil pathogens. It can be evaluated from easily from soil root and plant organic matters. Trichoderma species had competency to overcome the growth of fungal plant pathogens and appreciate plant growth & development. Trichoderma species has cultured on distinctive culture media to composed higher biomass of fungus product of biomass of fungi cereals, organic & non synthetics media have been used. *Trichoderma* species can use as seed treatment, applied direct to the soil before planting & added in organic fertilizers. Fungi (*Trichoderma viride***)** to be inoculated in soil as biopesticides need to be multiplied on artificial media to harvest on a large scale so that it can be supplied to farmers.

Present study organized to evaluate grain such cereals, potato and carrot as different source for media at temperature and naturally available solid media such as carrot both, potato extract broth, jwar +maize grain (20gms each) +10per liter gm dextrose for the biomass production of Entomopathogenic fungi.

Mass Production Techniques of *Trichoderma* species- Mass production is broad aspect of successful biological control techniques which include establishment of products, formulation and delivery system of Entomopathogenic fungi where used as efficient disease control.

Mass production of *Trichoderma viride* included Liquid state formulation- In Liquid state formulation many type of liquid media used for mass production of *Trichoderma viride*.

***Trichoderma viride* final formulation base on various carrier materials:**

Formulation of *Trichoderma viride*-

In general mass production form in solid, liquid or semisolid form. The carrier material which use in production, should be in inert base. *Trichoderma viride* can be formulated as dust & powder form following formulation generally used in industries.

TALC based formulation-

Trichoderma viride cultured & prepared stock culture which colony forming unit should be more than 2×106 per ml & stock culture mixed with sterile talc powder, moisture under shade self-life of species in talc based formulation is observed 3-4 month. This method has been popular in

India for management of several soil bone diseases of various crops through seed treatment at 4-5gm/kg of seed of various crops viability of treatment in talc formulation by 50% after 120 days storage.

PESTA granules- Trichoderma species biomass was done as pesta granules, 52ml *Trichoderma viride* inoculums added in wheat flour 100gms and mixed by hand gloved in sterile water to from adhesive dough. This dough is kneaded, pressed, flat and folded by hand several times 1mm thick sheets (pesta) is prepared and air dried till it breaks crispy. After drying dough sheet was ground and passed through mesh (1.0mm) sieve and granules was collected.

Press Mud formulation-
Press mud was used as a carrier substrate for production of *Trichoderma viride*. *Trichoderma viride* production on press mud was formulated & sold to farmers.

Oil Based Formulation- This formulation prepared by mixing of harvested conidia from solid /liquid state formulation with combination of vegetable/ mineral oil which is stable emulsion formulation. In oil based formulation spore is suspended in a water immiscible solvent like petroleum fraction (diesel, mineral oil, and vegetable oil). After the added surface active agent for this preparation require a high concentration of oil soluble emulsifying agent which provide homogenous emulsion on dilution in water. Oil which is used in formulation should not have toxicity to fungal spore, human, plant and animals. This type of preparation used in formulation of *trichoderma* species, *Beauveria bassiana* & *pseudomonas species* for foliar spray.

Trichoderma viride can be used as seed treatment (3-10 gm per kg seeds). Seed biopriming (seed with Trichoderma and incubated and warm & moist conditions until just period to radical emergence), root treatment, soil treatment, aerial spraying and wound dressing.

Standard of *Trichoderma* formulation- *Trichoderma viride* counts should be a maximum from 2×10^6 CFU per ml or gm in final formulation. Pathogenic contamination should not be present like salmonella, shigella or vibrio. Pathogenic contamination count should not be expressed 1×10^4 CFU ml/gm. In talc base formulation moisture should not present more than 8%. Quality of the products should be periodically checked by the identified agency and that will ensure availability of better product to formers.

Procedure

1. 1 gm soil suspension was prepared in 10 ml distilled water and mixed properly, Allowed to settling down and performed serial dilution.
2. Loopful suspension was taken from supernatant of last three aliquots and streaked on Martin Rose Bangle agar.
3. Petri plate Incubated at room temperature for seven days.

4. Colonies characters observed on Petri plate and prepared mount slide with the help of Lacto phenol cotton blue and observed in microscope
5. The samples are drawn aseptically for checking of purity

The isolated *Trichoderma* species cultured with following specific media ingredients.

1. Potato Dextrose Broth contents Potato extract 300gm per liter and Dextrose 25gm per liter.
2. Carrot barley broth contents Carrot Extract 100gms per liter and Barley 25gms per liter.
3. Carrot Glucose broth content Carrot Extract 100gms per liter and Glucose 25gm per liter.
4. Jwar Maize Glucose broth content Jwar Extract 100gms per liter, Maize Extract 100 gms per liter and Glucose 25gms per liter.
5. Yeast Extract +Glucose broth content Yeast Extract 20 gms per liter, Glucose 25gms per liter.
6. Peptone +Glucose broth content Peptone 20 gms per liter and Glucose 25gm per liter.
7. Optimized broth content Potato extract 200 gms per liter, Peptone 10 gms per liter, Yeast extract 5 gms per liter, Glucose 20 gms per liter, Saw dust 25gms per liter and Wheat bran 25 gms per liter.
8. Lid is placed in position and sterilized at 15lb pressure for 30 minutes in horizontal autoclave.
9. The medium is inoculated through inoculation point aseptically with 200 mL suspension of *Trichoderma viride.*
10. The rate of aeration is adjusted by manipulating air in-let valve and air bleeder to give a constant pressure.
11. The cultures are grown for 40-72 (fast growing strains) and 96-144 hours (slow growing strains).
12. After the complete process, culture should be recovered by releasing pressure.

Observations: The broth samples are drawn aseptically at 12 hours intervals after inoculation till the end of growth cultures.

Results

Mass production of Entomopathogenic fungi are very convenient step in commercial industry. It should be reached as higher counts and utilization as Biological control agent. Mass production may be inexpensive by this way we can obtain high counts by using various substrate wheat bran, saw dust, peptone, potato extract, carrot, maize, jwar and yeast extract which used as liquid or solid substrate. Consequential investigation borne out on larger production of Entomopathogenic fungi whichever exhibit broad activeness. The collected soil samples were analyzed by distinctive media like Martin Rose Bangol Agar, *Trichoderma* Selective Media and

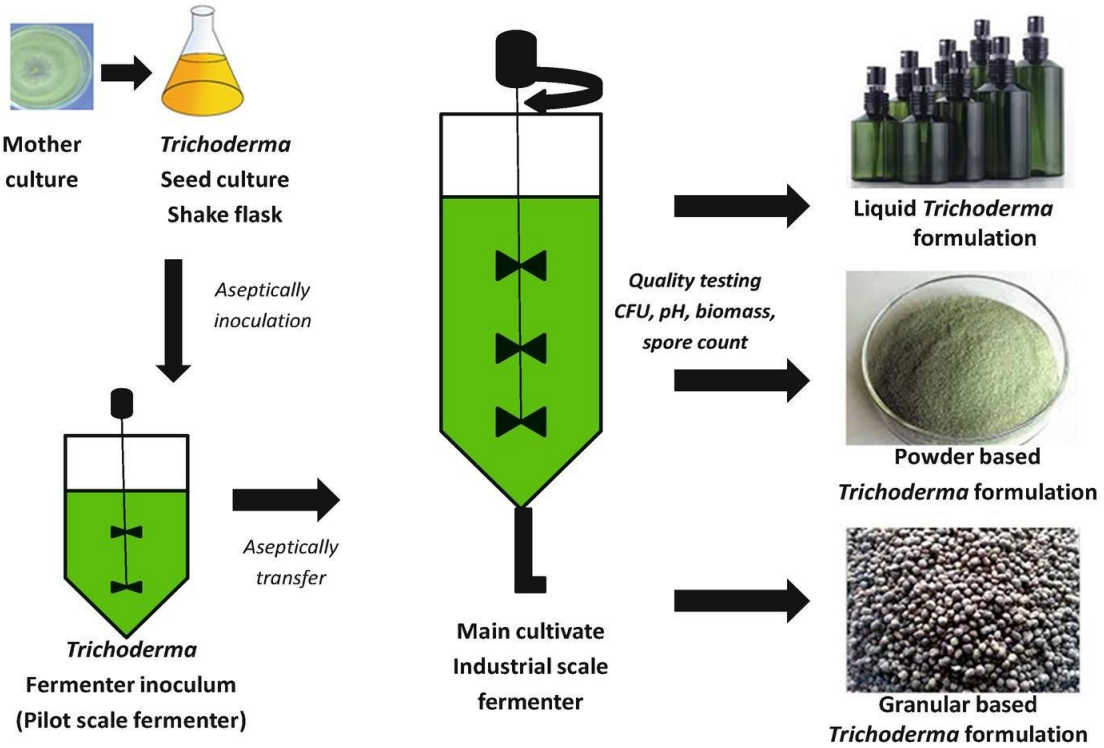
Oat meal agar by serial dilution. Selected fungi cultures were obtained on selective media. Fungi name were given on the basis of morphological and microscopic characteristic.

Future aspects

1. Formulation of *Trichoderma viride* with prolonged self-life and store ability for dry weather condition.
2. Quality control laboratories.
3. Identification of strain which suitable for various environmental factors.
4. Fast tract registration.
5. Development of liquid /oil formulation suitable for various environment factors.
6. Scaling up solid state production system with industrial collaboration.

Precautions

- Don't use chemical fungicide after application of Trichoderma for 4-5 days.
- Don't use *trichoderma* in dry soil. Moisture is an essential factor for its growth and survivability.
- Don't put the treated seeds in direct sun rays.
- Don't keep the treated FYM for longer duration.



Product development process of different formulation of *Trichoderma* sp. using submerged batch fermentation

Experiment 18

Aim: Methods of evaluation of biopesticides

Requirement: tube petriplates & inoculation bottles, potato-dextrose agar (PDA) and broth (PDB), malt extract agar (MEA), autoclave, aluminium foil, inoculating needle, spirit lamp, containers, CMC (carbon methyl cellulose)

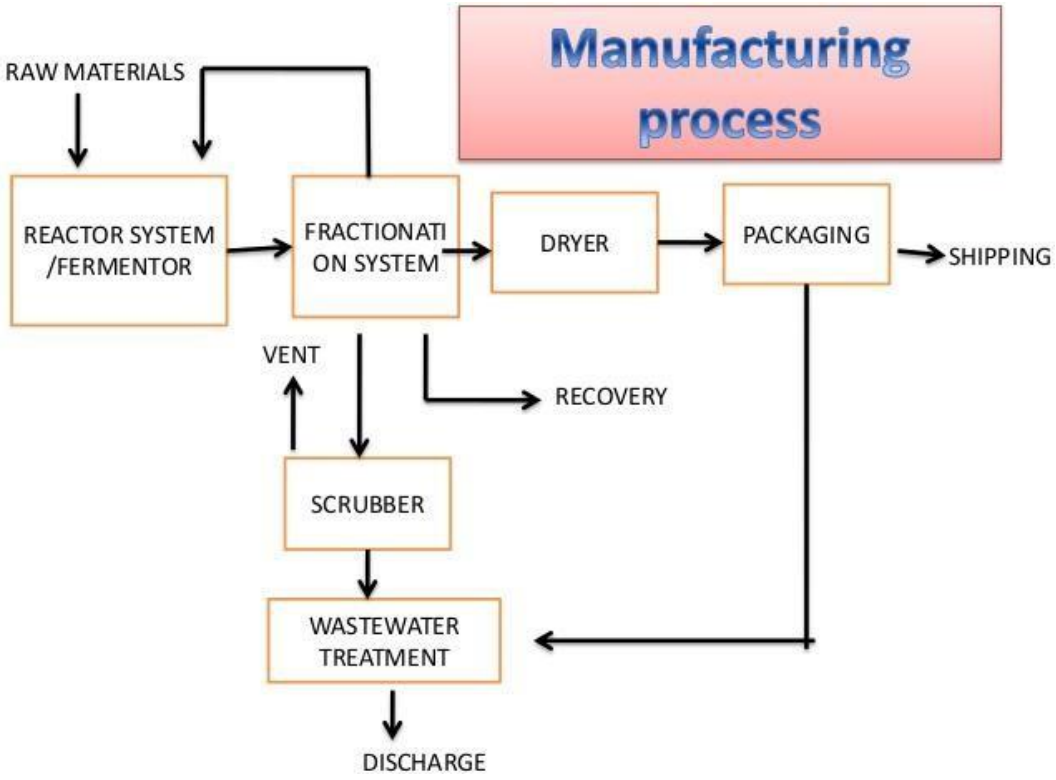
Theory

Biopesticides can be formulated in different forms according to their shelf life, stability, and microorganism. In terms of physical state, these formulations can be either dry or liquid. The active component can be produced with the help of stabilizers, additives, spreads, synergist, stickers, surfactants, coloring agents, nutrients, dispersants, melting, and ant freezing agents.

Procedure

1. All media are autoclaved at 121°C for 15 min, cooled to 50°C, and poured into 90 mm Petri dishes, unless otherwise stated. Standard media [e.g., potato-dextrose agar (PDA) and broth (PDB), malt extract agar (MEA) and broth (MEB) is prepared according to manufacturer's instructions]
2. Prepare POB broth media
3. Cooking of 200gm potato for 1 lit media than extract taken and add water & mix 20gm dextrose
4. Than make final volume 1 lit
5. Fill media in 1lit conical flask & plugged with aluminium foil than tied with rubber band
6. Sterilize media & required glassware like test tube petriplates & inoculation bottles for 15 min in 121^0c in autoclave
7. Kept these for cooling
8. Sterile LAF with ethyl alcohol than transfer glass wares & media in LAF
9. Pour media in bottle, Petridishes
10. Inoculated pure culture of *Trichoderma viridae* with the help of inoculating needle using spirit lamp to avoid contamination
11. Collect the fungus growth & crush these culture with the help of blander mix these with the help of blander
12. After that mix well with talcum powder for final product of *Trichoderma viridae*
13. Drying of product at room temp for 1 day
14. Mix CMC (carbon methyl cellulose) as sticky agent in dried powder form
15. Packed in dry and clean containers e.g., drums type depend on type of pesticide
16. Capacity 10, 25, 50, 100, 200 lits.

17. Temper-proof, closer to avoid leakage, sturdy
18. Packing product in polythene bags & labeling



Molecular characterization of biofertilizers and biopesticides

Experiment 19

Aim: To purify and preserve the biofertilizers and biopesticides

Purification- It is done to obtain the pure culture (Single distant colonies). Following techniques are used-

1. Quadrant method/streak
2. Radiant streak
3. T streak
4. Continuous

1. **Quadrant method/streak:** Microbes which we get from leaf with the help of inoculating loop. Make a line in new plate of nutrient agar. It is best method to obtain pure culture. Again red hot and cool it and again streak with respective line and again repeat respectively.
2. **Radiant streak:** Touch and make 4 to 5 lines continuously and again red hot and the cool and between the first line and make rays.
3. **T- streak:** From mother plate it is done starting from/corner and red hot the inoculating loop and cool and start and start from the centre of I^{st} line and again red hot and again cool.
4. **Continuous streak:** Make one line and make a continuous streak. It is simplest method for sub culturing.

Preservation: It is done for the future uses. There are 4 techniques:

1. Slant
    - Transfer the culture media in the test tube having nutrients agar
    - Autoclave the test tube containing media
    - Cool it
    - Keep the test tube in the slanting position
    - Incubate at 30 ºC for 24 hrs
    - Store at 4 ºC for 5-6 months

2. **Glycerol stock**
    - Pure culture should be transferred on new nutrients plate and incubate for 24 hrs
    - After 24 hrs, the 1 or 2 full loop culture may be transferred in cryovial containing 1 of the cryoprotectant such as glycerol, dimethyl sulphoxide (DMSO) and ethyl glycol.

3. **In mineral oil**
    - Transfer the pure culture in test tube containing nutrient agar
    - Pour mineral oil in the same test tube
    - Store at room temperature for 4-5 months

4. **Lyophilisation**
    - It is done to pressure microbes from generation to generation
    - Scrap the culture with the help of inoculating loop and transfer to glass vail
    - Cover the glass vail to lyophilize for 24 hrs at 80 ºC
    - After 24 hrs we will get powdered from the pure culture. Heat the cryovail under flame and break the seal
    - Preserve it under room temperature

Procedure

1. For purification of bacterial isolates different bacterial colonies were picked and streaked separately on nutrients agar plate with sterile inoculating loop. Plates were incubated at 25- 30 ºC in BOD incubator.
2. When pure cultures were obtained the culture were preserved on NA slant and glycerol stocks (25-30%).

Experiment 20

Aim: Isolation of DNA from the bacterial cells

Requirement: Tris base, Proteinase K, Phenol\chloroform, ethanol, RNAase, SDS, EDTA, Tryptone, Yeast extract, NaCl, LB medium, TE buffer, Lysis buffer, Eppendorf tube

Theory: Good quality DNA is a prerequisite for all experiments of DNA manipulation. All plant DNA extraction protocols comprise of the basic steps of disruption of the cell wall, cell membrane and nuclear membrane to release the DNA into solution followed by precipitation of DNA while ensuring removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites.

Procedure

1. Transfer 1.5 ml of the overnight *E. coli* culture (grown in LB medium) to a 1.5 ml Eppendorf tube and centrifuge at max speed for 1min to pellet the cells.
2. Discard the supernatant.
3. Resuspend the cell pellet in 600 µl lysis buffer and vortex to completely resuspend cell pellet
4. Incubate 1 h at 37 °C.
5. Add an equal volume of phenol/chloroform (25:1) and mix well by inverting the tube until the phases are completely mixed.
6. Spin at max speed for 5 min at 1000 rpm. There is a white layer (protein layer) in the aqueous: phenol/chloroform interface.
7. Carefully transfer the upper aqueous phase to a new tube by using 1 ml pipetman.
8. Steps 4-6 can be repeated until the white protein layer disappears.
9. To remove phenol, add an equal volume of chloroform to the aqueous layer. Again, mix well by inverting the tube.
10. Spin at max speed for 5 min.
11. Remove aqueous layer to new tube.
12. To precipitate the DNA, add 2.5 or 3 volume of cold ethanol (store ethanol at -20 °C freezer) and mix gently (DNA precipitation can be visible).
13. Incubate the tube at -20 °C for 30 min or more.
14. Spin at max speed for 15 min at 4 °C.
15. Discard the supernatant and rinse the DNA pellet with 1 ml 70% ethanol (stored at RT).
16. Spin at max speed for 2 min. Carefully discard the supernatant and air-dry the DNA pellet.
17. Resuspend DNA in TE buffer.
18. Check isolated Gemonic DNA on an agarose gel.

Precautions

Phenol is a very strong acid that causes severe burns. Chloroform is a carcinogen. Wear gloves, goggles and lab coat, and keep tubes capped tightly.

Experiment 21

Aim: Quantification of genomic DNA by agrose gel electrophoresis

Requirement: Gel casting instrument, microwave oven, beaker, ethidium bromide, pipette and tips

Theory: The quantity and quality of the genomic DNA can be checked by agarose gel electrophoresis. The principle of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. When an electrical potential is applied to the DNA, it moves towards the positive pole. The 1× TAE buffer was prepared by diluting 50×TAE buffer.

Procedure:

1. The quality and quantity of genomic DNA was checked on 0.8% agarose gel electrophoresis.
2. 0.8% agarose solution in 100mL TAE was made for typical DNA fragment carefully. To dissolve the agarose the solution was carefully bring first to boil in a microwave oven.
3. Solution is then cooled down to about 28 ˚C at room temperature or in water baths. The solution is stirred or swirl white cooling
4. 5 µL ethidium bromide stock (10 mg/µL) /100mL gel solution was added for a final concentration of 0.5 µg/mL.
5. Solution is stirred to disperse the ethidium bromide and then poured into gel rack.
6. The comb was inserted at one side of the gel, about 5-10 minute from the end of the gel. When gel gets cooled down then comb was removed carefully.
7. The gel along with rack was put into the tank with TAE. Ethidium bromide at the same concentration covered with TAE with the slots at the end electrode that have magnetic current.
8. After the gel was prepared, micropipette was used to inject about 4 µL of stained DNA. The lid of electrophoresis chamber was closed and current (typically 100 V for 30 minute) was applied with 15 mL of gel. The coloured dye in the DNA ladder and DNA samples act s front wave that runs faster than DNA itself. When the front wave approached the end of the gel, the current was stopped.
9. DNA was stained with ethidium bromide and was then visualized under ultraviolet light documented analyzer system.

Observation: Ethidium bromide stained DNA was visualized under UV light in gel electrophoresis system.

Experiment 22

Aim: PCR amplification of bacterial genomic DNA

Requirements

Genomic DNA, micropipette, eppendrof tube, PCR tubes, ice bucket, micropipette tips, Taq polymerase, dNTPs mixture, milliQ water, and primers (forward and reverse).

Theory

The development of PCR techniques has revolutionized the field of biotechnology beginning with molecules of DNA. PCR can generate millions of copies of DNA in few hours. Kary Mullis discovered this technique in 1983 and received Nobel Prize for this technique in 1993. The technique involves repeatedly round of *in vitro* DNA synthesis by a heat stable enzymes known as Taq DNA polymerase which is isolated from a bacterium *Thermus aquaticus*.

Under most conditions, DNA is double stranded consisting of two such nucleotide chains that bound around each other as the double helix. Primers are single stranded consisting of a string of the nucleotides in a specific order that will under the right conditions binds to a specific complementary sequence of nucleotise in another piece of single stranded DNA. DNA for primers must have complementary nucleotide sequence on either side of the piece of DNA. The PCR mixture is taken through the replication cycles consisting of

1. Denaturation: At 94-96 ºC, the DNA is denatured into single strand
2. Annealing: At 50-65 ºC, two primers i.e forward and reverse anneals to their complementary sequence on either side of the target sequence.
3. Extension: At 72 ºC, the polymerase binds and extends a complementary DNA strand from each primer.

Steps from 1-3 comprises one cycle. As amplification proceeds, the DNA sequence between the primer doubles after each cycle. Thus the amount of amplified product will be 2 copies starting from single molecules after number of cycles.

Procedure

Preparation of master mix

Buffer	2.5µl
$MgCl_2$	2.0µl
dNTPs mix	2.5µl
Primer F	1µl
Primer R	1µl
Taq polymerase	0.5µl
MilliQ water	14.5µl (variable)
DNA template	1-5µl (variable)

PCR amplification protocol

Initial denaturation	5 minutes at 94 ºC
Denaturation	94 ºC for 1 minutes
Annealing	52 ºC for 30 seconds
Elongation	72 ºC for 30 seconds
Final elongation	72 ºC for 10 minutes

1. 1.2% agarose gel made
2. Ladder of 500bp and PCR products was melted
3. Gel was run at 60V for 40 minutes
4. The pictures was seen in gel-doc

Observation and results

Quantification of PCR products was done through gel electrophoresis. Picture of gel having DNA bands was seed under gel doc

Precautions

The amount of reagent should be accurate.